INTRODUCTION
Mesenchymal stem cells in the periosteum have the potential to differentiate into neochondrocytes in vivo and in vitro (1, 2). Specific chondrocyte precursors have not yet been identified, but it is likely that they reside in the cambium layer, adjacent to the bone. To clarify the mechanisms regulating periosteal chondrogenesis for cartilage repair and for fracture healing, it will eventually be necessary to localize and identify the specific chondrocyte precursor. The purpose of this study was to determine the spatiotemporal gene expression pattern of Coll II (Col II) by using periosteal neochondrocytes.

MATERIALS AND METHODS
21 periosteal explants, isolated from the proximal tibiae of 17 two month-old NZ rabbits were cultured in agarose and DMEM with TGF-β1 as previously published for up to 6 weeks (2). We chose to acquire the rabbit-specific Coll II cDNA sequence by employing the strategy of “gene digging,” which involves the amplification of a gene segment by PCR using primers designed from the conserved nucleotide sequence. In situ hybridization for Coll II and safranin O/fast green staining ensure reproducibility and an average measurement was used. Examination was repeated twice separated by at least a 2 week period to juxtaosseous: the area adjacent to the original bone (Fig. 1-B). Each examination was divided into 2 regions; (A) juxtafibrous: the area adjacent to the fibrous layer and (B) juxtaosseous: the area adjacent to the original bone (Fig. 1-B). Each examination was repeated twice separated by at least a 2 week period to ensure reproducibility and an average measurement was used. Immunohistochemical analysis for Coll II and safranin O/fast green staining were performed in the serial sections. Data are expressed as means ± S.D. Statistical analyses were performed by repeated measures ANOVA and Duncan’s New Multiple Range post-hoc testing.

RESULTS
At 1 week, Coll II mRNA expression was evident in the most juxtaosseous region of the cambium layer (Fig. 1-A). At 2 weeks, Coll II mRNA expression had extended toward the fibrous layer from the juxtaosseous area (Fig. 1-B). At 3 weeks, Coll II mRNA expression was present throughout the whole cambium layer(Fig 1-B). However, at 4-6 weeks, Coll II mRNA expression in the juxtaosseous area had decreased compared to that in the juxtafibrous area (Fig. 1-C). Coll II mRNA expression was never seen in the fibrous layer of the periosteum, even after cartilage had been formed in the explants. The Coll II expression index gradually decreased in both (A) and (B) areas after 2 weeks, but significantly decreased in the juxtaosseous area (Fig. 2). Interestingly, morphologically immature flat chondrocytes were observed at the most juxtaosseous area and they continued to express Coll II mRNA even at the sixth week. However, there were no significant differences between the uptake of safranin O in these two areas nor in the intensity of the immunostaining with antibodies to Coll II throughout the culture period.

DISCUSSION
Chondrogenic activity during periosteal chondrogenesis was first evident in the juxtaosseous area, which had been in contact with bone, and then progressed toward the juxtafibrous area. This progression suggests that the cells in the juxtaosseous region are more differentiated, and those in the juxtafibrous region are less so. An alternative interpretation would involve a paracrine signaling process, resulting in a “wave” of chondrogenic activity progressing through the cambium layer (Fig. 3). If the latter is true, it should be possible to identify regulatory molecular mechanisms with in situ hybridization, and the identification of specific growth factors that are part of a cascade of events controlling chondrogenesis. At 3 weeks all of the cambium layer expressed the chondrogenic phenotype, however Coll II mRNA expression was not seen in the cells of the fibrous layer. These results indicate that the chondrocyte precursors are located in the cambium layer.

CONCLUSION
These data indicate that the chondrocyte precursors are located in the cambium layer which is where chondrogenic activity occurs.


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