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The role of the A2B adenosine receptor in the differentiation of mesenchymal stem cells to osteoblasts and chondrocytes: implications for bone development and fracture repair

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Boston University
THE ROLE OF THE A2B ADENOSINE RECEPTOR IN THE
DIFFERENTIATION OF MESENCHYMAL STEM CELLS TO OSTEOBLASTS
AND CHONDROCYTES: IMPLICATIONS FOR BONE DEVELOPMENT AND
FRACTURE REPAIR

by

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B.S., University of Arizona, 2004
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2013
ACKNOWLEDGMENTS

There are numerous people who have played a role in getting me to where I am today. This includes teachers and mentors, as well as friends and family. Here I will address a few.

First and foremost I want to thank Dr. Katya Ravid for seeing potential in me from the time of our first meeting. She brought out the best in me with equal parts demand and support and, most importantly, provided an example of a strong and successful woman scientist.

I want to thank Dr. Lou Gerstenfeld for being my bone expert. He was always patient with me in my acquisition of bone biology knowledge. Few PIs would take the time to teach tissue staining to a student who was not their own and I am endlessly appreciative.

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Finally, thank you to my family and friends. You were always there to encourage me and keep me sane.
THE ROLE OF THE A2B ADENOSINE RECEPTOR IN THE
DIFFERENTIATION OF MESENCHYMAL STEM CELLS TO OSTEOBLASTS
AND CHONDROCYTES: IMPLICATIONS FOR BONE DEVELOPMENT AND
FRACTURE REPAIR
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SHANNON H. CARROLL
Boston University School of Medicine, 2013
Major Professor: Katya Ravid, D.Sc./Ph.D., Professor of Medicine, Biochemistry

ABSTRACT

The development, maintenance and repair of the skeletal system are dependent on the differentiation of both chondrocytes and osteoblasts from their common progenitor, the mesenchymal stem cell (MSC). The A2B adenosine receptor (A2BAR) is a G-protein-coupled receptor that signals by increasing cAMP and/or activating phospholipase C signaling. Considering the published roles of cAMP on MSC differentiation, and our finding that the expression of the A2BAR is induced following injury, we hypothesized that ablation or activation of the A2BAR impacts the differentiation of osteoblasts and chondrocytes and that this would manifest as changes in skeletal development and bone fracture repair. Activation of the A2BAR increased the differentiation of bone marrow-derived MSCs to osteoblasts by increasing mRNA expression of the transcription factors runt-related transcription factor 2 (Runx2) and Sp7 transcription factor (Osterix), which are essential for osteoblast differentiation. To examine the effect of the A2BAR on bone formation in vivo, we subjected wild type
(WT) and A2BAR knockout (KO) mice to bone fracture. A2BAR KO mice had impaired bone formation during fracture repair with increased cartilage volume. As fracture repair recapitulates the events that occur during endochondral ossification, we compared the growth plates of WT and A2BAR KO mice. In comparison to WT, A2BAR KO mice had a shorter growth plate initially, but a taller growth plate at a later age. These results suggest that initiation of endochondral ossification may be delayed in the A2BAR KO mice. Finally, we investigated whether the A2BAR is involved in chondrocyte differentiation. A2BAR activation decreased mRNA expression of the key transcription factor for chondrocyte differentiation, SRY (sex-determining region Y)-box 9 (Sox9) and decreased the mRNA expression of the hypertrophic chondrocyte marker Collagen X. Taken together, these data demonstrate a previously unidentified role of the A2BAR receptor in regulating MSC differentiation to both osteoblast and chondrocyte lineages. Further, we showed that mice null for the A2BAR have dysregulated bone formation during development and after injury. The importance of this receptor during bone formation and fracture repair could have implications for A2BAR-based therapies for bone maintenance and repair.
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<tr>
<td>18s</td>
<td>18s ribosomal subunit</td>
</tr>
<tr>
<td>A1AR</td>
<td>A₁ adenosine receptor</td>
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<tr>
<td>A2AAR</td>
<td>A₂A adenosine receptor</td>
</tr>
<tr>
<td>A2BAR</td>
<td>A₂B adenosine receptor</td>
</tr>
<tr>
<td>A3AR</td>
<td>A₃ adenosine receptor</td>
</tr>
<tr>
<td>ADA</td>
<td>Adenosine deaminase</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
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<tr>
<td>AMP</td>
<td>adenosine monophosphate</td>
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<tr>
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<td>adenosine triphosphate</td>
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<tr>
<td>BAY 60-6583</td>
<td>2-[6-amino-3,5-dicyano-4-[4-(cyclopropylmethoxy)-phenyl]pyridin-2-ylsulfanyl]acetamide</td>
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<tr>
<td>BMP</td>
<td>bone morphogenetic protein</td>
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<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>cAMP</td>
<td>Cyclic 3'-5'-adenosine monophosphate</td>
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<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
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<tr>
<td>C/EBPβ</td>
<td>CCAAT/enhancer binding protein beta</td>
</tr>
<tr>
<td>Col</td>
<td>collagen</td>
</tr>
<tr>
<td>CRE</td>
<td>cAMP response element</td>
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<tr>
<td>CREB</td>
<td>cAMP response element-binding protein</td>
</tr>
<tr>
<td>CREM</td>
<td>cAMP response element modulator</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle's Medium</td>
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<td>Symbol</td>
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<tr>
<td>DNA</td>
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<td>DPCPX</td>
<td>1,3-Dipropyl-8-cyclopentylxanthine</td>
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<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<td>ENT</td>
<td>Equilibrative nucleoside transporters</td>
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<td>E-NTPDases</td>
<td>Ecto-nucleoside triphosphate dephosphorylases</td>
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<td>FBS</td>
<td>fetal bovine serum</td>
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<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
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<td>H$_2$O$_2$</td>
<td>Hydrogen peroxide</td>
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<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
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<tr>
<td>IκB</td>
<td>Inhibitor of NF-κB</td>
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<tr>
<td>Il</td>
<td>interleukin</td>
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<tr>
<td>kDa</td>
<td>kilodalton</td>
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<tr>
<td>kg</td>
<td>kilogram</td>
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<td>MEF</td>
<td>mouse embryonic fibroblast</td>
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<td>mg</td>
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<td>micro-CT</td>
<td>micro-computed tomography</td>
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<tr>
<td>ms</td>
<td>millisecond</td>
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<tr>
<td>Term</td>
<td>Full Form</td>
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<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
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<td>MSC</td>
<td>mesenchymal stem cell</td>
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<tr>
<td>NECA</td>
<td>5'-(N-ethylcarboxamido)-adenosine</td>
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<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa B</td>
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<tr>
<td>nM</td>
<td>nanomolar</td>
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<tr>
<td>Nox</td>
<td>NAD(P)H oxidase</td>
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<td>NPP</td>
<td>Ecto-pyrophosphatase/phosphodiesterase</td>
</tr>
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<td>PBS</td>
<td>phosphate buffered saline</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PTH</td>
<td>Parathyroid hormone</td>
</tr>
<tr>
<td>PTHrP</td>
<td>Parathyroid hormone-related protein</td>
</tr>
<tr>
<td>qPCR</td>
<td>quantitative or real-time polymerase chain reaction</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
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<tr>
<td>rRNA</td>
<td>ribosomal ribonucleic acid</td>
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<tr>
<td>RT-PCR</td>
<td>reverse transcriptase polymerase chain reaction</td>
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<tr>
<td>Runx2</td>
<td>Runt-related transcription factor 2</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
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<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
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<td>SDS-PAGE</td>
<td>SDS polyacrylamide gel electrophoresis</td>
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<td>Sox9</td>
<td>SRY (sex determining region Y) box 9</td>
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<table>
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<tr>
<th>Acronym</th>
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<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor alpha</td>
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<td>TRAP</td>
<td>Tartrate resistant alkaline phosphate</td>
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<td>units</td>
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CHAPTER I. INTRODUCTION

The development of the skeletal system, as well as its repair, are dependent on the differentiation of both chondrocytes and osteoblasts from their common progenitor, the mesenchymal stem cell (MSC). There are two processes from which bone is formed. During intramembranous osteogenesis, bone is formed directly from MSC differentiation to osteoblasts. This process gives rise to flat bones, such as those of the skull and clavicle. In contrast, endochondral osteogenesis requires MSC differentiation to chondrocytes and the formation of a cartilage template, which is followed by ossification by osteoblasts. This process is responsible for the formation of the long bones of the skeleton, and remains active in the growth plates of growing bones (reviewed in [1, 2]). Bone fracture repair recapitulates the events of skeletogenesis and is, therefore, used as an experimental model of bone formation [3]. These processes are regulated by paracrine actions between osteoblasts and chondrocytes and, thus, proper skeletogenesis requires precise control over the differential differentiation of the MSC to these lineages. Failure of chondrocyte or osteoblast precursors to proliferate and differentiate leads to various types of skeletal dysplasias, depending on the pathway involved (reviewed in [4]).

Skeletalogenesis: an overview

The osteochondroprogenitor

The majority of what is known about MSC differentiation along the skeletal lineage comes from embryology. Endochondral and intramembranous skeletogenesis begins with the proliferation and migration of mesenchymal cells to form condensations. These condensations are characterized as tightly packed cells that express specific
condensation markers, and will determine the position, shape and size of the skeletal elements [5]. The transforming growth factor-β (TGFβ) and Hox family of molecules, derived from the epithelium, have been implicated in directing the formation of condensations. These molecules, along with the cell-cell and cell-matrix interactions that result from the compact nature of these cells, are thought to trigger MSC differentiation [5, 6].

As the osteochondroprogenitor is multipotent, lineage fate decisions must be made and maintained. The transcription factor runt-related transcription factor 2 (Runx2) is believed to determine osteoblast versus chondrocyte differentiation. Various studies have shown that upregulation of Runx2 induces osteoblastic differentiation, while its persistent expression in chondrocytes causes premature maturation and mineralization [7]. Runx2 is expressed in prechondrogenic and preosteogenic condensations, but during early embryogenesis, outside signals regulate its expression [5]. Homeobox protein A2 (Hoxa-2) and Wnt (wingless-type MMTV integration site) signaling have both been implicated in this process. Through specific inactivation in the developing limbs of mice, β-catenin, a downstream effector protein of Wnt signaling, was found to be essential to bone development as these embryos had impaired osteoblast differentiation [8]. Also, it was found that if β-catenin is activated, it upregulates Runx2 expression and MSCs differentiate to osteoblasts, rather than chondrocytes [8-10]. These results implicate β-catenin in the determination of osteo- versus chondrogenesis.

*Chondrogenesis*
Chondrocytes are the first skeletal cells to arise, and their differentiation depends on the expression of the transcription factor sry (sex determining region Y)-box 9 (Sox9). The importance of Sox9 was discovered when a genetic mutation in humans was found to be the cause of campomelic dysplasia, characterized by severe cartilage abnormalities [11, 12]. Genetic deletion of Sox9 in mice blocks chondrocyte differentiation at the point of mesenchyme condensation, indicating that it is necessary for the induction of chondrocyte differentiation [13]. In the prechondrogenic mesenchyme, Runx2 is expressed along with Sox9 [14]. However studies show Sox9 to be dominant over Runx2 [15] by promoting Runx2 protein degradation as well as inhibiting its transcriptional activity [16]. Additionally, Nkx3.2 inhibits Runx2 transcription, enforcing the differentiation to chondrocytes [17]. Sox9, along with co-activators Sox5 and Sox6, binds and activates promoters of chondrocyte specific genes. These include collagen 2α1 (Col2α1) and aggrecan [5].

Also important to chondrogenesis is chondrocyte maturation. This involves the transition of chondrocytes from proliferating, to non-proliferating and hypertrophic, and eventually apoptotic [2]. The expression of Sox9, along with the activation of cAMP response element binding protein (CREB) and c-Fos, maintains the chondrocytes in a proliferative state [2]. In order for the chondrocytes to exit the cell cycle and become hypertrophic, Runx2 must be upregulated and Sox9 suppressed. The exit from the cell cycle and apoptosis is necessary for the eventual invasion by osteoblasts and ossification of the bone matrix [2].

