Modulation of c-Myb during chondrogenesis

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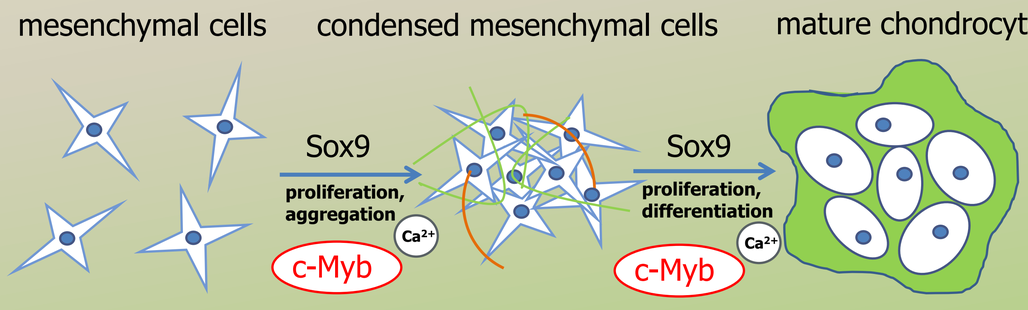
Introduction

Chondrogenesis is a multi-step cellular event: first, the mesenchymal cells differentiate into chondrocytes which proliferate, mature, and undergo hypertrofy and matrix calcification. This process is important in embryonic skeletal development as well as in postnatal skeletal growth.



Transcription factor c-Myb is known as associated with control of cell proliferation, survival, and cell death. It plays a critical role in hematopoietic development with *c-Myb* null mice dying around day 15 of embryogenesis. Our recent work has shown that c-Myb could be important in cell differentiation of undifferentiated cells, not only in proliferation. Transcription factor Sox9 is critical for chondrocyte differentiation and function (Long & Ornitz, 2013). Homozygous mice for *Sox9 (Sox9-/-)* are perinatally lethal.

Aims

1. To determine whether overexpression of c-Myb in micromass cultures would affect initiation of cartilage nodules.
2. To test, whether inactivation of c-Myb would cause downregulation of creating cartilage.
3. To follow, if there is any connection between c-Myb and Sox9 transcription factors in signalling pathways.

Material and methods

Micromass culture:
The mesenchymal micromasses were established from mouse front limbs in embryonic day 12 (ED12) and then were transiently transfected using the Fugene transfection reagent. We performed gain-of-function experiments used cells transfected by construct carrying c-Myb. Down-regulation was achieved by c-Myb siRNA. Cells were lyzed and analyzed by qPCR after 24 hour.

qPCR:
Mesenchymal cells were cultivated 24 hour and then were lyzed. RNA was isolated and used for first-strand cDNA synthesis. The samples were processed using qPCR and analyzed by the ΔΔCt method with normalization against *actin* mRNA levels, used as an internal control.

Alcian-blue staining:
Micromasses were cultured for 7 days, fixed with 10% paraformaldehyde and were stained in AB (AB – 5% Alcian-blue in 95% EtOH) in 0.1M HCl, overnight at RT. Data were analyzed in pixels of the total area using Adobe Photoshop 6.0 software.

Organ cultures:
The front limbs were dissected from the mouse embryos at embryonic day 12.5 (ED12.5) and cultured on a supportive metal grid covered by a membrane to allow transport of the culture medium (Fig. 1A). Limbs were cultured for 72 hours.

