INTRODUCTION
Fracture-nonunion is one of the most problematic conditions following orthopaedic trauma, however there has been little investigation of the molecular basis for its development. Bone morphogenetic proteins (BMPs) are generally recognized to have significant roles in osteogenesis, however, little is known about the expression patterns of BMPs in abnormal bone healing that results in nonunion formation. Moreover, little attention has been directed at investigating the potential role of BMP antagonists in abnormal bone healing. These facts lead us to investigate and compare the gene expression patterns of BMPs and their antagonists in normal and abnormal bone healing using rat experimental models.

METHODS
Animal model: Three-month-old male Long Evans rats were used in this study under a research protocol approved by the Institutional Animal Care and Use Committee. Forty-eight animals, with half assigned to standard stabilized closed femoral mid-shaft fracture and half assigned to experimental atrophic nonunion, were used. For experimental atrophic nonunion, the fracture site was exposed and the periosteum was cauterized circumferentially for a distance of 2mm on each side of the fracture. Four animals from each group were sacrificed at the following time points: post-fracture days 3, 7, 10, 14, 21, and 28.

Tissue harvest and RNA extraction: The newly generated tissues, i.e., fracture callus for the standard fracture and fibrous tissue surrounding the fracture site for the nonunion were harvested. Total cellular RNA was extracted from the harvested tissue.

GeneChip® microarray analysis: RNA samples of post-fracture day 14 for both groups were studied by a Genechip® microarray analysis. Affymetrix® Rat Genome 230 2.0 oligonucleotide arrays were hybridized with fragmented, biotin-labeled cRNA. After hybridization, the arrays were stained with an Affymetrix® fluidics station. The arrays were scanned on the Genechip® Scanner 3000 and analyzed with the GeneChip® Operating Software v1.1.1. To select differentially expressed genes of interest, dChip model-based expression analysis was used.

Real-time quantitative RT-PCR: RNA samples were reverse-transcribed to synthesize cDNA samples. TaqMan® primer and probe pairs for target genes and GAPDH were from Applied Biosystems. Real-time quantitative PCR was done in triplicate on the cDNA with an ABI 7700 Sequence Detector. Results were normalized to GAPDH levels using the formula \( \Delta \Delta Ct \) (threshold cycle) = Ct of target gene – Ct of GAPDH. The comparative Ct method was used to investigate the amount of target gene relative to a calibrator. Of forty-eight animals, an arbitrary animal was selected as a calibrator. The \( \Delta \Delta Ct \) value was calculated as follows: \( \Delta \Delta Ct \) of an animal = \( \Delta Ct \) of an animal – \( \Delta Ct \) of the calibrator. The amount of target gene normalized to GAPDH and relative to a calibrator of an animal was given by the formula 2\(^{-\Delta \Delta Ct}\).

Statistical analysis: The values of gene expressions of standard fracture and nonunion were compared at each time point and evaluated with Student’s t-test. Significance was defined as p-values less than 0.05.

RESULTS
Genechip® microarray analysis revealed that several genes related to BMPs are up- or down-regulated in the nonunion compared with the standard fracture. Up-regulated genes include follistatin-related protein and Tob 1. Down-regulated genes include BMP-2, 3, 4, 6, 7, 3B, 4, 6, 7, noggin, drm, sclerostin, Tsg 2, BAMBI, Smad 7, and distal-less homeobox 5.

Real-time PCR analysis revealed that gene expressions of BMP-2, 3, 4, 6, 7, 3B, 4, 6, 7, noggin, drm, sclerostin, and BAMBI were significantly lower in the nonunion than in the standard fracture at several time points. We found unique gene expression patterns for GDF-5, GDF-7, and chordin. The gene expressions of GDF-5 and GDF-7 were lower in the nonunion at the early time points and higher in the nonunion at the later time points. Chordin gene expression was significantly higher in the nonunion than in the standard fracture at the later time points.

DISCUSSION
It is probable that excessive BMPs are detrimental to normal fracture healing; therefore, negative feedback loops to temper BMP activities are needed for normal fracture healing to progress. More BMPs are expressed in the standard fracture than in the nonunion, therefore more BMP antagonists are also necessary to temper BMP activities.

CONCLUSION
Decline in BMP gene expression is implicated in the nonunion formation. The balance between BMPs and their endogenous antagonists is critical for fracture healing.