Osteoblastogenesis
The transcription factor Runx2 is absolutely necessary for osteoblast differentiation. Runx2 knockout (KO) mice show a total absence of differentiated osteoblasts and, therefore, lack any bone [18, 19]. As Sox9 suppresses Runx2 activity, osteoblast differentiation occurs when Runx2 is upregulated and stabilized. Twist proteins, transcription factors with important roles in embryogenesis, also negatively regulate Runx2, by blocking its DNA binding domain and inhibiting its ability to upregulate osteoblast specific genes [20]. Therefore, the inhibition of Twist proteins, specifically Twist-1 and/or Twist-2, is thought to initiate osteoblastogenesis [20]. Members of the distal-less (Dlx) family of homeobox proteins (Dlx5 and Dlx6) are important for endochondral ossification in the developing appendicular and axial skeleton [21]. Dlx5 has been shown to act directly upstream of Runx2, by binding its gene promoter, [22] and Dlx5/Dlx6 double knockout mice exhibit delayed ossification [23]. Other homeobox transcription factors are msh homeobox 1 and 2 (Msx1/Msx2), though whether their regulation of osteoblast differentiation is positive or negative is still controversial [24]. Msx1/Msx2 may be necessary for the expression of Runx2, as the Msx1/Msx2 double KO fails to express Runx2 [25], although more direct evidence is needed (Schematic 1 summarizes major transcriptional regulators of osteoblastogenesis). Wnt signaling has also been implicated in triggering osteoblast differentiation (reviewed in [26, 27]). There is a β-catenin responsive TCF/Lef binding site on the Runx2 gene promoter, and therefore, may upregulate Runx2 expression [28]. Once upregulated, Runx2 triggers osteoblast differentiation and bone development by binding promoters of osteoblast specific genes, including Osterix, alkaline phosphatase and Osteocalcin. Once
its expression is stabilized, Runx2 inhibits Sox9 transcriptional activity [29], further pushing the MSC to the osteoblast lineage. In addition to regulating osteoblast differentiation, Wnt signaling also regulates osteoblast number and function. Post-natal deletion of β-catenin in Osterix-expressing cells causes osteopenia, however the number of osteoblasts is increased [30]. Therefore a continued investigation of the role of Wnt signaling in osteoblast function, particularly osteoclast regulation, is needed.

In summary, it appears that the differentiation to chondrocytes may occur by default, when Runx2 and osteoblast differentiation are suppressed. It is also possible that signals that upregulate Sox9 are responsible for the suppression of the osteoblast differentiation program. Schematic 2 illustrates these two paths of differentiation.
Schematic 1: Transcriptional regulators of MSC differentiation to skeletal lineages. Osteochondroprogenitors arise from MSCs and express both Runx2 and Sox9. Multiple transcriptional regulators (including Dlx5, Nkx3.2 and Msx1/Msx2) have been found to modify the expression of Runx2 and Sox9 and, therefore, drive differentiation to chondrocytes or osteoblasts.
Schematic 1

MSC

Nkx3.2

Dlx5

Msx1/Msx2

Sox9

Runx2

osteochondroprogenitor

chondrocyte

Sox9

collagen2a1 aggrecan

osteoblast

collagen 1 osteocalcin

Runx2

Osterix
Schematic 2: Osteoblast versus chondrocyte differentiation. Differentiation to the chondrocyte lineage may occur, by default, with suppression of Runx2 and osteoblastogenesis. Conversely, osteoblast differentiation may be actively suppressed by Sox9 and/or its regulators.
Schematic 2

Osteoblast-Chondrocyte Precursor
(expresses Runx2)

- Hoxa-2
- NKX3.2
- SOX9

Runx2

- Wnt Signaling
  - β-catenin
  - activates

Hallmarks of Differentiation as Default
(upon Runx2 downregulation)

Chondrocytes

Osteoblasts

- Osterix
- Stabilizes
- cAMP/PKA

Targeted Differentiation
Cyclic-AMP as a regulator of osteoblastogenesis and chondrogenesis

Though there are master regulators that direct MSC differentiation, various other molecules have been demonstrated to modulate the process. Here, we will focus on those that signal through cyclic-AMP (cAMP). cAMP is a ubiquitous second messenger that is synthesized from ATP by adenylyl cyclases. cAMP levels are regulated by different stimuli, one major effector being G-protein-coupled receptors. These receptors are classified either as stimulatory (Gαs) or inhibitory (Gαi) of adenylyl cyclase. Changing levels of cAMP is translated to the cell through cAMP's action on cyclic nucleotide-gated ion channels, on exchange proteins known as Epacs, and on Protein Kinase A (PKA) (reviewed in [31]). PKA activation further perpetuates the signal by phosphorylating different target proteins. Ultimately, cellular transcription can be modified through the cAMP-dependent transcription factors CREB, CREM and ATF-1 (reviewed in [31-33]).

Cyclic AMP is degraded by phosphodiesterases, which remove a phosphodiester bond and produce AMP. These enzymes play a major role, not only in terminating the signal, but also in regulating the amplitude and duration of the signal [34].

The role of Gαs signaling in osteoblast differentiation was spurred by the finding that individuals with mutations in the Gαs gene have a bone phenotype. Inactivating mutations in the Gαs gene (GNAS) cause Albright hereditary osteodystrophy [35], whereas activating mutations cause fibrous dysplasia of bone [36]. To explore its role in bone development, Hsiao et al., created a mouse expressing an engineered Gαs receptor in osteoblasts and found it to have highly increased bone mass [37]. Conversely, complete knockout of Gαs receptors in osteoblasts reduced the number of osteoblasts and
impaired bone formation [38, 39]. Though interpretation of these types of experiments is limited, they support a potential role for cAMP signaling in MSC differentiation. In accordance, parathyroid hormone (PTH), a hormone critical for bone development and homeostasis, signals through a G-protein-coupled receptor (reviewed in [40]), reinforcing the importance of cAMP signaling in skelatogenesis. The PTH receptor binds PTH, as well as PTH related protein (PTHrP), and is coupled to Gαs as well as Gαq [41]. There is a long history of PTH’s effect on osteoblast differentiation. Studies in PTH receptor KO mice show that the signaling is not required for osteoblast differentiation [42], however PTH has been demonstrated to enhance osteoblast differentiation (reviewed in [43]). Recently, it was found that PTH interacts with canonical Wnt signaling and FGF-2 signaling and that this interaction enhances osteoblast differentiation (reviewed in [44]). Also, PTH signaling was found to interact with bone morphogenetic protein signaling, which additionally enhances osteoblast differentiation [45-48].

The direct effect of cAMP on chondrogenesis has not been fully investigated. In one study, treatment of rabbit chondrocyte cultures with cAMP analogs was found to suppress terminal differentiation and hypertrophy of chondrocytes. Similarly to osteoblasts, PTH is not required for chondrocyte differentiation [42]. However, PTH treatment of C3H10T1/2 cells can enhance the early stages of chondrocyte differentiation while suppressing chondrocyte maturation [49]. Additionally, limb explants from PTHrP KO mice displayed accelerated chondrocyte maturation [50].

cAMP effect on transcriptional regulators of osteoblastogenesis
Studies have shown that increasing intracellular levels of cAMP in cell lines or in primary mouse and human MSCs, through cAMP analogs or forskolin (a direct adenyl cyclase activator), has a positive effect on Runx2 expression [51-54]. In addition, activation of receptors that signal through cAMP increases Runx2 expression in MSC cell lines and in primary rodent MSCs. These include the PTH receptor [55] and the A2B adenosine receptor [54, 56]. This increase in expression may be due to cAMP activation of CREB, as the Runx2 promoter contains a functional CREB domain [57]. Another likely effect of cAMP is its modulation of Runx2 activity. Runx2 has putative PKA phosphorylation sites in its activation domain, and treatment with a PKA-specific cAMP analog increases its phosphorylation [58, 59] and activation of the collagenase-3 promoter [58] and the matrix metalloproteinase-13 promoter [59]. The role that PKA phosphorylation of Runx2 has in vivo during osteoblast differentiation remains to be determined.

As mentioned earlier, Osterix is another transcription factor essential for osteoblast differentiation and bone development. As Runx2 KO mice do not express Osterix [18, 19] but Osterix KO mice do express Runx2, it was deduced that Osterix is downstream of Runx2 [60]. Analysis of the Osterix gene promoter identified a Runx2 binding site, and Runx2 was reported to upregulate Osterix expression [61]. A putative CREB site was also found in this gene promoter, suggesting cAMP may be able to regulate Osterix expression [61]. In support of this, treatment of cells lines or primary mouse MSCs with cAMP analogs increases Osterix expression [51, 54, 62]. However, exposure to relatively high cAMP (1 mM) inhibits its expression in UM-106-01 cell line.
or in mouse primary osteoblasts [63]. Similarly to Runx2 expression, Gαs receptor activation causes an increase in Osterix expression [54], the mechanism of which remains to be demonstrated.

Dlx5, an upregulator of Runx2 [22], is phosphorylated by PKA, which increases Dlx5 protein levels by augmenting its stability. PKA signaling also increases Dlx5 transcriptional activity. Therefore, PKA signaling enhances Dlx5-induced osteoblast differentiation [64].

Of note, some have reported a negative effect of cAMP on osteoblast differentiation. Yang et al., found that the PKA inhibitor, PKI, increased the expression of Runx2 in a human MSC-derived cell line while forskolin, 3-isobutyl-1-methylxantine (IBMX) and a cAMP analog decreased the expression of osteopontin [65]. Koh et al., found forskolin to decrease Osteocalcin expression in MC3T3-E1 cells. Forskolin also decreased the number of mineralized nodules formed by rat primary calvarial cells [66]. Tintut et al., reported that treatment of MC3T3-E1 cells with forskolin decreased the activity of alkaline phosphatase and inhibited mineralization, as well as decreased the expression of alkaline phosphatase, Bone sialoprotein, Osteocalcin and Osteopontin [67]. The discrepancy in reported effects of cAMP on osteoblastogenesis may be due, in part, to the relatively high concentration of forskolin or cAMP analog used (10-100 uM). For instance, Turksen et al., found that treatment with 10 μM of forskolin inhibited osteoblast differentiation while 1 nM increased it [68].

cAMP effect on Sox9 and chondrogenesis
Multiple studies have suggested synergism between cAMP signaling and Sox9 expression. The Sox9 promoter contains a CRE site [69] and, using a Sox9 gene promoter reporter construct in various MSC cell lines, it was reported that binding of this site by CREB increase Sox9 promoter activity [70].

Sox9 interacts with CREB binding protein (CBP) and p300 to increase its transcriptional activity. Using a Col2α1 gene promoter reporter construct in a chondrocyte cell line, as well as a Gal4-Sox9 fusion protein, it was reported that co-transfection with CBP and/or p300 increased Sox9 activity as a transcriptional activator [71].

A yeast two-hybrid screen of Sox9 binding partners identified the PKA catalytic subunit as interacting with Sox9 [72]. This prompted the investigation of PKA-dependent Sox9 phosphorylation and two serine residues were found to be phosphorylated [72]. Phosphorylation of these sites by PKA increased Sox9 activation of the Col2α1 gene promoter [72]. Zhao et al., investigated this finding further by mutating the serine residues. The absence of PKA phosphorylation partially reversed PKA enhancement of Sox9 activity, suggesting that PKA augmented Sox9 activity through multiple mechanisms. The investigators found an interaction between Sox9 and CBP in chondrogenic differentiating C3H10T1/2 cells by using co-immunoprecipitation, and this interaction increased Sox9 transcriptional activity. The importance of this interaction was confirmed by mutating a CREB site in the region of the Sox9 interaction, which reversed the effect [73]. Therefore, cAMP signaling, by means of PKA, enhances Sox9 expression and transcriptional activity through direct phosphorylation and activation of CREB. In
addition to findings in a cell line, differentiation of chick limb bud chondrocytes in the presence of the PKA inhibitor H89 blocked chondrogenesis. The authors found PKA to promote chondrogenesis by down-regulating N-Cadherin through a PKC-dependent pathway [74].

Taken together, it appears that cAMP signaling is capable of upregulating Runx2, a master regulator of osteoblasts, and Sox9, a master regulator of chondrocytes. Since Sox9 is an inhibitor of Runx2 expression, it is possible that the sum effect of cAMP on lineage determination depends on its concentration, cellular localization and a possible regulatory loop.

