SoxC Transcription Factors Promote Chondrocyte Proliferation And Survival In The Growth Plate

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Disclosures:

Introduction:
Tissue engineering that uses mesenchymal stem cells is a promising tool to repair injured or degenerated growth plate and articular cartilage. To improve the efficiency of tissue engineering, it is important to understand the molecular mechanisms underlying skeletogenesis. Most bones are built during development upon a cartilage template. This template features growth plates, i.e., highly organized structures in which chondrocytes sequentially proliferate in columns, undergo prehypertrophy, hypertrophy, and eventually terminally maturation and death. Sry-related HMG box-containing (Sox) transcription factors have master roles in the fate determination of specific cell types in many tissues. Among them, the SoxC group is composed of Sox4, Sox11 and Sox12. SoxC genes have important roles to promote survival of many cell types, including skeletogenic mesenchymal cells (Ref.1). However, the roles of SoxC in differentiated skeletal cell types remain largely unknown. We investigated here the importance of SoxC genes in the cartilage growth plate by conditionally deleting these genes in differentiated chondrocytes in mouse embryos.

Methods:
Mice were used according to federal guidelines and as approved by the Cleveland Clinic Institutional Animal Care and Use Committee. Since SoxC genes have redundant roles in many processes, we investigated their roles in the growth plate by analyzing mice in which all SoxC alleles were inactivated in chondrocytes. These mice were carrying Sox4 and Sox11 conditional null alleles, Sox12 null alleles and an ATC (SoxCATC) or Col2Cre (SoxCCol2Cre) transgene (Refs. 2 and 3). To activate ATC, doxycycline (D9891, Sigma) was administered at 2 mg/ml in drinking water with 5% sucrose from gestation day 12.5 (E12.5) to the time that embryos were collected. Paraffin sections of mouse tissues were stained with the cartilage-specific dye Alcian blue and counterstained with nuclear fast red or hybridized in situ with 35S-labeled RNA antisense probes. Immunostaining was performed on frozen sections. TUNEL staining was performed using In Situ Cell Death Detection Kit (Roche). EdU incorporation was performed using Click-iT EdU Cell Proliferation Assay Kit (Life Technologies). For EdU analysis, pregnant females were injected intraperitoneally with EdU solution 2 h before being killed. Data were obtained from at least three control and three mutant littermates. A p-value < 0.05 was considered statistically significant by the Student's t-test.

Results:
RNA in situ hybridization of E18.5 fetus sections showed that the SoxC genes have overlapping expression patterns in the cartilage growth plate. Skeleton preparations of newborn pups revealed that cartilage and bone elements were well formed in SoxCATC pups, but most were about 10% shorter than in control littermates (Table). Histology analysis of growth plates of E18.5 and E16.5 SoxCATC fetuses showed that epiphyses had a normal...
length, but that the proliferating zone, hypertrophic zone and endochondral bone were 10% shorter than normal (Fig.1). Close inspection of chondrocyte morphology uncovered a growth plate organization defect. In controls, proliferating chondrocytes were piled up in columns that were straight and often exceeding 6-cell layers. However, in SoxC\textsuperscript{ATC} fetuses, fewer columns were well formed and chondrocytes were present in reduced number and were often enlarged. We obtained the same results with SoxC\textsuperscript{Col2Cre} fetuses. RNA in situ hybridization of stage-specific differentiation markers showed that Sox9, a master regulator of chondrocytes, was expressed at a normal level in proliferating chondrocytes of SoxC\textsuperscript{ATC} fetuses, but hypertrophic chondrocyte markers, such as Col10a1 and Bmp6, were expressed at a reduced level. The EdU cell proliferation assay indicated that SoxC\textsuperscript{ATC} chondrocytes were proliferating at a normal rate in epiphyses, but at a reduced rate in the columnar zone (Fig.2). The TUNEL assay identified dying cells only in the terminal layers of control growth plates, but detected a significant proportion of dying chondrocytes in SoxC\textsuperscript{ATC} columnar and hypertrophic zones (Fig.3). This result was confirmed in SoxC\textsuperscript{Col2Cre} fetuses and by immunostaining of cleaved caspase 3.

**Discussion:**
We deleted the SoxC genes with the ATC and Col2Cre transgenes specifically in differentiated chondrocytes of growth plates and other cartilage types. SoxC\textsuperscript{ATC} newborn pups that were treated with doxycycline from E12.5 were mildly dwarf, as revealed by a 10% reduction in size of all long bones, compared to control littermates. The same phenotype was already detected at E16.5 and E18.5. Growth plates of mutant fetuses were malformed, showing disorganized columns of proliferating chondrocytes, a lower rate of cell proliferation and a higher rate of cell death. Therefore, this study uncovers that SoxC transcription factors contribute to long bone elongation during skeletogenesis by directly affecting the ability of chondrocytes to form fully functional growth plates.

**Significance:**
Here we showed that SoxC transcription factors are necessary to promote chondrocyte proliferation and survival in the cartilage growth plate. These factors thus have important in the developing skeleton beyond their essential roles previously demonstrated in mesenchymal progenitor cells. These results suggest that proper expression of SoxC transcription factors should be guaranteed at all stages of cell differentiation in mesenchymal stem cell-based tissue engineering approaches designed to repair injured or degenerated cartilage in human patients.

**Acknowledgments:**
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**References:**

<p>| Length of long bones of newborn pups |
|-------------------------------|----------------|-----|-----|-----|
| long bone | control | SoxC\textsuperscript{ATC} | Mut/cont | p value |</p>
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<th>Bone</th>
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Figure 1. E16.5 fetus tibias stained with Alcian blue and nuclear fast red. Right, high-magnification pictures of the proliferating zone.
Figure 2. EdU incorporation assay in tibia proximal growth plates of E16.5 fetuses. Left, proliferating cells are seen as green dots; cell nuclei are seen as blue dots. Cells were counted in indicated areas, 1; epiphysis 2; early columnar zone and 3; late columnar zone. Right, the percentage of positive cells in each area. Data on graph show relative number of mutants compared to controls.

Figure 3. TUNEL cell death assay in tibia proximal growth plates of E16.5 fetuses. Left, dying cells are seen as green dots; cell nuclei are seen as blue dots. Right, the percentage of TUNEL-positive cells in the columnar (CZ) and hypertrophic (HZ) zones. Data are shown as the averages with SD.