Introduction: Chondrocytes and osteoblasts derive from a common precursor cell. Establishment of cell lineages involves several differentiation steps. These differentiation steps are controlled by specific transcription factors. For instance, the differentiation of precursor cells into osteoblasts was blocked in mice deficient in runx2 resulting in the lack of endochondral and intramembranous bone formation (1). Recently, another transcription factor, osterix, was identified which acts downstream of runx2. Osterix deficient mice also show no intramembranous and endochondral bone formation. Osterix, in contrast to runx2, however, controls the differentiation of preosteoblastic cells into mature osteoblasts (2). Since these transcription factors act as master regulators of cell differentiation, it is important to determine the factors regulating the expression of these transcription factors. The progressive ankylosis gene (ank) encodes a transmembrane protein that transport intracellular pyrophosphate, PPi, to the extracellular milieu. Extracellular PPi can directly act as a signaling molecule regulating cellular events or it can be hydrolyzed into phosphate (P) (3). P, also can act as a signaling molecule regulating cellular differentiation events (4). Interestingly, mutations in human ank were discovered resulting in an overgrowth of craniofacial and long bones (5,6). In addition, Ank is expressed in osteoblastic cells (7). These findings suggest that Ank may play a role in osteoblast differentiation. To determine the role of Ank in osteoblast differentiation, we suppressed Ank expression in an osteoblastic cell line, MC3T3 and determined their differentiation into mature osteoblasts. In addition, we analyzed the osteoblastic differentiation of bone marrow stromal cells isolated from ank/ank mice, which express a non-functional Ank protein, and wild type littermates (8).

Materials and Methods: MC3T3-E1 cells were cultured at confluence in Dulbecco’s modified Eagle's medium with 10% FCS and then cultured in the presence of ascorbate (50 μg/ml) and β-glycerophosphate (10 mM) to induce collagenous matrix release and mineralization (differentiation medium). Cells were cultured for up to 20 days in differentiation medium in the absence or presence of ascorbate (50 μg/ml) to induce collagenous matrix release and mineralization (differentiation medium). Bone marrow stromal cells were isolated from femurs of 4 week-old ank/ank mice or wild type littermates and cultured at 2x10^6 cells per 10 cm^2 well in αMEM supplemented with 15% FCS. After cells reached confluency, 100 μM β-glycerophosphate was added and cells were cultured for up to 35 days. APase staining of these cultures were performed after 16 days of culture, whereas van Kossa staining (mineralization) was performed after 35 days.

Results: To determine the role of Ank in osteoblast differentiation, we suppressed Ank expression in osteoblastic MC3T3 cells using siRNA and studied the osteoblastic differentiation of stromal cells isolated from the bone marrow of ank/ank mice or wild type littermates. MC3T3 cells mineralized within 20 days after the addition of ascorbate and β-glycerophosphate. Ank gene and protein expression in these cells was the highest at 3 days and declined afterwards, whereas alkaline phosphatase (APase) expression and activity increased on day 8 and was the highest between day 17 and day 20. Suppression of Ank expression in MC3T3 cells using siRNA led to a decrease of APase expression and activity. It also resulted in a decrease of the expression of osteoblast-related marker genes, including type I collagen, bone sialo protein, and osteocalcin. The expression of osterix was down-regulated in Ank expression suppressed MC3T3 cells, whereas runx2 expression was upregulated. PFA (blocker of phosphate transport proteins) treatment of MC3T3 cells resulted in a decrease of APase activity compared to the activity in untreated cells. Bone marrow stromal cells isolated from bone marrow of ank/ank mice and wild type littermates were cultured in the presence of 100 μg/ml ascorbate-2-phosphate for various days and analyzed by staining for APase enzyme activity and van Kossa to determine mineralized colonies. The number of APase activity positive and van Kossa stained colonies was markedly reduced in bone marrow stromal cell cultures from ank/ank mice compared to the number of APase activity and van Kossa positive colonies in bone marrow stromal cell cultures from wild type littermates.

Discussion: In this study we show that the progressive ankylosis gene (ank) is a positive regulator of osteoblast differentiation. Ank is highly expressed in the pre-osteoblastic cell line MC3T3. Ank expression is the highest in the immature cells and decreases when these cells differentiate into mature osteoblasts. Suppression of Ank expression resulted in an inhibition of differentiation of these cells into mature osteoblasts as indicated by the decrease in the expression of mature osteoblast-related marker genes. Interestingly, osterix expression was decreased in Ank expression suppressed MC3T3 cells, whereas runx2 expression was increased in these cells. Furthermore, our study shows that osteoblastic differentiation of bone marrow stromal cells isolated from ank/ank mice, which express a truncated non-functional Ank, is suppressed. These findings suggest that Ank regulates osteoblast differentiation by controlling the expression of osterix. Since osterix has been shown to be a major transcription factor acting downstream of runx2 and regulating the differentiation of preosteoblastic cells into mature and functional osteoblasts, whereas runx2 appears to control the differentiation of precursor cells into pre-osteoblastic cells, we propose that Ank regulates differentiation of preosteoblastic cells into mature osteoblasts by regulating osterix expression (1,2). In addition, our findings that PFA, which inhibits phosphate transporter proteins, inhibited APase activity in MC3T3 cells, suggest that the extracellular PPi resulting from the Ank transport, is being hydrolyzed into phosphate and that phosphate is then being transported back into the cell acting as a signaling molecule controlling osteoblast differentiation. Inorganic phosphate has been previously suggested to be involved in the regulation of osteoblast differentiation (4). In conclusion, our findings demonstrate that Ank via controlling PPi/Pi homeostasis in preosteoblastic cells regulates their differentiation into mature osteoblasts.


The Progressive Ankylosis Gene (ank) Regulates Osteoblast Differentiation
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