Introduction
Chondrocyte differentiation, typically described by sequential changes in cell morphology and stage-specific gene expression patterns, is a far more complex process than it appears, requiring a variety of tightly coordinated regulatory events. Chondrocyte differentiation involves complex processes, such as cell proliferation, hypertrophy, terminal differentiation, mineralization and cell death (apoptosis). In growth plate cartilage, these events are necessary to ensure normal growth and development of the skeleton. On the other hand, based on studies from our and other laboratories, if the same hypertrophic and terminal differentiation processes are activated in articular chondrocytes, articular cartilage will experience devastating destructive changes, as seen in osteoarthritis (1,2). In this study, we asked the following questions: (i) What triggers terminal differentiation of growth plate chondrocytes? Since differentiation of growth plate chondrocytes is accompanied by increases in cytosolic calcium concentration \([\text{Ca}^{2+}]\), and changes in \([\text{Ca}^{2+}]\), have been shown to play important roles in differentiation events of various cell types, we especially asked whether changes in \([\text{Ca}^{2+}]\), play a role in terminal differentiation and mineralization. Furthermore, we asked (ii) how terminal differentiated chondrocytes regulate the mineralization process. To address these questions, we used a cell culture system in which terminal differentiation and mineralization of hypertrophic growth plate chondrocytes were induced by treatment wit retinoic acid (RA).

Methods
Chondrocytes were isolated from day 19 old chick embryonic growth plate cartilage. After cells have reached confluency, cells were treated with RA (35nM) or RA (35nM) and BAPTA-AM, a specific cell-permeate Ca\(^{2+}\) blocker. Chelation of cytosolic calcium with BAPTA results in the release of matrix vesicles. While matrix vesicles isolated from RA-treated cultures contained significant amounts of annexin II, V and VI, matrix vesicles isolated from untreated and RA/BAPTA-treated cultures contained only little of these annexins. RA/BAPTA-treated cultures showed no or little Ca\(^{2+}\) uptake. Annexin II, V and VI mediated Ca\(^{2+}\) influx into fura-2 loaded liposomes reconstituted from lipids isolated from authentic matrix vesicles. Annexin II, V or VI-mediated Ca\(^{2+}\) influx into these liposomes was inhibited by the 1,4-benzothiazepine derivative K-201, which specifically binds to annexins. More interestingly, K-201 also inhibited Ca\(^{2+}\) uptake and mineralization by authentic matrix vesicles in a dose-dependent manner (Fig.2).

Results
Treatment of hypertrophic growth plate chondrocytes with RA caused a significant increase in \([\text{Ca}^{2+}]\), (Fig.1), followed by a relocation of annexin II, V and VI from the cytoplasm to the plasma membrane, and the release of mineralization-competent matrix vesicles containing alkaline phosphatase, annexin II, V and VI.

Discussion
The results of this study demonstrate that increases in \([\text{Ca}^{2+}]\), play a crucial role in the terminal differentiation and mineralization processes of growth plate chondrocytes. Increases in \([\text{Ca}^{2+}]\), lead to the relocation of annexin II, V and VI from the cytoplasm to the plasma membrane, followed by the release of annexin II, V and VI-containing matrix vesicles, which after being released into the extracellular matrix initiate the mineralization process. Chelation of cytosolic calcium with BAPTA results in the release of matrix vesicles which contain less of annexin II, V and VI than matrix vesicles isolated from RA-treated cultures. Furthermore, this study shows that annexin II, V and VI form Ca\(^{2+}\) channels in matrix vesicles, enabling Ca\(^{2+}\) influx into these vesicles. Interfering with the Ca\(^{2+}\) channel activities of annexin II, V and VI leads to a loss of Ca\(^{2+}\) uptake and mineralization ability of matrix vesicles. Thus, after matrix vesicles are released into the extracellular space, the annexins form Ca\(^{2+}\) channels in these particles leading to the rapid Ca\(^{2+}\) influx into the vesicles and initiation of mineralization.

References