Rab23 regulates differentiation of ATDC5 chondroprogenitor cells

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Introduction: Chondrocyte differentiation can be mimicked in vitro by insulin treatment of the mouse chondroprogenitor cell line ATDC5. Utilizing this property of ATDC5 cells, we carried out a large scale screening of random retroviral insertions to identify novel regulators of chondrogenesis. In this study we report the characterization of two ATDC5 clones (H8-5 and H8-6) that were unable to undergo chondrogenic differentiation after insertional mutagenesis. Sequencing of retroviral integration sites in these two clones revealed that the retroviral DNA was inserted into the promoter region of Rab23 (Ras-related in brain 23), a gene encoding a member of the Rab family of small GTPases. Interestingly, Rab23 mutations have been identified both in mice and humans, and the mutant phenotypes include defects in bone development and limb patterning. Our study provides evidence for the first time that Rab23 mutations may exert their effects on the skeletal system through down-regulation of the essential transcription factor Sox9.

Materials and Methods: Lentivirus-mediated siRNA knockdown - Lentiviral siRNA constructs were based on the pLL3.7 lentiviral vector that also co-expresses green fluorescence protein (GFP). Lentivirus produced by the 293FT packaging cells was used to infect ATDC5 cells. Infected GFP+ cells were first enriched through sorting by flow cytometry then expanded for further analysis.

Results: Inverse PCR analysis revealed that in clones H8-5 and H8-6, the retroviral DNA was inserted into the 5’ promoter region of the Rab23 gene. We noted that the level of Rab23 protein in these two clones was higher than in normal controls, indicating that retroviral insertion activated the Rab23 gene. To investigate the effects of Rab23 down-regulation on differentiation, we constructed a lentiviral vector for delivery of siRNA to achieve stable and long-term knockdown of Rab23 in ATDC5 cells.

While ATDC5 cells infected with a non-specific siRNA exhibited no change in Rab23 expression, lentivirus-delivered siRNA targeting Rab23 mRNA was able to significantly knock down expression of Rab23 (Fig. 1A). Such knockdown of Rab23 is sustainable as a similar effect was observed in cells even with 12 days of insulin treatment. RT-PCR analysis revealed that knockdown of Rab23 was accompanied by a significant inhibition of Col2 expression when compared with mock or non-specific siRNA samples (Fig. 1B). This inhibitory effect on matrix genes was not limited to Col2 as expression of aggrecan was also down-regulated following Rab23 knockdown. However, expression of the housekeeping gene GAPDH was not affected, suggesting a specific effect of Rab23 knockdown on matrix genes.

To further confirm that production of the cartilage-like extracellular matrix was negatively impacted by Rab23 knockdown, ATDC5 cells were first infected with lentivirus for siRNA knockdown then cultured in insulin medium for 12 days before staining with Alcian blue. The intensity of Alcian blue staining did not differ significantly between mock-infected cells and cells with non-specific siRNA. However, Alcian blue staining was significantly diminished in cells with Rab23 knockdown (Fig. 1C), reflecting a reduced production of the cartilage-like extracellular matrix by these cells.

How could a knockdown in Rab23 protein lead to down-regulation of matrix genes? We reasoned that a specific level of Rab23 might be critical for the functions of chondrocyte-regulatory genes such as Sox9 that is known to control expression of Col2 and aggrecan. As shown by western blotting analysis, siRNA knockdown of Rab23 in these cells also resulted in a decrease in Sox9 (Fig. 1D). As the level of Sox9 protein is critical to chondrogenesis, the inhibitory effects of Rab23 knockdown on Col2 and aggrecan are likely mediated through a concomitant down-regulation of Sox9.

Discussion: Mutations in Rab23 were originally found to be associated with the mouse open brain phenotype. These mutations are single-nucleotide substitutions in both mutant alleles that introduce early stop codons into the open reading frame of Rab23. In addition to neural tube defects, mouse Rab23 mutations are also associated with malformations in axial skeleton and limbs. In humans, homozygous nonsense mutations in Rab23 were recently linked to Carpenter Syndrome, a genetic disorder characterized by premature closure of cranial sutures between certain bones of the skull. Individuals with Carpenter syndrome may also have unusual short fingers and toes, polydactyly or physical abnormalities such as short stature.

Our study shows that normal differentiation of ATDC5 chondroprogenitor cells requires a specific level of Rab23 protein. Too much or too little Rab23 protein leads to down-regulation of Sox9 expression and subsequent failure of chondrogenic differentiation. Our results also suggest a potential mechanism to explain the skeletal defects associated with mutations of Rab23. Specifically, Rab23 mutations might exert their effects by down-regulating Sox9 protein leading to defective/incomplete cartilaginous anlagen.

In patients with Carpenter syndrome, it is possible that Rab23 mutations result in decreased expression of Sox9 in osteochondral progenitor cells that eventually differentiate into flat bones of the skull. Formation of flat bones of the skull does not require a cartilage precursor but instead is mediated through intramembranous ossification. In this process, osteochondral progenitor cells directly differentiate into bone-forming osteoblasts. It has already been known that downregulation of Sox9 coincides with terminal differentiation of osteoblasts in developing craniofacial bones. Our study suggests that Rab23 mutations leads to decreased levels of Sox9 protein in these osteochondral progenitor cells this in turn leads to accelerated osteoblast differentiation resulting in premature closure of the cranial sutures.

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