**Adenosine receptors**

Adenosine is a regulatory metabolite and its receptors are comprised of a family of seven transmembrane domain G protein-coupled receptors. A1 and A3 adenosine receptors (A1AR and A3AR) are coupled to Goi and are, therefore, adenylyl cyclase inhibiting, whereas A2A and A2B adenosine receptors (A2AAR and A2BAR) are coupled to Gos, which stimulates adenylyl cyclase and produces a cAMP signal. The A2BAR may also be coupled to Goq, which activates phospholipase C [75]. A2BAR has a relatively low affinity for adenosine. High extracellular concentrations of adenosine can be achieved during cell injury or stress [76]. A2AAR and A2BARs are widely expressed to varying degrees, with high expression in the vasculature [77]. Our laboratory and others have shown that A2BAR expression is inducible under stress conditions such as inflammation and hypoxia (reviewed in [78]). A2AAR and A2BAR are expressed in
MSCs [56, 79], and interestingly, high expression of the A2AAR and A2BAR receptors can be found in cartilage [80].

**Adenosine receptors and osteoblast and chondrocyte differentiation**

Only recently has the role of adenosine receptors in osteoblast differentiation been examined. In a broad sense, purinergic signaling was looked at in the context of bone, and ATP receptors (P2X and P2Y) were found to promote differentiation and proliferation in an osteoblast cell line (MC3T3-E1) [81]. It was found that after bone injury and when exposed to hypoxic conditions, rat osteoblasts secrete ATP in the high nM to μM range [82]. This extracellular ATP is available for catabolism to adenosine by ectonucleotidases, which are expressed on osteoblasts [79]. In accordance, it was recently demonstrated that genetic ablation of the ectonucleotidase CD73 results in osteopenia and decreased osteoblast differentiation in mice [83].

In vitro studies found adenosine receptors to be expressed in both human [79] and rodent MSCs [56]. Based on its expression and measurements of cAMP levels after agonist treatments of rat MSCs, Gharibi et al., concluded that the A2BAR is the dominant receptor, relative to other adenosine receptors, and that its expression increases during osteoblast differentiation [56]. In human MSCs, A2BAR activation increased osteoblast differentiation, as determined by an increase in alkaline phosphatase activity [79]. Similarly, in rat MSCs, activation of the A2BAR increased Runx2 and alkaline phosphatase expression, as well as the number of mineralized nodules [56]. We found bone marrow-derived MSCs from A2BAR KO mice to have decreased osteoblast differentiating potential, with diminished expression of Runx2 and Osterix. Activation of
the A2BAR with pharmacological agonists increased the expression of these transcription factors, as well as caused an increase in the number of mineralized nodules. Treatment with a cAMP analog also increased the expression of Osterix, suggesting cAMP may be the mechanism of action for A2BAR effect on differentiation. Additionally, the A2BAR KO mouse had mild osteopenia, and a delayed or impaired bone fracture healing response [54].

There have been few reports on the effect of the cAMP inhibitory adenosine receptors, A1AR and A3AR on osteoblast differentiation. Overexpression of the A1AR in an osteoblast precursor cell line led to inhibition of osteoblast differentiation, and instead promoted differentiation to adipocytes [84]. However, treatment of human MSCs with the A1AR agonist, N6-cyclopentyladenosine (CPA), caused an increase in osteoblast differentiation [79]. In rat MSCs, very little A3AR was found [56] and in human MSCs, treatment with the A3AR agonist, 1-Deoxy-1-[6-[(3-iodophenyl)methyl]amino]-9H-purin-9-yl]-N-methyl-β-D-ribofuranuronamide (IB-MECA), had no effect on osteoblast differentiation [79].

A role for adenosine receptors in chondrocyte differentiation has not yet been examined and/or reported. However, as A1 and A2-type ARs are known to be expressed in MSCs [56, 79], and given the published influence of cAMP on Sox9 (as described above), it is likely that a role for these receptors on the differentiation of this lineage will be found.

Inflammation and osteoblast and chondrocyte differentiation: implication for adenosine receptors
Inflammation plays a significant role in bone development (reviewed in [85]) and regeneration (reviewed in [86]). While adenosine receptor signaling through cAMP has the potential to directly influence osteo- or chondrogenesis, cAMP signaling through these receptors can also affect the level of inflammatory cytokines systemically or at the cellular level (reviewed in [87]). Here, we will focus on a brief survey of the effects of inflammatory processes on bone cell differentiation, followed by a summary of A2-type adenosine receptors effects on inflammation and its potential influence on bone cell lineages.

**The effect of inflammation on osteoblast differentiation**

The effect of inflammation on the skeletal system, including osteoblast differentiation, has been well studied, particularly the effects of tumor necrosis factor-α (TNF-α) (reviewed in [88]). Experiments by Gilbert et al., in both fetal calvarial cells and the osteoblast precursor cell line MC3T3-E1 show that TNF-α inhibits osteoblast differentiation, specifically at the early stage of lineage commitment [89]. Further experimentation showed this inhibition to be associated with downregulation of Runx2 transcription and a subsequent decrease in nuclear Runx2 [90]. Though these authors did not find NFkB signaling to be involved, Huang et al., found that inhibition of NFkB signaling by overexpression of IkB in ST2 cells, a mesenchymal stem cell line, abolished the inhibitory effect of TNF-α on Runx2 gene expression [91]. The inhibitory effect of TNF-α on mRNA expression and osteoblast differentiation was confirmed in primary mouse MSCs. Here, the authors also found interleukin-1β (IL-1β) to have a similar effect [92].
These studies on mRNA expression were complimented by Kaneiki et al., who found TNF-α to promote Runx2 protein degradation in C2C12 and 2T3 osteoblast precursor cells by upregulating the E3 ligases Smurf1 and Smurf2 [93]. In order to examine this phenomenon in primary MSCs, Zhao et al., isolated an MSC-enriched fraction from the bone marrow of TNF-α overexpressing mice. In these cells they found upregulation of the E3 ligase Wwp1 relative to cells from WT mice. Further experimentation found Wwp1 to be upregulated by TNFα and responsible for inhibiting osteoblast differentiation. However, in these primary cells, the inhibition was caused by the degradation of JunB, a promoter of osteoblast differentiation, rather than affecting Runx2 directly [94].

In addition to their studies on Runx2, Lu et al., found TNF-α treatment to inhibit Osterix mRNA expression, and claim this to be a direct effect of TNF-α signaling through MEK and inhibition of the Osterix gene promoter [95]. Additionally they found TNF-α-stimulated binding of paired mesoderm homeobox protein 1(Prx1), causing inhibition of Osterix gene promoter activity and transcription in MC3T3 and C3H10T1/2 cells [96]. Interestingly, Prx1 is a developmental regulator of skeletogenesis that was previously thought to be silenced after embryogenesis [96].

Other regulators of osteoblast differentiation are affected by TNF-α. In culture, Msx2 has been shown to inhibit osteoblast differentiation [97]. Treatment of C2C12 or fetal calvarial cells with TNF-α caused increased expression of Msx2 and reduced expression of alkaline phosphatase. This effect was independent of Runx2, as the phenomenon was maintained in Runx2 null cells, and overexpression of dominant
negative IκB showed NFκB signaling to be involved. To model inflammation induced by wear of artificial limbs, macrophages were activated with titanium particles and their media, enriched in inflammatory cytokines, was used to treat MC3T3-E1 cells. Treatment with this conditioned media or TNF-α inhibited Runx2 expression and osteoblast differentiation and this was attributed to NFκB activation and increased expression of sclerostin, an inhibitor of the Wnt pathway [98].

The effect of inflammation on chondrocyte differentiation

Relative to osteoblastogenesis, little is known about the effect of inflammation on chondrocyte differentiation. IL-1 and TNF-α decreased Sox9 mRNA and protein expression in a chondrocytic cell line, MC615, and in primary mouse chondrocytes through the NFκB signaling pathway [99, 100]. However, in these studies differentiation was not directly addressed. To study chondrocyte differentiation, Nakajima et al., differentiated a chondrocyte progenitor cell line, ATDC5, in the presence of IL-6. IL-6 decreased collagen 2α1 and collagen 10 mRNA expression in a dose-dependent manner and inhibited the formation of cartilage nodules [101]. Wehling et al., differentiated human MSCs to chondrocytes in the presence of either IL-1 or TNF-α. They found that both cytokines decreased the size of the cartilage pellet and lowered the amount of glycosaminoglycan accumulated. IL-1 treatment decreased the expression of Col2α1 and aggrecan mRNA [102].

Taken together, it is then quite possible that adenosine receptor modulation of inflammatory cytokines affect directly or indirectly the process of MSC differentiation.
into bone cell lineages. Schematic 3 illustrates a proposed mechanism of A2BAR effects on osteoblast differentiation involving CREB and/or TNF-α.
Schematic 3: Proposed mechanism for A2BAR action on osteoblast differentiation. Activation of the MSC A2BAR triggers cAMP signaling, which may modulate the expression and/or activity of a key osteoblast transcription factor, Runx2, and promote osteoblast differentiation. A2BAR activation also decreases TNF-α levels, which have an inhibitory effect on osteoblast differentiation.
Adenosine receptors and inflammation

One of the principal attributes of adenosine and its receptors is its pro- or anti-inflammatory effects, most of which are mediated via cAMP signaling. Both pro- and anti-inflammatory effects of the A2BAR have been described, and these depend on the cell type and stimulus. Additionally, these effects have been ascribed either protective or deleterious roles, depending on the context, e.g., chronic versus acute pathology (reviewed in [103]). Complete KO of the A2BAR gene results in a slight systemic inflammation, as KO animals have elevated plasma levels of TNF-α at baseline, and elevated levels of TNF-α and IL-6 expression in macrophages [104]. These differences in cytokine levels are exacerbated upon stress or injury. Treatment of A2BAR KO mice with lipopolysaccharide causes an exceptional increase in TNF-α and IL-6 plasma levels and in macrophage expression, relative to WT mice [104].

The ability of the A2BAR to dampen inflammation in response to a stimuli highlights its protective role during stress or injury. The importance of this role is confirmed by findings that the expression of the receptor is also induced by these stimuli. Treatment of vascular smooth muscle cells with TNF-α causes an upregulation of A2BAR expression. Further, this increase was shown to be mediated by NADPH oxidase 4 (Nox4) signaling [105].

As NFκB has an important role in the regulation of cellular inflammation it has been a target of investigation in relation to A2BAR signaling. Recently, we found the A2BAR to directly bind p105, an inhibitor of NFκB, stabilizing it and preventing its
degradation [106]. This mechanism helps explain the inflammatory phenotype in the A2BAR KO mice and the ability of the A2BAR to down-regulate inflammation.

Considering the above described effects of TNF-α, IL-1 and IL-6 on bone cell differentiation, it is possible that some of the A2BAR's recently described protective effect in a mouse bone injury model [54] is related to changes in cytokine levels.

**Adenosine receptors as therapeutics for osteoporosis, skeletal injury and arthritis**

A role for adenosine receptors in osteoporosis is gaining increasing momentum. In mouse bone marrow-derived cells, antagonism of the A1AR with 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) inhibited the differentiation of osteoclasts (bone reabsorbing cells) [107]. Also in these cells, treatment with an A2AAR agonist, 4-[2-[[6-Amino-9-(N-ethyl-β-D-ribofuranuronamidosyl)-9H-purin-2-yl]amino]ethyl]benzene propanoic acid hydrochloride (CGS21680), inhibited the differentiation of osteoclasts, as well as inhibited their activity. Additionally, the A2AAR KO mouse has an increased number of osteoclasts, as well as reduced bone volume [108]. Similarly, A2BAR KO mice display a mild reduction in bone density [54]. In our study, we also found that A2BAR KO mice have changes in bone fracture healing with decreased callus bone formation and an apparent delay in healing [54]. Though the anti-inflammatory effects of adenosine likely have an important role in improving skeletal injury repair, we contend that adenosine, through the A2BAR, may also improve healing by promoting osteoblast differentiation [54]. Therefore, agonism of the A2-type ARs may be useful as a therapeutic for osteoporosis and bone injury.
As caffeine is an antagonist of adenosine receptors, its effects on bone may be relevant here. In epidemiological studies of risk factors for osteoporosis in humans, caffeine was either found to be negatively associated with [109, 110] or not associated with bone mineral density [111-114]. It is possible that effects of caffeine are confounded by factors such as age, estrogen levels and calcium intake. In a study of over 3,000 individuals using the Framingham Cohort, it was found that caffeine intake was associated with a higher relative risk of hip fracture [115]. In experimental animals, caffeine has been shown to inhibit bone formation. When demineralized bone particles were implanted subcutaneously, rats that were treated with caffeine had decreased chondrogenesis and decreased mineralization [116], suggesting that caffeine impairs new endochondral bone formation by inhibiting the proliferation and differentiation of chondroprogenitor cells. In addition, chick osteoblasts treated with caffeine had decreased collagen expression and alkaline phosphatase activity, resulting in reduced matrix formation [117]. Finally, differentiation of osteoclasts from mouse bone marrow-derived cells was enhanced with caffeine treatment [118]. Whether any or all of these negative effects of caffeine on bone formation and maintenance is solely due to antagonism of adenosine receptors has not been determined.

