INTRODUCTION
Anti-inflammatory agents exhibit its effect by suppressing the enzymes called cyclooxygenase (COX), which converts arachidonic acids to prostaglandins. COX, which plays an important role in bone metabolism, promotes osteoclastogenesis when induced by IL-1 in stromal cells. Moreover, studies with COX-2 knockout mice have demonstrated the role of these enzymes in bone remodeling. In addition to bone resorption, COX-2 may also have a role in bone formation. Zhang et al. have reported that inducible COX-2 increases the expression of core-binding factor α1 (Runx2/Cbfα1) and osteo, which are required for bone formation. Up to date, no in vitro study has been conducted to identify whether the COX inhibitors such as nonsteroidal anti-inflammatory drug (NSAIDs) suppress osteogenic differentiation of human bone marrow cells. Moreover, little is known about the relationship between inflammatory condition and osteogenic differentiation. The purpose of this study is to identify whether osteogenic differentiation in human bone marrow-derived mesenchymal stem cells (hBMSCs) is inhibited after treatment with NSAIDs.

METHODS
Cell culture, Osteogenic differentiation, and Drug treatment: MSCs were isolated from bone marrow aspirates of adult donors aged 13–60 years under the approval of Institutional Review Board (IRB). To induce osteogenic differentiation, cells were incubated in an osteogenic medium supplemented with 10% FBS, 0.1μM dexamethasone, 50μg/ml ascorbic acid, and 10mM β-glycerophosphate (Sigma). After 7 days, cells were analyzed by alkaline phosphatase (ALP) staining.

As non-selective and COX-2 selective inhibitor, we used Celecoxib (Pfizer, NY, USA) and Naproxen (Myung Moon, Seoul, Korea). To examine the effect of NSAID on osteogenesis of hBMSCs, cells were treated with drugs in different concentration (Celecoxib: 10μM-therapeutic dose, 20μM or 40μM; Naproxen: 100μM-therapeutic dose, 200μM or 300μM). The drug-treated conditioned media were replaced once every 2 days during osteogenic differentiation. All cultures except the control groups were treated with 1% of IL-1β to enhance the expression of COX-2 and provoke inflammatory condition in vitro.

Reverse transcription-polymerase chain reaction (RT-PCR): Total RNA was isolated from cells using RNeasy kit (Qiagen, Valencia, CA), and reverse-transcribed using an Omniscript kit (Qiagen). For RT-PCR, cDNA was amplified in a total volume of 50 μl containing 1X PCR buffer, 0.4 μM of each primer, 0.2 mM dNTP mix, and 1 U of Taq DNA polymerase (Qiagen) at optimal temperature. PCR products were analyzed in a 1.5% agarose gel.

RESULTS
In hBMSCs, we confirmed that the expression of COX-2 protein was significantly increased after the treatment of IL-1β (1 ng/ml), whereas COX-1 remained constant (data not shown). We then examined whether mRNA levels of prostanoid synthesis-related enzymes (COX-1, COX-2, ePGES, and mPGES) were reduced in cells treated with Celecoxib or Naproxen. Among these, only mRNA level of mPGES was reduced in the Celecoxib-treated cells, suggesting that mPGES, a downstream molecule of COX-2, was selectively inhibited by Celecoxib. In contrast, both ePGES and mPGES were reduced in a concentration-dependent manner in cells treated with Naproxen. COX-1 and COX-2 expression were not changed in cells treated with Celecoxib or Naproxen (data not shown). On day 7 after inducing osteogenic differentiation of hBMSC, ALP, an earlier marker of osteogenic differentiation, was expressed very weakly in the non-inflammatory conditioned MSCs, and there was no significant differences in the extent of ALP staining between the doses of the two NSAIDs (Fig. 1 upper). However, in the inflammatory-conditioned MSCs, ALP expression was significantly reduced by the over-therapeutic doses of NSAIDs (40 μM of celecoxib and 300 μM of naproxen) compared to the control osteogenic MSCs or the therapeutic dose groups (10 μM of celecoxib and 100 μM of naproxen) (Fig. 1 lower). This finding suggests that high dose of NSAIDs may inhibit osteogenic differentiation in inflammatory-conditioned MSCs.

We then investigated the effects of NSAIDs on the expression of osteogenesis-related genes during osteogenic differentiation of hBMSCs. The mRNA expression of Runx2/Cbfα1, Dlx5, and osteocalcin was decreased by the two NSAIDs in inflammatory-conditioned MSC and showed a significant decrease for the over-therapeutic dose while they remained constant or irregular in the non-inflammatory-conditioned MSCs. (Fig. 2).

DISCUSSION
The present study revealed that the treatment of overtherapeutic dose of COX-2 inhibitor during in vitro osteogenesis of inflammatory-conditioned MSC resulted in a significant reduction in ALP expression but not in non-inflammatory-conditioned cells. Inhibited ALP expression in overtherapeutic-dose NSAID-treated MSC might be correlated with decreased PGE2 synthesis and osteogenesis-related genes, suggesting that the inhibitory effect of NSAIDs during osteogenesis in inflammatory-conditioned MSCs may proceed via pathways different from those in osteogenesis under non-inflammatory condition. Interestingly, osteogenesis of hBMSC was not affected by conventionally accepted in vitro therapeutic dose of 10μM celecoxib or 100μM naproxen, but rather at much higher (over-therapeutic dose) of 40 μM for Celecoxib and 300 μM for Naproxen. Consequently, precautions must be taken when considering administration of greater than recommended dosage of NSAIDs as its effect on hBMSCs at these higher dosages remain unknown.

REFERENCES
1. Phinney DG et al., J cell Physiology; 2004; 200; 400–406

ACKNOWLEDGEMENT
This research was supported by a grant IG-KOR-006-2007 (8379) from Pfizer and by a grant (code: SC3210) from the Stem Cell Research Center of the 21st Century Frontier Research Program funded by the Ministry of Education, Science and Technology, Republic of Korea.

Figure 1. Comparison of the extent of ALP staining on osteogenic differentiation of IL-1β-treated and/or untreated MSCs with NSAIDs treatment.

Figure 2. RT-PCR analyses of mRNA expression for transcription factors and markers required for osteogenic differentiation.