INTRODUCTION
Postmenopausal osteoporosis with fragility in bone is widely recognized disease caused by estrogen depletion. Significant efforts has been paid to explore cellular and molecular mechanisms underlying bone loss in these patients and numbers of new options for bone loss prevention have been developed. However, it is hard to decrease fracture risk dramatically in these patients. Furthermore, researchers including our group demonstrated that estrogen deficiency impaired fracture healing in rats. It seems important to understand how estrogen regulate fracture healing at cellular and molecular level for the development of better treatment of these fractures.

Chondrogenesis, the initial steps in cartilage formation from non-cartilaginous cells, has been documented to play a pivotal role in embryonic skeletal development and skeletal repair after injury. In fracture healing, chondrogenesis generate cartilage tissue that bridges fracture gap and restores mechanical stability by endochondral ossification. A regulatory role for estrogen on chondrogenesis has only been reported in embryonic duck syrinx, a cartilaginous vocal organ for birds that expresses sexual dimorphism in the duck. Further investigations using established in vitro model for chondrogenesis are needed to clarify the regulatory role of estrogen during chondrogenesis.

In this study, we focused on the direct regulatory role of estrogen on chondrogenesis using in vitro chondrogenesis model, ATDC5 that undergoes chondrogenesis in response to insulin stimulation. We also examine the possibility for involvement of insulin receptor substrate (IRS)-1, downstream signaling molecule of insulin and type 1 IGF receptors, expression in estrogen action in ATDC5 cells.

MATERIALS AND METHODS
Cell culture: ATDC5 cells were obtained from RIKEN Cell Bank (Tsukuba, Japan). ATDC5 cells were maintained as previously described. All experiments were performed on cells prior to passage fifteen. Effect of 17beta-estradiol on cell growth and alcian blue staining: Cells were cultured in 6 or 24-well plates with the medium without phenol red, supplemented with 5% dextran-coated-charcoal treated FBS. After the confluence, the medium was changed to medium containing 10 µg/ml insulin with or without 17beta-estradiol and ICI 182,780. On the day of harvest, cell number was measured using CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay, following the manufacturer’s instruction. For alcian blue staining, cells were fixed and stained with 0.1% alcian blue dye. Dye was extracted from the cells and absorbance of the extracted dye was measured at 650 nm. RNA extraction, cDNA synthesis, and reverse-transcriptase PCR (RT-PCR): Total RNA was extracted from ATDC5 cells using TRIZOL. cDNA was synthesized and PCR amplification of estrogen receptor alpha and beta, IRS-1, and GAPDH were carried out. Amplified PCR products were separated by electrophoresis in 3.5% agarose gel. For IRS-1: Cell lysate was prepared with RIPA buffer as described previously. Protein was separated by 8% SDS-PAGE and transferred to PVDF-membrane. Anti-IRS-1 antibody and anti-phosphotyrosine antibody were obtained from Upstate Biotechnology. IRS-1 and tyrosine phosphorylated IRS-1 was visualized by enhanced chemiluminescence.

RESULTS
Cell number and alcian blue staining were increased under insulin stimulation by 16 days along with changes in morphological characteristics of the cells; multi-layering and then, cartilage-like nodule formation (Fig. 1). Physiological concentrations of 17beta-estradiol (10^-10~10^-8M) alone did not induce chondrogenesis but enhanced insulin-induced cell growth and alcian blue staining in a dose-dependent manner. The estrogen receptor antagonist, ICI 182,780 did not alter insulin-induced cell growth and alcian blue staining, however, it antagonized the effect of 17beta-estradiol. RT-PCR confirmed expression of estrogen receptors alpha and beta in both un-stimulated and differentiated ATDC5 cells. Short-term treatment with 17beta-estradiol revealed that first four days were more sensitive to 17beta-estradiol stimulation compared to the later days. To investigate the involvement of IRS-1 in 17beta-estradiol enhancement of insulin-induced chondrogenesis, expression of IRS-1 and its activation were analyzed. Semi-quantitative PCR showed that insulin treatment decreased expression of IRS-1 by 1 day but 17beta-estradiol inhibit the down-regulation by insulin (Fig. 2). The amount of IRS-1 and tyrosine phosphorylated IRS-1 protein was also increased by 17beta-estradiol treatment for 3 days.

DISCUSSION
Despite the existence of a large body of information on the role of estrogen in skeletal homeostasis, the regulatory role of this hormone in chondrogenesis remains unclear. Based on reported histological changes in chondrogenesis observed in the fracture callus of O VX rats, we hypothesized that estrogen, in addition to regulating chondrocyte maturation and function, also plays a regulatory role in chondrogenesis. In this study, we tested this hypothesis using ATDC5 cells, a mouse EC cell line which is known to undergo chondrogenesis with insulin stimulation. Profound effects on cell growth and proteoglycan accumulation strongly suggest that 17beta-estradiol enhanced insulin-induced chondrogenesis in these cells via receptor mediated mechanisms. As far as we are aware, this is the first report which identifies a direct and positive effect of 17beta-estradiol on chondrogenesis in mammalian cells. Although further support of our results need to be obtained in vivo models of chondrogenesis, it can be inferred that lack of the direct and positive effect of estrogen is one of the reasons for the delayed chondrogenesis observed in the fracture callus of O VX rats. Estrogen may affect ATDC5 cells via insulin-signaling dependent mechanism because of absence of the effect in un-treated cells. Recent studies have shown that IRS-1, which plays a critical role in metabolic and mitogenic action of insulin and IGF-I, was up-regulated by estrogen in the breast cancer cell line, MCF-7. The same cross-talk in signal transduction was observed in ATDC5 cells by day 3, the period when the cells were sensitive to 17beta-estradiol stimulation. Given the profound effect of insulin on chondrogenesis in ATDC5 cells, the modulation of insulin signaling by estrogen is an appealing concept. Further studies are needed to clarify physiological relevance of estrogen on insulin-induced chondrogenesis.

ACKNOWLEDGEMENTS
This study was supported by Mayo Foundation and Eisai Company Ltd., Japan.

REFERENCES