Adenosine receptors have been found to be protective against a variety of injuries, including but not limited to cardiovascular (reviewed in [119]), kidney (reviewed in [120]), lung (reviewed in [121]), and gastrointestinal (reviewed in [122]). As arthritis is an inflammatory disease of the joints, adenosine receptors have been investigated in the context of this disease. Direct infusion of adenosine into the joint in a
rat arthritis model reduced the pathogenesis of the disease [123]. Methotrexate is a drug commonly used for the treatment of rheumatoid arthritis and ameliorates the condition by decreasing the inflammatory response in the joints. At least part of its inflammatory action is attributed to its ability to increase adenosine release in the joints [124]. Currently, the principal receptor to be implicated is the A3AR. An A3AR receptor agonist, CF-101, has been undergoing clinical trials for the treatment of rheumatoid arthritis, however the improvement in rheumatoid arthritis has not yet reached statistical significance [125].

Conclusions

There are a multitude of signaling molecules and pathways that converge during chondrocyte and osteoblast differentiation. The coordination of the spatial and temporal pattern of these molecules is necessary for correct bone formation. Through exploration of the downstream signaling of G-protein-coupled receptors, cAMP was found to be an important component of the signaling pathways needed for full differentiation of MSCs along the skeletal lineage. Continued investigation into the contribution of these receptors, adenosine receptors among them, to bone homeostasis and regeneration could lead to important discoveries with clinically therapeutic implications.
Hypothesis of Thesis Research

Considering the published roles of cAMP on mesenchymal stem cell differentiation, and our finding that the expression of the cAMP-elevating A2BAR is induced following injury, we hypothesized that ablation or activation of the A2BAR impacts the differentiation of osteoblasts and chondrocytes and that this could manifest as changes in endochondral ossification during development of the growth plate and during bone fracture repair.

Specific Aims of Research

1. To examine the role of the A2BAR in bone repair and in the differentiation of bone marrow mesenchymal stem cells to osteoblasts;
2. To examine the impact of A2BAR deletion on skeletal development;
3. To determine how the A2BAR affects the differentiation of mouse embryonic fibroblasts to chondrocytes
CHAPTER II. MATERIALS AND METHODS

Animals and experimental setup

All procedures were performed according to the Guidelines for Care and Use of Laboratory Animals published by the National Institutes of Health. Throughout these studies, all animals received humane care that was in agreement with the guidelines of and approved by the Institutional Animal Care and Use Committee of the Boston University School of Medicine. A2BAR KO mice were originally generated and bred onto C57BL/6J (confirmed by Bax Max) in our laboratory [104]. Age- and sex-matched C57BL/6J wild type (WT) mice were used as controls. All bone fracture studies were performed on adult males between the ages of 15-18 weeks old and all bone marrow for in vitro studies was isolated from 6-7 week old mice. Age and sex selection were based on previous data and the time course of fracture healing had been established in this fracture model by both histological and selected candidate gene profiling [126, 127]. Growth plate analysis was performed on femurs from WT (generated in-house) and A2BAR KO male mice at exactly 2, 3 and 4 weeks of age.

Quantitative micro-computed tomography (micro-CT)

Bone analysis post-fracture

Fractured femurs were collected, fixed in 4% formaldehyde for 5 days at 4°C, and then stored in phosphate buffered saline (PBS) at 4°C. Scans were performed using a Scanco micro-CT 40 system (Scanco Medical, Basserdorf, Switzerland) located in the Orthopaedic and Developmental Biomechanics Laboratory at Boston University. Scans were performed using a 12-micron voxel size resolution with 200 ms integration time,
under conditions of 55 E(KVp) and 145 I(μA). Reference lines were manually adjusted on each individual bone to include the entire callus area. Transverse images scanned by the micro-CT were then traced manually with a computer program and stacked to render a 3-D image of the callus.

Subsequent analysis of the scans was carried out using the software program supplied by the manufacturer of the micro-CT instrument. Measurements of callus density (volume fraction and mineral density) were made directly from the micro-CT image data of each specimen. For these measurements, the callus (including the portion of the callus in the medullary canal) was first isolated using a semi-automated segmentation procedure that excludes the original cortex. A global threshold algorithm was then used which applies a fixed, constant threshold to all specimens. A constrained 3-D Gaussian filter (filter width = 0.8, filter support = 1 voxel) was used to partially suppress image noise. The total callus volume, length, mineralized volume fraction, and mineral density were then calculated using standard algorithms provided by the system manufacturer. The mineral density was calculated with the aid of a standard curve obtained from weekly scans of a set of hydroxyapatite phantoms of five different mineral densities. The mediolateral and anteroposterior callus diameters are calculated as the maximum width of the callus in each of the frontal and sagittal planes along the length of the callus.

**Bone analysis at baseline**

Cortical and trabecular bone of WT (bred in-house) and A2BAR KO mice were analyzed at 4, 8 and 15 weeks of age. Bones were fixed in 4% formaldehyde for 5 days at 4°C, and then stored in PBS at 4°C. Scans were performed using a 12-micron voxel size resolution.
with 200 ms integration time, under conditions of 55 E(KVp) and 145 I(μA). For cortical bone analysis, the mid-diaphyseal region was found by measuring the total length of the bone and dividing in half. From that mid-point, 27 slices (at 12 μm/slice) were taken above and below, for a total of 54 slices and a scanned region of 0.648 mm. The cortical bone was isolated using a semi-automated segmentation protocol with a fixed threshold of 263. The total bone volume, cortical bone volume, cortical thickness, average mineral density and minimum and maximum cross-sectional area were calculated using standard algorithms provided by the system manufacturer. For trabecular bone analysis the metaphysis was identified by starting the scans 4.162 mm from the distal end of the bone and taking 134 slices (at 12 μm per slice) distally for a totaled scanned region of 1.6 mm. The trabecular bone was isolated using a semi-automated segmentation protocol with a fixed threshold of 263. The total volume, bone volume, trabecular number, trabecular thickness and trabecular spacing were calculated using standard algorithms provided by the system manufacturer.

**Cartilage analysis**

Femurs from WT (bred in-house) and A2BAR KO mice were collected at 2 and 3 weeks of age, wrapped in PBS-soaked gauze and frozen -20°C. Bones were further processed by the lab of Dr. Mark Grinstaff at Boston University’s Department of Biomedical Engineering as described previously [128]. Briefly, the distal femur was scanned by micro-CT, incubated for 1 hour at room temperature in CA4+ contrast agent and re-scanned. Baseline and stained images were subtracted to yield total cartilage.

**The mouse femoral fracture model**
A closed mid-diaphyseal fracture of the left femur was generated by controlled blunt trauma using a scaled-down modification of the apparatus described by Bonnarens and Einhorn [129]. Mice were anesthetized via isoflurane and a medial parapatellar skin incision was made in the right leg, followed by a medial parapatellar incision into the quadriceps to the patellar ligament insertion, preserving the patellar ligament intact. An intramedullary pin entry point was created in the femur using a 25G needle. Then, an intramedullary pin (the guide pin of a 25G spinal needle (Becton Dickson, cat. #405181). The pin was cut and the patellar ligament was returned to the original position. The skin was closed with 5.0 nylon suture (Ethicon, cat. # 698G). A subcutaneous dose of 0.5mg/kg buprenorphine hydrochloride (American Regent, Inc. NCD 0517-0725-05) and 0.1mg/kg penicillin (Butler, cat. #035001) was given to each mouse. For enrollment in our studies, the location and quality of fractures were assessed immediately after the fractures had been generated, while animals were still anesthetized, by X-ray analysis. A fracture whose configuration was inconsistent with our standardized placement criteria was not used in our studies. Mice were monitored and checked daily for well-being.

**Specimen harvesting and histology**

Femurs were fixed in 4% formaldehyde (1-5 days depending on age of bone) at 4°C, then decalcified in 14% w/v EDTA dissolved in water for 5 days at 4°C, then sent to Boston University’s Experimental Pathology Laboratory Services Core (Cheryl Spencer http://www.bume.bu.edu/eplsc/) for embedding in paraffin and sectioning (5 um sections).

*Safranin O and Fast Green*
Slides were deparaffinized and rehydrated as follows: xylene for 10 minutes, repeated 3x, 100% ethanol for 10 minutes, 90% ethanol for 5 minutes, 75% ethanol for 5 minutes and water for 5 minutes. The slides were then stained with Fast Green FCF (Sigma, cat. #F7258) for 8 minutes at room temperature to visualize bone, with Safranin O for 10 minutes at room temperature to visualize cartilage (Sigma, cat. #S8884), and counterstained with hematoxylin for 1 minute (Fisher Healthcare, cat. #22-220-109). Slides were dehydrated and coversliped with Cytoseal 60 mounting media (Fisher Scientific, cat. #NC9527348). Slides were visualized and images were taken using a Nikon Eclipse 50i light microscope and a Spot Insight 2 Mega-pixel color camera with Spot version 5.0 imaging software (Diagnostic Instruments Inc.).

Tartrate resistant acid phosphatase (TRAP) staining

Bone sections were deparaffinized, rehydrated (as above) and stained for TRAP positive cells using a Leukocyte Acid Phosphatase Kit (Sigma, cat. #386A) according to the manufacturers instructions. Slides were then dehydrated and coversliped with Cytoseal mounting media.

Growth plate analysis

Growth plate height was measured by analyzing sections of WT and A2BAR KO distal femurs stained with Fast Green and Safranin O. The total height of the growth plate at the midline of the bone was measured using NIH software ImageJ (version 1.62; http://rsb.info.nih.gov/ij/) and heights were averaged for 4-8 sections per bone.

Immunohistochemistry
Immunohistochemistry for Aggrecan was performed on paraffin sections that were deparaffinized and rehydrated (as above). Antigen retrieval was performed by treating the sections with a chondroitinase (Sigma, cat. #C2905) solution (0.5 U/ml in 50 mM Tris, pH 8.0, 60 mM sodium acetate and 0.02% BSA) at 37°C for 30 minutes. Endogenous peroxidase activity was eliminated by treating sections with 3% H₂O₂ in methanol at room temperature for 10 minutes. Sections were blocked in 10% goat serum for 1 hour at room temperature. Rabbit anti-mouse Aggrecan antibody (Millipore, cat. #AB1031) was added at a 1:50 dilution overnight at 4°C. Goat anti- rabbit HRP secondary antibody (Santa Cruz Biotechnology, cat. #SC-2004) was added at a 1:200 dilution at room temperature for 1 hour. Staining was visualized using DAB Substrate Kit for Peroxidase (Vector Laboratories, cat. #SK-4100) according to the manufacturer’s protocol.

Immunohistochemistry for Collagen type II α1 (Col2α1) was performed as for Aggrecan with the following changes: antigen retrieval was performed by treating sections with a pepsin (Sigma, cat. #P6887) solution (1 mg/ml in 50mM Tris-HCl, pH 2.0) for 10 minutes at 37°C. Sections were treated with mouse anti- chicken Col2α1 antibody (Millipore, cat. # MAB8887) at a 1:50 dilution overnight at 4°C. Goat anti- mouse secondary antibody (Santa Cruz Biotechnology, cat. #SC-2005) was added at a 1:200 dilution at room temperature for 1 hour. Slides were visualized and images were taken using a Nikon Eclipse 50i light microscope and a Spot Insight 2 Mega-pixel color camera with Spot version 5.0 imaging software (Diagnostic Instruments Inc.).

**Whole skeleton preparation and staining**
Mice were sacrificed at 1 day of age by isoflurane followed by decapitation and were prepared and stained according to a protocol from the lab of Andrew McMahon. Animals were skinned and eviscerated and skeletons were fixed in 100% ethanol for 24 hours at room temperature, on a rocker. Skeletons were transferred to 100% acetone for 24 hours at room temperature, on a rocker. Skeletons were stained with a combination of 1 volume of 0.3% alcian blue (in 70% ethanol), 1 volume of 0.1% alizarin red (in 95% ethanol), 1 volume of glacial acetic acid and 17 volumes of 70% ethanol. Skeletons were stained for 4 days at 37°C, on a rocker. Excess stain was removed by rinsing skeletons in water, followed by incubation in 1% potassium hydroxide [66] for 3 hours at room temperature, followed by incubation in fresh 1% potassium hydroxide overnight at room temperature, on a rocker. Skeletons were then taken through washes, composed of 20% glycerol/1% KOH for 24 hours, 50% glycerol/1% KOH for 24 hours and finally stored in 80% glycerol.

