Title: Tusc2/Fus1 regulates osteoclast differentiation through NF-κB and NFATc1

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Running Title: The role of Tusc2 in osteoclasts.

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Supplementary Materials and Methods

Reagents

Recombinant human macrophage colony-stimulating factor (M-CSF) was a gift from Dr. David Fremont (Washington University, St. Louis, MO, USA), while recombinant human sRANKL was purified from bacteria. Recombinant human bone morphogenetic protein 2 (BMP2) was purchased from Cowellmedi (Seoul, Republic of Korea). Alizarin red and -glycerophosphate were obtained from Sigma-Aldrich (St. Louis, MO, USA). Ascorbic acid was purchased from Junsei Chemical (Nihonbashi-honcho, Japan). Antibodies specific for Flag and actin were purchased from Sigma-Aldrich. Antibodies against c-Fos, NFATc1, and lamin B1 were obtained from Santa Cruz Biotechnology (Dallas, TX, USA). Antibodies against phospho-I B (nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor), p38, phospho-p38, c-Jun N-terminal kinase (JNK), phospho-JNK, extracellular signalregulated kinase (ERK), phospho-ERK, phospho-CREB, and CREB were obtained from Cell Signaling Technology (Beverly, MA, USA). Antibodies against phospho-CaMKIV and CaMKIV were purchased from Abcam (Cambridge, UK).

Pit formation assay

BMMs were cultured on Osteo assay plates (Corning, NY, USA) in the presence of M-CSF (30 ng/ml) and RANKL (100 ng/ml) for 5 days. Resorption lacunae were vis ualized using bright-field microscopy.

Retroviral gene transfection

Retroviral vectors were transfected into the packaging cell line, Plat E, using FuGEN E 6 (Promega, NC, USA) according to the manufacturer's instructions. Viral supernata nts were harvested from the culture medium 48 h after transfection. BMMs or osteobl asts were then incubated with viral supernatants for 6 h in the presence of polybrene (Sigma-Aldrich).

Quantitative real-time PCR

Cultured cells were lysed in Qiazol lysis reagent (Qiagen GmbH, Germany) for total RNA extraction. Purified RNA was then reverse transcribed into cDNA using Superscr ipt II Reverse Transcriptase (Thermo Fisher Scientific), according to the manufacturer' s protocol. Quantitative real-time PCR was performed in triplicate using the SYBR mi xture (Qiagen GmbH). The relative mRNA amounts were then calculated by normalizi ng them to the level of the endogenous housekeeping gene, GAPDH.

Short interfering RNA (siRNA) transfection

Control siRNAs and Tusc2 siRNAs were purchased from Dharmacon (GE Healthcare). The siRNAs were transfected into BMMs using Lipofectamine 2000 (Invitrogen, CA, USA), according to the manufacturer's protocol.

Statistical analysis

Statistical analyses were performed using an unpaired Student t test. All data are pres ented as the mean ± SD. P values < 0.05 were considered statistically significant.