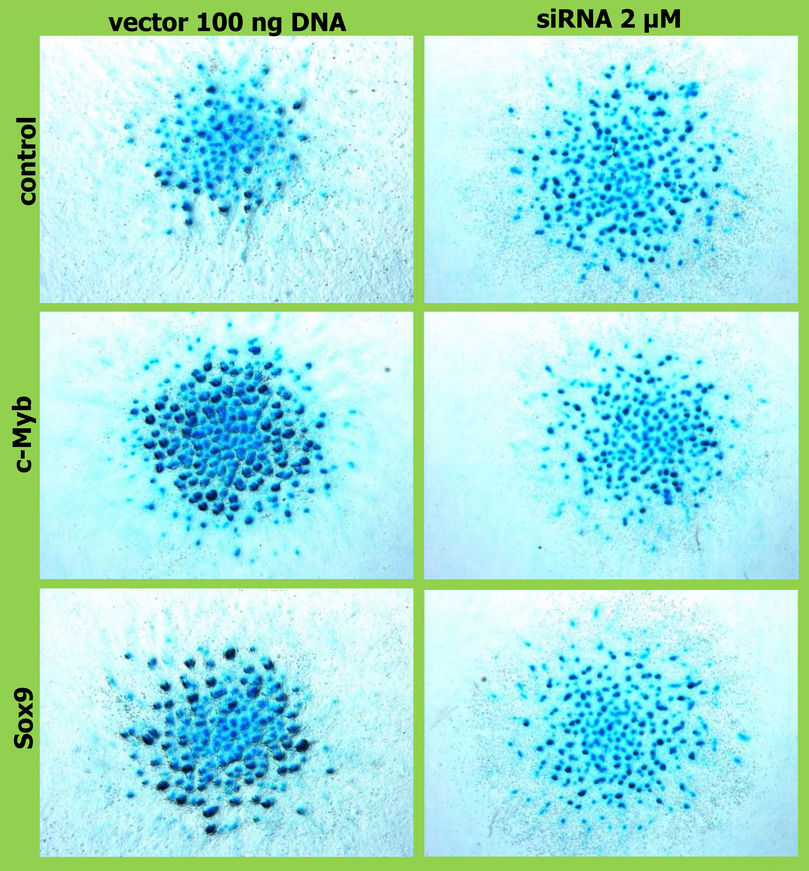
Electroporation:
The DNA (c-Myb and GFP, respectively) solution was injected with a micropipette into the limb mesenchyme. Both electrodes were inserted into the mesenchyme and 2-3 sets of 5 square pulses of 45 volts were applied. As control for succesful electroporation GFP was used (Fig. 1B, 1C).

Fig. 1: Electroporated organ cultures

Immunohistochemistry:
After deparaffinization and rehydration of the sections, mouse organs cultures were processed by immunohistochemical staining of PCNA (anti-PCNA, Santa Cruz, sc-7907) and c-Myb (anti-c-Myb, Abcam, ab59233).

Results: Overexpression c-Myb/Sox9 enhanced chondrogenesis **Results: Modulation of c-Myb influenced Sox9 expression**

c-Myb ➡ Sox9



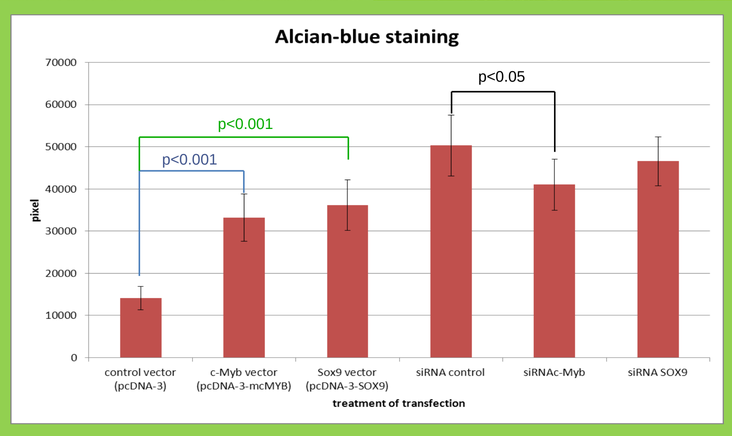


Fig. 2: Micromass cultures formed cartilage nodules after 7-day culture period. Overexpression of c-Myb and Sox9 enhanced chondrogenesis while downregulation of c-Myb and Sox9 inhibited it. The level of pixels shows statistically significant diference between treated cells and controls based on one-way ANOVA test after Alcian blue staining.

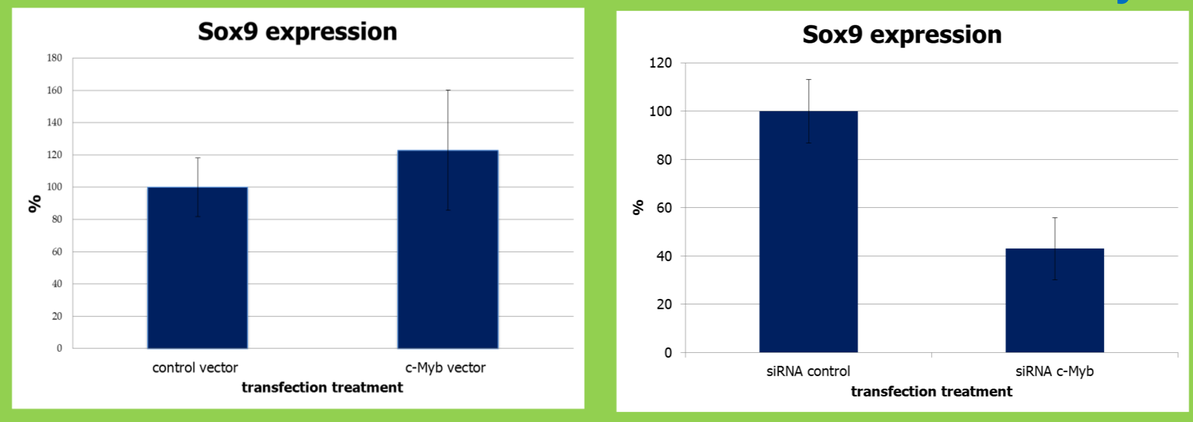


Fig. 3: Sox9 expression was increased to 122 % after c-Myb overexpression. In comparison, the level of Sox9 expression was decreased after downregulation of c-Myb using siRNA.