Cells and treatments

Isolation, culture and osteoblast differentiation of bone marrow-derived mesenchymal stem cells (MSCs)

Mice were euthanized by isoflurane administration, followed by cervical dislocation. Femurs and tibiae of mice were dissected and soft tissue removed. Using scissors, the ends of each bone were cut enough to reveal the marrow cavity. A 23G needle was inserted into the marrow cavity and a 10 ml syringe was used to flush the bones with growth media (αMEM supplemented with 10% mesenchymal stem cell qualified fetal bovine serum (MSC-FBS; Invitrogen, cat. #12662-029) and 1% penicillin/streptomycin).
Cells were plated at 6x10^6 cells/ml in growth media and were grown at 37°C with 5% CO₂. Cells were left undisturbed until day 4 of culture when half of the media was removed and replaced. On day 6 of culture all media was removed and cells were treated with osteo-inductive reagents (10 nM dexamethasone, 0.04 μM L-ascorbic acid and 8 mM β-glycerophosphate, disodium salt), as described by Edgar et al., [130]. Thereafter, media was changed every two days and osteo-inductive reagents added.

*Generation and culture of primary osteoclasts*

Bone marrow was isolated from mice as described above. Cells were plated at 6x10^6 cells per ml (day 0). The following day (day 1) non-adherent cells were collected and washed 1x with PBS. Cells were re-plated at 4x10^6 cells per ml in media containing 30 ng/ml recombinant mouse monocyte-colony stimulating factor (M-CSF, R and D Systems, cat. #416-ML). On day 3, media was replaced with media containing 30 ng/ml M-CSF and 30 ng/ml recombinant mouse receptor activator of nuclear factor κ-B ligand (RANKL, R and D Systems, cat. #462-TR). On days 5 and 7, 50% of media was replaced and M-CSF and RANKL was added to a final concentration of 30 ng/ml each. On day 8, cells were fixed in 0.5% glutaraldehyde and stained for osteoclast marker, using TRAP staining as described above. Osteoclasts were additionally identified based on morphology (large size and multiple nuclei).

*C3H10T1/2 cell culture and differentiation*

C3H10T1/2 cells were obtained as a gift from Dr. Stephen Farmer, Boston University School of Medicine, Department of Biochemistry. Cells were originally obtained from ATCC (cat. # CCL-226). Cells were grown in αMEM with 5% FBS (Invitrogen, cat.
and 1% penicillin/streptomycin. Chondrocyte differentiation was induced as in Denker et al., [131]. $10^5$ cells were plated in a 10 μl drop and allowed 1 hour to attach. After the incubation, chondro-induction medium was added (αMEM with 10% FBS, 1% penicillin/streptomycin and 100 nM recombinant human bone morphogenetic protein-2 (BMP-2, Sigma, cat. #H4791). Chondro-induction medium was replaced every other day. Cultures were maintained for up to 6 days.

**Generation of mouse embryonic fibroblasts (MEFs)**

Embryos were collected at age e13. Embryos were removed from the uterus and placed in PBS on ice. Embryos were cleaned of extra-embryonic tissue and the head and fetal livers were removed. Each embryo was minced and placed in 1 ml of 0.25% Trypsin-EDTA at 37°C for 15 minutes. The solution was pipetted up and down during the incubation to dissociate aggregates. 2 ml of MEF growth medium (DMEM with 10% FBS and 1% penicillin/streptomycin) was added and pipetted up and down to disperse cells. Cells were centrifuged for 5 minutes at 470 x g and the cell pellet was resuspended in growth media at $2 \times 10^5$ cells per ml. Cells were frozen as stocks and thawed as follows: as follows: $2 \times 10^6$ cells were resuspended in 1 ml of cryopreservation medium (10% DMSO and 40% FBS in growth medium) and vials were placed in a cryopreservation container and placed at -80°C. After 24 hours, vials were moved to liquid nitrogen. To thaw, the frozen vial was placed in a 37°C water bath until contents began to thaw. Then, 1 ml of growth media was added to the vial and the entire contents transferred to 9 ml of growth media. The cells were centrifuged at 270 x g at 4°C for 5 minutes, the supernatant
was removed and the cell pellet was resuspended and cells were plated at $2 \times 10^5$ cells/ml.

*Mouse embryonic fibroblast culture and differentiation into chondrocytes*

WT C57BL/6 MEFs were purchased from GlobalStem (cat. #GSC-6002) and cultured in DMEM with 10% MSC-FBS and 1% penicillin/streptomycin. Chondrocyte differentiation was performed as described by Saeed et al., [132]. $1 \times 10^5$ cells were plated (24-well plate) in a 10 µl drop and allowed 1 hour to attach. After the incubation, chondro-induction medium was added (1:1 DMEM:F12 with 10% MSC-FBS, 1% penicillin/streptomycin, 100 nM BMP-2, 10 mM β-glycerophosphate, disodium salt and 250 mM L-ascorbic acid). Chondro-induction medium was replaced every other day.

*Assessment of ex vivo mineralization*

To assess mineralized nodules culture plates were washed three times with PBS, and then fixed with 10% formaldehyde at room temperature for 10 minutes. Following fixation, cells were washed with deionized water and cell layers were covered with alizarin red solution (Millipore, cat. #ECM815) and incubated at room temperature for 30 minutes. Following staining, cell layers were rinsed with deionized water until washes ran clear. Digital photographs of stained cell layers were then taken with a digital camera from a fixed distance on a light box for illumination. The stained area per well was quantified using a computerized imaging system, Image-Pro Plus 4.1 (Media Cybernetics). For mineral analysis, mouse bone marrow cultures were grown in osteo-inductive media for 6 days. Cultures were washed in PBS then lyophilized and shipped to the Musculoskeletal Repair and Regeneration Core Center at the Hospital for Special Surgery, NY for analysis. Freshly dried ($120^\circ$C, 24 h) KBr (200 mg) was mixed with the samples and
pellets were made for spectroscopic analysis. For Fourier Transformed Infrared Spectroscopy (FT-IR), samples from tissue culture wells were pooled. Specimens were scanned in transmittance on a Nicolet 4700 FT-IR spectrometer. Spectra were collected from 400-2000 cm⁻¹. Spectra were first baselined and the mineral to matrix ratio was determined as the area under the phosphate peak (900–1,200 cm⁻¹) divided by the area under the amide I peak (1,585–1,720 cm⁻¹).

**Assessment of in vitro cartilage accumulation**

Cell cultures were washed with PBS and then fixed in 4% glutaraldehyde for 15 minutes at room temperature. Cultures were rinsed 2x with 0.1N HCl and stained with alcian blue solution (1% alcian blue in 0.1N HCl) at room temperature for 30 minutes. Cultures were rinsed with 0.1N HCl until washes were clear. Cultures were photographed at 1x magnification and images were analyzed for mean intensity of stain using NIH software ImageJ (version 1.62; http://rsb.info.nih.gov/ij/). A standard minimal threshold was determined using negative control cultures stained with alcian blue.

**Pharmacological treatments**

*Treatment concurrent with differentiation*

Immediately after chondro-inductive media was added, cells were treated with 1 μM Bay 60-6583 (provided to Dr. K. Ravid by Bayer HealthCare AG, Wuppertal, Germany), 1 μM Forskolin (Sigma, cat. # F6886) or vehicle (DMSO). Cells were subsequently treated at the first media change (day 2 of culture).

*Pharmacological treatment and cAMP measurement*
Cells were treated with 1 U/ml adenosine deaminase (Roche, cat. #11936921) 20 minutes prior to agonist treatment. Cells were treated with 20 μM Papavarine hydrochloride (a phosphodiesterase inhibitor) (Sigma cat. # P3510) for 10 minutes prior to agonist treatment. Cells were treated with 1 μM Bay 60-6583, 10 μM 5’-N-ethylcarboxamido adenosine (NECA; Sigma, cat. # E2387), 100 μM 8-bromo-cAMP (Tocris Bioscience, cat. #1140), 1 μM Forskolin or vehicle (DMSO) for 10 minutes. Cells were washed 3 times with ice cold PBS then lysed by adding 120 μl of cold 0.1N HCl and scrapping. For normalization, an aliquot of the lysate was assayed for total protein using the BioRad Protein Assay Kit (Bio-Rad Laboratories, cat. #500-0001). To measure cAMP, the lysates were first methylated and analyzed using a Direct cAMP ELISA Kit (Enzo Life Sciences, cat. #ADI-900-066) according to the manufacturer’s instructions.

Quantitative reverse transcription-based polymerase chain reaction (RT-PCR)

Fractured calluses were collected on days 3, 7, 14 and 21 post-injury and were cut at a distance of 5 mm on either side of the fracture. For baseline measurements (day 0), femurs of WT and A2BAR KO mice were collected and the mid-diaphyseal region was dissected out. For other bone analyses, the entire bone was collected. All bone samples were snap-frozen in liquid nitrogen and stored at -80°C until they were used for RNA isolation.

For RNA isolation, bones were pulverized in liquid nitrogen using a mortar and pestle and added to 600 μl of RLT buffer supplied in the RNeasy Mini Kit (Qiagen, cat. #74134). This sample was homogenized 2x for 15 seconds each, using a Fisher Scientific Power Gen 1000 S1 homogenizer, then placed on ice. Lysates were placed on a rocker at
4°C for 30 minutes, and then centrifuged for 10 minutes at 16,000 x g at 4°C. Supernatant was collected and used immediately for reverse transcription.

For cell culture, Buffer RLT was added and the cells were scraped. Lysate was collected and stored at -80°C. Lysates were homogenized using Qiashredder columns (Qiagen, cat. #79654) and RNA was isolated with the RNeasy Mini Kit (Qiagen, cat. #74134).

RNA was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, cat. #4368814) according to the manufacturer’s protocol. Gene expression was measured using TaqMan Gene Expression Master Mix (Applied Biosystems, cat. #4370048) on an Applied Biosystems 7700 Sequence Detector. The following Applied Biosystems Taqman Gene Expression Assays (primer/probe sets) were used; 18s rRNA (cat. #4319413E), β-actin (Mm00607939_s1), Runx2 (Mm00501580_m1), Osterix (Mm00504574_m1), Col2α1 (Mm01309565_m1), Aggrecan (Mm00545794_m1), GAPDH (cat. #4352339E), Sox9 Mm00448840_m1), and C/ebp-β (Mm00843434_s1).

**Western blot analysis**

Femurs were snap-frozen in liquid nitrogen at the time of collection and stored at -80°C. On the day of Western blot analysis, bones were pulverized in liquid nitrogen using a mortar and pestle and added to 600 μl of RIPA buffer (1% NP-40, 0.5% sodium cholate, 0.1% SDS) supplemented with 1X proteinase inhibitor (Roche, cat. #11697498001) and 1X phosphatase inhibitor (Roche, cat. #04906837001). This was homogenized 2x for 15 seconds each using a Fisher Scientific Power Gen 1000 S1 homogenizer, then placed on ice. Lysates were placed on a rocker at 4°C for 30 minutes, and then centrifuged for 10
minutes at 16,000 x g at 4°C. Supernatant was collected and total protein concentration was measured using the BioRad Protein Assay Kit (BioRad Laboratories, cat. #500-0001). After normalizing protein concentration between samples, protein lysate was reduced with 4X SDS loading buffer and resolved by 8%-SDS (Aggrecan; molecular weight greater than 250 kD) or 10%-SDS (Sox9; molecular weight of 65 kD) gels. Proteins were transferred overnight at 34 V at 4°C on 0.2 μm PVDF membrane (BioRad Laboratories, cat. #162-0177) with transfer buffer (25 mM Tris and 192 mM glycine) supplemented with 10% methanol and 0.1% SDS (for Aggrecan) or 20% methanol (for Sox9). After transfer, membranes were blocked with 10% milk in 1X PBST (1X PBS and 0.05% Tween) for 1 hour at room temperature and blotted with primary antibody as follows: anti- Aggrecan (Millipore, cat. #AB1031) at 1:1,000 dilution for 1 hour at room temperature, anti- Sox9 (Santa Cruz Biotechnology, cat. #SC-20095) at 1:1,000 dilution for 1 hour at room temperature, anti- β-actin (Sigma, cat. #A5441) at 1:5,000 dilution for 1 hour at room temperature. Membranes were washed and incubated with the appropriate secondary antibodies: goat anti- rabbit (SantaCruz Biotechnology, cat. #SC-2004) or goat anti- mouse (SantaCruz Biotechnology, cat. #SC-2005) at dilutions of 1:10,000, except for β-actin (1:100,000) for 1 hour at room temperature. Proteins were visualized with Immobilon Western Chemiluminescent horseradish peroxidase (HRP) Substrate (Millipore, cat. #WBKLS0500).

Transfection of C3H10T1/2 cells with a RUNX2-responsive promoter

The following plasmids were obtained as a gift from Dr. Xaralabos Varelas, Department of Biochemistry, Boston University School of Medicine: p6OSE2-luc, pCMV5-β-gal and
flag-Runx2, all of which confer ampicillin resistance. Optimal transfection efficiency of C3H10T1/2 cells was found with transfection of 0.5-1.0 μg of DNA with 1 μl of Lipofectamine 2000 transfection reagent (Invitrogen, cat. #11668) per well of a 24-well plate. Optimal transfection was assessed by the percentage of β-galactosidase positive cells upon transfection with pCMV5-β-gal (yielding approximately 40% positive cells). For concurrent transfection and chondrocyte differentiation, C3H10T1/2 cells were grown in 10 cm plates until 95% confluent. Cells were transfected with p6OSE2-luc reporter and pCMV5-β-gal reporter (2:1 ratio) and 1 μl Lipofectamine 2000 per 1 μg of DNA was used. After 24 hours, cells were trypsinized, replated in micromass cultures (10⁵ cells/10 μl) and differentiated to chondrocytes (as described above). 48 hours post-chondro-induction, cell lysates were collected using the Luciferase Assay System with Reporter Lysis Buffer (Promega, cat. #E4030) and stored at -80°C for subsequent analysis.

**Statistical analysis**

Data are presented as means ± standard deviation. Data were analyzed using Student’s T-test at a level of significance of p<0.05.

**Generation of the CD68-A2BAR transgenic mouse**

The CD68-A2BAR transgenic mouse, created by Drs. Milka Koupenova and Alexia Eliades, were genotyped and bred under my active participation and guidance. The mice were created as follows: Starting with an SM22α-hA2BAR-β-globin construct (created by Dr. Zhao in our laboratory), the SM22α promoter was excised with NotI (New England Biolabs, cat. #R0189S) and HindIII (New England Biolabs, cat. #R0104S). The
human CD68 promoter was obtained from a pcDNA-CD68-intron-A2AAR vector (gift from Dr. Chen, BUSM) by excising the promoter and intron region with ClaI (New England Biolabs, cat. #R0197S) and BsrGI (New England Biolabs, cat. # R0575S). These two regions were ligated and the final construct was verified with Stul (the correct insert: 2 fragments- 0.7 kb and 8.5 kb; no insert: linearizes the vector- 8.2 kb). The correct orientation was verified with SalI and NcoI (correct orientation: 2.6 kb and 6.6 kb). The final construct was confirmed by DNA sequencing.

The CD68-A2BAR construct was purified via electroporation and microinjected into the pronuclei of fertilized A2BAR-ApoE double KO mouse oocytes to produce transgenic mice on an A2BAR-ApoE double KO background. Transgenic founders were generated by the Boston University Transgenic Core Facility. Potential founders were screened for the CD68 promoter-hA2BAR sequence by tail DNA isolation and PCR as described below.

**DNA isolation and genotyping by PCR**

Mouse tails were cut (approximately 0.1 cm) and digested in 300 µl of digestion buffer (100 mM NaCL, 10 mM Tris pH 7.6, 25 mM EDTA pH 8.0, 0.5% SDS) and 6 µl of 10 mg/ml Proteinase K (American Bioanalytical, cat. #AB00925) at 55°C overnight. Samples were centrifuged at 16000 x g at 4°C for 10 minutes and supernatant was collected. To the supernatant, 100 µl of Protein Precipitation Solution (Qiagen, cat. #10455697) was added and left on ice for 5 minutes. Samples were centrifuged at 1600 x g at 4°C for 10 minutes and the supernatant was collected. To the supernatant, 300 µl of 100% isopropanol was added and samples were gently mixed. The samples were
centrifuged at 16000 x g at 4°C for 10 minutes, the supernatant was discarded and the pellet was washed with 300 μl of 70% ethanol. After centrifugation (16,000 x g at 4°C for 10 minutes) the supernatant was discarded and the pellet air-dried. The DNA was resuspended in 30 μl of H₂O and the DNA concentration was measured by spectrophotometry.

To screen for CD68-A2BAR, primers were designed to span the hA2B-β-globin interface. For PCR, 100 ng of DNA was added to 12.5 μl of GoTaq Green Master Mix (Promega, cat. #M7123), 9.5 μl H₂O, 1 μl of 1 μM sense primer 5'-CCACAAGAAACAAAGAGGACACG-3' and 1 μl of 1 μM anti-sense primer 5'-GGGGAAAGAAAACATCAAGGGTC-3' for 45 cycles at 57°C to generate a fragment of 613 nucleotides, as detected by electrophoresis on a 1.5% agarose gel. A2BAR KO was similarly confirmed with the sense primer 5'-GGCACCTCTCCCTC-3' and the anti-sense primer 5'-CAGCCTCTGTCCACATACACT-3' and ApoE KO was confirmed with the primers 5'-GCCTAGCCGAGGGAGAGCCG-3' , 5'-TGTGACTTGGAGCTCTGCAGC-3' and 5'-GCCGCCCCGACTGACATCT-3'.

Two founder lines (denoted #2 and #3) were identified and bred to homozygocity and heterozygocity respectively. Homozygotes and heterozygotes were identified by qPCR genotyping as described below. Transgenic mice were then crossed with A2BAR KO mice to regain the WT ApoE gene.

**DNA isolation and genotyping by qPCR**

Mouse tails were cut (approximately 0.1 cm) and DNA isolated with the DNeasy Blood and Tissue Kit (Qiagen, cat. #69504). DNA concentration was measured by
spectrophotometry. For the qPCR reaction, 1 μl (approximately 100 ng) of DNA was added to 10 μl TaqMan Gene Expression Master Mix (Applied Biosystems, ca. t#4369016), 8 μl of H₂O and 1 μl of human A2BAR primer/probe Gene Expression Assay (Applied Biosystems, Hs00386497_m1) or genomic A2BAR primer/probe Gene Expression Assay (Applied Biosystems, Mm01285229_s1) as an endogenous control, as the primers/probe are within exon 2 of the mouse A2BAR gene. Data were analyzed using the ΔΔCT method. Genotype was determined by comparing CT values with those of known heterozygotes.
CHAPTER III: RESULTS

Aim 1: The role of the A2BAR in bone repair and in the differentiation of bone marrow mesenchymal stem cells to osteoblasts


MSC differentiation to osteoblasts is impaired in A2BAR KO mice

MSCs were prepared from the bone marrow of A2BAR KO mice and age-, sex- and strain-matched wild type (WT) mice. They were then induced to undergo osteogenic differentiation by growing the cultures in standard osteo-inductive media (see methods). Cultures from WT and A2BAR KO mice showed no differences in total protein content at the time of osteo-induction or at subsequent times days when they were analyzed (Fig. 1). At 9 and 12 days after osteo-induction, bone marrow from A2BAR KO mice showed fewer mineralized nodules, demonstrating a reduction in osteoblast differentiation in the absence of the A2BAR (Fig. 2A). Using FT-IR spectroscopic analysis (see methods), we validated that the accumulated mineral in both the WT and A2BAR KO cultures had the typical characteristics of poorly crystalline hydroxyapatite that is seen in newly formed bone tissues (Fig. 3).

We next examined the expression of the two transcription factors that are central to osteoblast differentiation, Runx2 and Osterix. As anticipated in WT cultures, osteo-
induction significantly increases the expression of Runx2 and Osterix after 24 hours (Fig. 4). Prior to osteo-induction, cells cultured from A2BAR KO mice have a trend towards reduced Runx2 expression and significantly reduced Osterix expression. With osteo-induction, the increase in the expression of both Runx2 and Osterix was significantly decreased in the A2BAR KO bone marrow cultures (Fig. 2B).
Figure 1: Bone marrow cells from WT and A2bAR KO mice have similar growth *in vitro*. Cells from WT and A2BAR KO bone marrow cultures were lysed just prior to osteo-induction (d0), 24 hours (d1) and 5 days post-osteo-induction and total protein was measured by Bradford Assay. A t test was performed. N=3, *p>0.05*
Figure 1

- wT - (L1 0.05)

- A2BAR KO

- Total protein (mg)

- Day 0 cell number (x10^6)

- WT

- A2BAR KO
Figure 2: Genetic ablation of the A2BAR leads to impaired osteoblast differentiation. A. Bone marrow cells were collected from WT and A2BAR KO mice and cultured in osteo-inductive medium (see methods). Cultures were stained with alizarin red 9 and 12 days (d9, d12) after osteo-induction to visualize mineralized nodules. Nodules were counted using ImagePro software. At each time point, A2BAR KO samples were compared to WT, set at 100%, N=4, *p<0.01. B. Measurement of osteoblast differentiation markers, Runx2 and Osterix, before adding the osteo-inductive reagents (d0) or 24 hours post-treatment (d1). Expression was measured by qRT-PCR and normalized to 18s rRNA. Data is presented relative to WT d0, and a t test was applied to each WT and A2BAR KO pair at each time point. N=5, *p<0.05.
Figure 2

A

WT

A2BAR KO

d9  d12

Change in nodule number (%)

B

WT

A2BAR KO

d0  d1

Runx2

Osterlix

Relative expression

*
Figure 3. Bone marrow MSC-derived osteoblasts from WT and A2BAR KO mice produce hydroxyapatite in vitro. Representative Fourier Transformed Infrared (FT-IR) spectra of cultures 10 days post-osteo-induction. Both WT and A2BAR KO cultures showed similar peaks in the mineral region.
Figure 4. Osteoblast transcription factors are upregulated upon in vitro osteo-induction. Runx2 and Osterix expression was measured by qRT-PCR prior to (d0) and 24 hours post (d1)-addition of osteo-inductive reagents. Expression was normalized to 18s rRNA. A t test was performed for each gene to show a significant increase in expression on d1. N=5, *p<0.05.
Figure 4

The figure shows a bar chart comparing the relative expression of Runx2 and Osterix under different conditions (d0 and d1). The expression levels are indicated by the height of the bars, with error bars showing the variability. Significant differences are indicated by asterisks (*) above the bars.

- **Runx2**:
  - d0: Low expression
  - d1: Increased expression

- **Osterix**:
  - d0: Low expression
  - d1: High expression

The chart suggests that Osterix expression increases significantly more than Runx2 under the d1 condition.
A2BAR activation increases osteoblast differentiation

As ablation of the A2BAR impairs osteoblast differentiation, we next tested whether activation of the A2BAR could increase differentiation. We treated bone marrow from WT and A2BAR KO mice with NECA, an A2-type receptor agonist, or vehicle, concurrently with osteo-induction. After 10 minutes of treatment, NECA significantly elevated cAMP levels in the WT cells, relative to treatment with vehicle (Fig 5A). NECA had no significant effect on cAMP levels in the A2BAR KO cells, indicating that there is little contribution of the A2AAR to the overall cellular levels of cAMP, consistent with the relatively low expression of the A2AAR in MSCs [56, 133]. To test the effect of A2BAR activation on nodule formation, NECA or vehicle was added at the time of osteo-induction (d0) and at the second media change (d2). Nodules were counted on day 6 of osteo-induction. We found that treatment with NECA caused a significant increase in the number of mineralized nodules in the WT bone marrow in contrast to the A2BAR KO cells. Though treatment of A2BAR KO cells with NECA showed a small and non-significant increase in the number of nodules (Fig. 5B) this is likely due to activation of other adenosine receptors after long-term exposure to the ligand. In accordance, NECA treatment of WT cells caused a significant increase in the expression of Runx2 and Osterix as compared to treatment with vehicle (Fig. 5C). There was no effect of NECA on Runx2 or Osterix expression in the A2BAR KO cultures (Fig. 5C), indicating that the influence of NECA on gene transcription is due primarily to activation of the A2BAR receptor. Since the A2BAR can signal through activation of adenylyl cyclase and the upregulation of cAMP, we predicted that cAMP is at least one of the signals responsible
for upregulation of Runx2 and Osterix. Treatment of WT cultures with the cAMP analog, 8-br-cAMP, highly increased intracellular cAMP levels (8 fold over vehicle treated; data not shown), and significantly augmented Osterix and tended to augment Runx2 expression (Fig. 5C). Considering these latter data, we are not ruling out the possibility that signaling pathways other than cAMP are needed for full upregulation of Runx2, and/or that finer tuning of cAMP levels is important for the control of Runx2 expression. Whereas treating A2BAR KO cultures with NECA did not upregulate Osterix expression, treating these cultures with 8-br-cAMP, and therefore bypassing the receptor signaling, did significantly increase Osterix expression (Fig. 5C). This supports our hypothesis that cAMP signaling upon A2BAR activation is, at least partially, responsible for the augmented Osterix expression.