Results: Electroporation of c-Myb increased amount of c-Myb positive cells

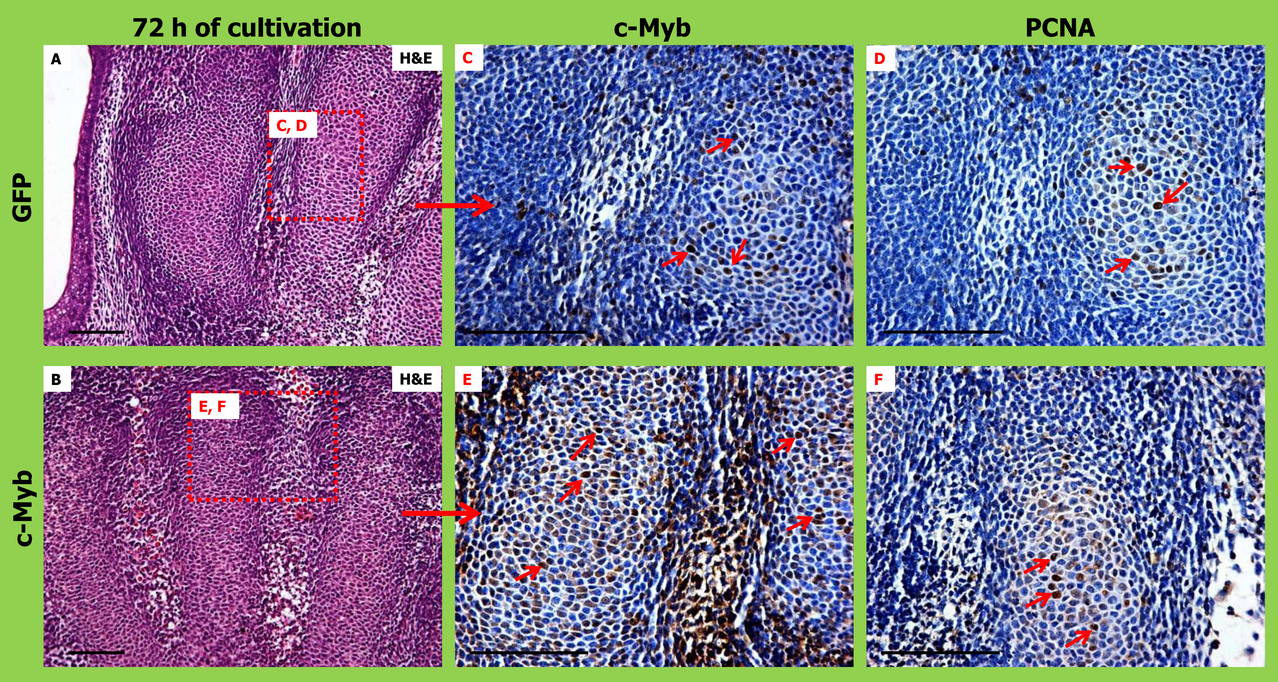


Fig. 4: The endochondral ossification in the front limbs was investigated after electroporation of c-Myb vector at 72 hour point. The higher number of c-Myb positive cells (brown) in c-Myb treated explants was apparent (E) vs. GFP control (C). The positive cells were identified in developing cartilage and perichondrium (E). PCNA was used as marker of proliferation, but the changes in number of positive cells were not obvious. PCNA positivite cells were found in the developing cartilage. With increasing number of c-Myb positive cells, the number of PCNA positive cells did not increase.

Conclusions:

The critical role of c-Myb during embryogenesis, especially in proliferation, was confirmed by many studies (Oh & Reddy, 1999; Sandberg *et al.*, 2005). Endochondral ossification is a highly organized process and gives rise to the majority of bone in the skeleton, which evolves via successive step of mesenchymal condensation, chondrogenesis and chondrocyte maturation.

Our results showed that the initiation of cartilage nodules was higher when c-Myb was overexpressed. This could be explained by a novel function of c-Myb in cell differentiation. Increased Sox9 expression after c-Myb overexpression and decreased Sox9 level after c-Myb siRNA silencing suggest a conclusion, that c-Myb could be an important regulator in transition between proliferation and differentiation of cells.

The authors declare no conflict of interest.

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