Of note, despite an increase in Osterix expression after a 24 hour exposure to 8-br-cAMP (Fig. 5C), longer treatment (at day 0 and day 2 of osteo-induction) caused a significant decrease in nodule formation (Fig. 6A). However, we found that this long exposure led to a decrease in total cell number and in total protein within the culture (Fig. 6B), suggesting that the inhibition in nodule formation is due to a secondary inhibition of cell proliferation that masks the effect on Osterix upregulation. These results are in agreement with previous studies showing high cAMP levels to inhibit proliferation (e.g.,[134]).

Taken together, these data show that A2BAR ablation has a profound effect on osteoblast differentiation (Fig. 2), and that A2BAR agonism can modulate osteoblast
transcription factor expression, and enhance the number of osteoblasts generated \textit{in vitro} (Fig. 5).
Figure 5. Activation of A2BAR increases osteoblast differentiation. A. Bone marrow
cells from WT and A2BAR KO mice were treated with vehicle (DMSO) or 10 μM
NECA for 10 minutes. cAMP was measured by ELISA and normalized to total protein.
N=4, *p<0.05. B. Bone marrow cells from WT and A2BAR KO mice were treated with
vehicle (DMSO) or 10 μM NECA at the time of osteo-induction and at the next media
change. Six days after osteo-induction, cultures were stained with alizarin red and
nodules were counted using ImagePro software (as in Fig. 1). The values for each
condition were compared to vehicle-treated cells, and each such pair was subjected to a t
test. N=4, *p<0.05. C. Bone marrow cell cultures from WT and A2BAR KO mice were
treated with vehicle (DMSO), 10 μM NECA or 100 μM 8-br-cAMP at the time of osteo-
induction. Lower doses of 8-br-cAMP were also attempted, yielding variable results (data
not shown). RNA was collected after 24 hours. Runx2 and Osterix expression was
measured by qRT-PCR and normalized to 18S rRNA. N=4, *p<0.05.
Figure 5

A

Runx2

Osterix

Relative expression

WT

A2bAR KO

d0
d1

B

Runx2

Osterix

Relative expression

WT

A2bAR KO

vehicle

NECA

8-br-cAMP

WT

A2bAR KO
Figure 6. Treatment with 8-br-cAMP decreases nodule formation and cell number in vitro. Cells were treated with vehicle (DMSO) or 100 μM 8-br-cAMP at the time of osteo-induction and at the next media change. A. Cultures were stained with alizarin red six days after osteo-induction to visualize mineralized nodules. Nodules were counted using ImagePro software. At each time point, A2BAR KO samples were compared to WT, set at 100%, N=4, *p<0.01. B. Six days after osteo-induction, cultures were trypsinized and cell number counted in duplicate. Cells were lysed and total protein measured using a Bradford Assay. N=4, *p<0.05.
Figure 6

A

<table>
<thead>
<tr>
<th></th>
<th>vehicle</th>
<th>8-br-cAMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A2BAR KO</td>
<td></td>
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</tbody>
</table>

Change in nodule number (%)

B

|            | vehicle | NECA      | 8-br-cAMP |
|------------|---------|-----------|
|            |         |           |

Change in total protein

Change in cell number
A2BAR KO mice exhibit impaired bone fracture physiology

In order to assess the role of A2BAR in a model of skeletal injury response, we examined callus formation after fracture. Since fracture healing is dependent on the initial MSC differentiation into osteoblasts and chondrocytes [135], we hypothesized that the reduced potential of A2BAR KO MSCs to differentiate into osteoblasts would be manifested as reduced bone formation after fracture. The progression of tissue formation and mineralization within the callus was first quantified using micro-CT imaging. Micro-CT revealed that fracture calluses of A2BAR KO mice showed a smaller overall total volume, as well as a decrease in the ratio of bone volume to total callus volume at 14 and 21 days post-fracture (Fig. 7A). Analysis of tissue sections supported these findings, showing that the A2BAR KO sections had larger areas of cartilage staining that had not transitioned into mineralized bone tissue (Fig. 7B).

In order to assess the progression of skeletogenic differentiation during fracture healing, the fracture callus was dissected and analyzed by qRT-PCR. Consistent with prior findings [136] for this injury model, a significant upregulation of Osterix and Runx2 was observed, both during early periods of MSC differentiation at day 3 post-fracture, and later starting on day 7 post-fracture, when mineralized cartilage resorption and primary bone formation are initiated in the WT animals. In contrast, the A2BAR KO mice show less expression of Runx2 and Osterix mRNA at 3 and 7 days post-fracture, as compared to WT (Fig. 7C). Although the level of expression of these factors equalized in the WT and A2BAR KO samples by day 14-post fracture, there was still a difference in the ratio of mineralized tissue volume to total callus volume 14 and 21 days post-fracture.
Such findings suggest that the A2BAR is involved in aspects of the skeletal repair process and its absence leads to delayed progression of bone development during fracture repair in the A2BAR KO mice.

Interestingly, the day 14 post-fracture sections from the A2BAR KO mice are dominated by cartilage, compared to WT (Fig. 7B), which is expected to delay normal fracture physiology [137]. The increased abundance of cartilage seen in A2BAR KO sections was validated by assessing the expression levels of chondrocyte markers, collagen type II, alpha 1 (Col2a1) and Aggrecan, which are elevated in the A2BAR KO fractures (Fig. 8A). Further analysis with immunohistochemistry verified that the increased mRNA expression is translated into greater protein contents, based on the increased total amount of Col2a1 and Aggrecan staining in the A2BAR KO as compared to WT (Fig. 8B).
Figure 7. Analysis of callus formation post-fracture in A2BAR KO mice. A. Micro-CT analysis of bone volume/total volume (BV/TV) and TV of fracture calluses from WT and A2BAR KO mice 14 and 21 days post-fracture. B. Representative sections of the fracture callus of WT and A2BAR KO mice at d14 and d21 post-fracture. Sections were stained with fast green (which depicts bone), safranin O (which depicts cartilage) and counter stained with hematoxylin. Composite of 4x magnified images. C. The mid-diaphyseal region (d0), or the fracture callus (d3, 7, 14 post-fracture) of WT and A2BAR KO mice were dissected and qRT-PCR was performed for the osteoblast transcription factors Runx2 and Osterix and normalized to β-actin. Data are presented as fold change relative to d0. In examining statistical significance, we focused on WT vs. A2BAR KO in each set, using a t test. N=3-5, *p<0.05.
Figure 7

A

- WT
- A2BAR KO

B

C

- WT
- A2BAR KO

Runx2

Caterix

Relative expression

d0  d3  d7  d14

d0  d3  d7  d14

*
Figure 8. Analysis of chondrocyte markers in fracture calluses of WT and A2BAR KO mice. A. The fracture callus (14 post-fracture) of WT and A2BAR KO mice was dissected and qRT-PCR was performed for the chondrocyte markers collagen type II α1 (Col2a1) and Aggrecan and normalized to β-actin. N=5, *p<0.05. B. Representative immunohistochemical staining of d14 fracture calluses for Col2a1 and Aggrecan. Arrows depict staining. 4x magnified composite images (left) and 10x magnified images (right).
Figure 8

A

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
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<tbody>
<tr>
<td>Col2a1</td>
<td>1.2</td>
<td>2.3</td>
</tr>
<tr>
<td>Aggrecan</td>
<td>2.9</td>
<td>3.3</td>
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B

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<tr>
<th></th>
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<tbody>
<tr>
<td>Col2a1</td>
<td>Image 1</td>
<td>Image 2</td>
</tr>
<tr>
<td>Aggrecan</td>
<td>Image 3</td>
<td>Image 4</td>
</tr>
</tbody>
</table>
A2BAR KO mice exhibit an osteopenic phenotype

As the differentiation of MSCs to osteoblasts and chondrocytes during the formation of the fracture callus recapitulates skeletogenesis [127, 138, 139], we explored the role of the A2BAR on bone development. There is no difference in body weight between 15 week old WT and A2BAR KO mice (Table 1). Micro-CT analysis of the cortical bone of the mid-diaphyseal region of 15 week old femurs shows a statistically significant reduction in cortical thickness (Fig. 9A,B), which correlates with a lower bone volume relative to total volume (BV/TV), as total volume was not different (Table 1). Additionally, these mice display a reduction in tissue mineral density (TMD) (Fig. 9A, Table 1). This shows that at 15 weeks of age, A2BAR KO mice display a mild osteopenic phenotype. Additionally, femurs of 15-week-old A2BAR KO mice were significantly shorter than WT, as measured with calipers (Table 1). Shorter femurs may be the result of impaired osteoblast differentiation during development, however, we cannot rule out other mechanisms, such as impairment in chondrocyte and/or growth plate development.

There are no significant differences in the cortical bone between WT and A2BAR KO mice at 4 or 8 weeks of age (Table 2). However, at 4 weeks of age, A2BAR KO mice have fewer trabeculae, a decrease in trabecular thickness and an increase in trabecular spacing, as compared to WT mice (Table 2). Additionally, as in the 15 week-old mice, femurs of 4 and 8 week old A2BAR KO mice are somewhat shorter, as compared to WT (Table 2).

Analysis of the growth plates of 4-week-old WT and A2BAR KO mice shows no statistically significant difference in the total growth plate thickness, proliferating zone
thickness or hypertrophic zone thickness, but A2BAR KO growth plates are slightly larger (Fig. 10, Table 3).
TABLE 1
Analysis of WT and A2BAR KO bone growth and homeostasis at 15 weeks of age
Bones were collected from male age-matched WT and A2BAR KO mice at 15 weeks of age. Micro-CT cortical analysis was done on the femur mid-diaphysis and trabecular analysis was done on the distal metaphysis.

<table>
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<th>n/p-value</th>
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<tr>
<td></td>
<td>WT</td>
<td>A2BAR KO</td>
</tr>
<tr>
<td><strong>Body Weight (g)</strong></td>
<td>30.92 +/- 1.65</td>
<td>30.42 +/- 1.03</td>
</tr>
<tr>
<td><strong>Femur Length (mm)</strong></td>
<td>16.12 +/- 0.14</td>
<td>15.50 +/- 0.20*</td>
</tr>
<tr>
<td><strong>Cortical Analysis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Volume (TV) (mm³)</td>
<td>1.32 +/- 0.09</td>
<td>1.26 +/- 0.02</td>
</tr>
<tr>
<td>Bone Volume (BV) (mm³)</td>
<td>0.70 +/- 0.06</td>
<td>0.64 +/- 0.04</td>
</tr>
<tr>
<td>TV/BV</td>
<td>0.54 +/- 0.03</td>
<td>0.51 +/- 0.03</td>
</tr>
<tr>
<td>Cortical Thickness (mm)</td>
<td>0.25 +/- 0.01</td>
<td>0.23 +/- 0.01*</td>
</tr>
<tr>
<td><strong>Mean Density (mg HA/ccm)</strong></td>
<td>1222.40 +/- 23.92</td>
<td>1190.72 +/- 25.78</td>
</tr>
<tr>
<td><strong>Min Cross-sectional area (mm²)</strong></td>
<td>1.17 +/- 0.09</td>
<td>1.12 +/- 0.09</td>
</tr>
<tr>
<td><strong>Max Cross-sectional area (mm²)</strong></td>
<td>1.52 +/- 0.10</td>
<td>1.45 +/- 0.12</td>
</tr>
<tr>
<td><strong>Trabecular Analysis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trabecular Number</td>
<td>4.73 +/- 0.37</td>
<td>4.45 +/- 0.65</td>
</tr>
<tr>
<td>Trabecular Thickness (mm)</td>
<td>0.05 +/- 0.01</td>
<td>0.05 +/- 0.01</td>
</tr>
<tr>
<td>Trabecular Spacing (mm)</td>
<td>0.21 +/- 0.02</td>
<td>0.23 +/- 0.03</td>
</tr>
</tbody>
</table>
Figure 9. Effect of the A2BAR on cortical bone parameters. A. Mid-diaphyseal cortical bone of 15 week-old WT and A2BAR KO mice were analyzed by micro-CT. A2BAR KO mice exhibit decreased cortical bone volume (BV) as a percent of total volume (TV) of the mid-diaphyseal region analyzed, decreased cortical thickness, and decreased cortical tissue mineral density (TMD) (mg hydroxyapatite (HA)/cm$^3$) as compared to WT controls. N=5, *p<0.05. B. Representative micro-CT images of mid-diaphyseal cortical bone of WT and A2BAR KO mice, showing small differences in overall shape and size.
Figure 9

A

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>A2BAR KO</th>
</tr>
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<tbody>
<tr>
<td>BV/TV (%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>A2BAR KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>TMD (mg HA/cm²)</td>
<td></td>
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</table>
### TABLE 2
Analysis of WT and A2BAR KO bone growth and homeostasis

Bones were collected from male age-matched WT and A2BAR KO mice at 4 and 8 weeks of age. Micro-CT cortical analysis was done on the femur mid-diaphysis and trabecular analysis was done on the distal metaphysis.

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<th>8 weeks</th>
<th>n/p-value</th>
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<td></td>
<td>WT</td>
<td>A2BAR KO</td>
<td></td>
<td>WT</td>
</tr>
<tr>
<td><strong>Body Weight (g)</strong></td>
<td>17.58 +/- 1.41</td>
<td>16.98 +/- 0.92</td>
<td>4/0.39</td>
<td>25.33 +/- 1.23</td>
</tr>
<tr>
<td><strong>Femur Length (mm)</strong></td>
<td>12.63 +/- 0.41</td>
<td>11.81 +/- 0.48*</td>
<td>4/0.02</td>
<td>15.23 +/- 0.17</td>
</tr>
<tr>
<td><strong>Cortical Analysis</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Volume (TV) (mm^3)</td>
<td>0.92 +/- 0.03</td>
<td>0.92 +/- 0.05</td>
<td>5/0.87</td>
<td>1.21 +/- 0.08</td>
</tr>
<tr>
<td>Bone Volume (BV) (mm^3)</td>
<td>0.34 +/- 0.03</td>
<td>0.36 +/- 0.02</td>
<td>5/0.36</td>
<td>0.60 +/- 0.05</td>
</tr>
<tr>
<td>TV/BV</td>
<td>0.37 +/- 0.03</td>
<td>0.39 +/- 0.02</td>
<td>5/0.31</td>
<td>0.50 +/- 0.06</td>
</tr>
<tr>
<td>Cortical Thickness (mm)</td>
<td>0.14 +/- 0.01</td>
<td>0.15 +/- 0.01</td>
<td>5/0.27</td>
<td>0.22 +/- 0.02</td>
</tr>
<tr>
<td>Mean Density (mg HA/ccm)</td>
<td>978.05 +/- 30.11</td>
<td>967.65 +/- 40.78</td>
<td>5/0.52</td>
<td>1103.00 +/- 6.79</td>
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<tr>
<td>Min Cross-sectional area (mm)</td>
<td>0.87 +/- 0.15</td>
<td>0.91 +/- 0.10</td>
<td>5/0.36</td>
<td>1.09 +/- 0.05</td>
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<tr>
<td>Max Cross-sectional area (mm)</td>
<td>1.05 +/- 0.22</td>
<td>1.09 +/- 0.22</td>
<td>5/0.95</td>
<td>1.25 +/- 0.19</td>
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<td><strong>Trabecular Analysis</strong></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>Trabecular Number</td>
<td>5.10 +/- 0.50</td>
<td>3.68 +/- 0.50*</td>
<td>4/0.01</td>
<td>5.22 +/- 0.24</td>
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<tr>
<td>Trabecular Thickness (mm)</td>
<td>0.04 +/- 0.01</td>
<td>0.05 +/- 0.01*</td>
<td>4/0.01</td>
<td>0.05 +/- 0.01</td>
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<tr>
<td>Trabecular Spacing (mm)</td>
<td>0.20 +/- 0.02</td>
<td>0.28 +/- 0.04*</td>
<td>4/0.01</td>
<td>0.19 +/- 0.01</td>
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</table>
Figure 10. Representative images of the growth plate of WT and A2BAR KO mice. Growth plates of the distal femur of 4-week-old WT and A2BAR KO mice were sectioned and stained with fast green (which depicts bone), safranin O (which depicts cartilage) and counter stained with hematoxylin. 4x and 20x magnifications.
Figure 10

WT

A2BAR KO
TABLE 3
Quantification of the growth plate of WT and A2BAR KO mice.
Heights of the total growth plate, the proliferating zone and the hypertrophic zone of 4-week-old WT and A2BAR KO mice were calculated by averaging 20 separate measurements within the middle 3/4 of the growth plate. 20 sections per mouse were quantified.

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>A2BAR KO</th>
<th>n/p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth plate height (mm)</td>
<td>330.43 +/- 35.84</td>
<td>356.26 +/- 24.73</td>
<td>3/0.36</td>
</tr>
<tr>
<td>Proliferating zone height (mm)</td>
<td>158.81 +/- 12.86</td>
<td>163.95 +/- 9.77</td>
<td>3/0.61</td>
</tr>
<tr>
<td>Hypertrophic zone height (mm)</td>
<td>123.51 +/- 14.84</td>
<td>150.51 +/- 32.63</td>
<td>3/0.26</td>
</tr>
</tbody>
</table>
Together, our results demonstrate that the A2BAR has an important role in the promotion of osteoblast differentiation and bone formation with development and after fracture. This role is, at least partially, through the control of the expression of osteoblast differentiation transcription factors.

**Aim 2: The impact of A2BAR deletion on skeletal development.**

**The A2BAR impacts growth plate dynamics**

We previously found A2BAR KO mice to have slightly shorter femurs and significantly less trabecular bone [54]. Therefore, we examined the growth plate of these mice. Femurs of 1, 2 and 4 week old mice were sectioned and stained with safranin O and fast green, indicative of cartilage and bone, respectively. Histology of the distal growth plate of the femur showed that the growth plate height at 1 week of age is similar in WT and A2BAR KO mice (Fig. 11A; N=2). At 2 weeks of age, A2BAR KO mice have a significantly shorter growth plate, relative to WT (Fig. 11B). However, at 4 weeks of age, the growth plate of A2BAR KO mice is taller relative to WT (Fig. 11B). To determine whether the resting and proliferating chondrocytes or the hypertrophic chondrocytes were accounting for the changes in height, each zone height was measured and normalized to total growth plate height. We found no difference in the resting and proliferating zone or hypertrophic zone between A2BAR KO and WT bones (Fig. 11C). These results suggest that the rates of growth plate development and/or maturation are altered in the A2BAR KO mice but that this alteration is not due to changes in one of the zones of the growth plate.
To examine how potential differences in chondrocyte differentiation and maturation may be contributing to the altered growth plates of A2BAR KO mice we measured Sox9, Collagen II type α1 (Col 2a1), Aggrecan and Collagen X type α1 (Col 10a1) mRNA expression in entire femurs at 2 weeks of age. We chose to utilize the entire femur rather than the growth plate so as not to introduce variability in sample collection. At 2 weeks of age there tended to be a decrease in Sox9 expression ($p=0.07$), as well as a tendency towards decreased Col 2a1, Col 10a1 and Aggrecan expression in the A2BAR KO (Fig. 12). It is possible that significance would be reached with a larger sample size, as there is a considerable amount of variability within different biological samples. Sox9 is a transcription factor expressed early in chondrocyte differentiation, Col 2a1 and Aggrecan are expressed in proliferating chondrocytes and Col 10a1 is expressed in hypertrophic chondrocytes [140]. That the expression of these factors tends to be lower in the A2BAR KO might support the histology data, showing shorter growth plates at 2 weeks of age (Fig. 11B).
Figure 11: Growth plates of WT and A2BAR KO mice. Femurs from 1, 2 and 4 week old male WT and A2BAR KO mice were sectioned and stained with safranin O (red: depicts cartilage) and fast green (blue/green: depicts bone). Growth plates are outlined in black. Growth plate height was measured in 4-7 sections per bone, at the mid-line (represented by dashed line), using SPOT imaging software and an average height was calculated for each bone. At 1 week of age, A2BAR KO mice tended to have similar growth plate height to WT (N=2). At 2 weeks of age, A2BAR KO mice had significantly shorter growth plate height (N=6, *p<0.05) while at 4 weeks of age the growth plate was significantly taller (N=3, *p<0.05). 4x magnification. Values represent averages +/- standard deviation.
Figure 11

A

1 week old

WT

A2BAR KO

B

2 weeks old

4 weeks old

WT

A2BAR KO

C

R + P

H

2 weeks old

4 weeks old
Figure 12: Chondrocyte mRNA expression in femurs of WT and A2BAR KO mice. RNA was isolated from whole femurs of WT and A2BAR KO male mice at 2 weeks of age. Sox9, Col 2a1, Aggrecan and Col 10a1 expression was measured by qRT-PCR and expression was normalized to GAPDH mRNA expression. A2BAR KO mice tended to have lower expression of Sox9 (p=0.07), Col 2a1 (p=0.4), Aggrecan (p=0.1) and Col 10a1 (p=0.2). Values represent averages +/- standard deviation (N=4).
Figure 12

Sox9

Col2a1

Aggrecan

Col10a1

relative expression

WT

A2BAR KO

relative expression

WT

A2BAR KO

relative expression

WT

A2BAR KO

relative expression

WT

A2BAR KO
We also examined tibiae of younger mice (1-day old), when the bone is composed predominantly of cartilage. We chose to analyze the tibia, rather than the femur, as it is easier to remove consistently from the post-natal mice. WT and A2BAR KO mice showed no statistically significant differences in expression of Sox9, Aggrecan, and Col 10α1 (Fig. 13A). At the protein level, there was no difference in Sox9 between the experimental groups, and there tended to be more Aggrecan in the A2BAR KO samples (Fig. 13B). Analysis of this data is limited as the possible increase in Aggrecan protein suggests more cartilage in the entire tibiae of A2BAR KO mice at one week of age, and is not restricted to the growth plate. A decrease in osteoblast differentiation and ossification in the A2BAR KO mice (as seen previously [54]) could account for the increased Aggrecan content. As to potential differences in WT and A2BAR KO samples at 2 weeks of age, a larger sample size is needed to reach firmer conclusions.

**Cranial bone development is somewhat impaired in A2BAR KO mice**

As we have previously found a role of the A2BAR in osteoblast differentiation [54], as well as some difference in growth plate dynamics in the A2BAR KO mice, we also carried out a gross analysis of the entire skeletons of 1-day old mice. Overall, there were no obvious changes in the spinal column, ribs, or forelimbs of the A2BAR KO mice, compared to WT (Fig. 14A). However, closer examination of the skulls showed impaired ossification of the parietal and interparietal bones of the A2BAR KO mice (Fig. 14B,C). This observation is in line with our finding that A2BAR KO mice have impaired osteoblast differentiation but does not give information about chondrocyte differentiation/maturation, as the bones of the skull form independent of cartilage [1].
This interesting observation related to decreased ossification of the parietal and interparietal bones in the A2BAR deleted mice will need further examination.

Taken together, through analysis of the post-natal skeletal development of the A2BAR KO mice, we have found differences in the dynamics of the growth plate, which likely explain the differences previously seen in total femur length and trabeculae number. Additionally, we found impaired ossification of some bones of the skull, possibly due to diminished osteoblast differentiation in the A2BAR KO mouse. However, the skeletal effects of lack of the A2BAR are minor, without overt or pathological influence on the animal.
Figure 13: Chondrocyte expression and protein levels in 1-day old WT and A2BAR KO mice. A. RNA was isolated from whole tibiae of 1-day old WT and A2BAR KO mice. Sox9, Aggrecan, and Col 10a1 expression was measured by qRT-PCR and expression was normalized to GAPDH mRNA expression. Data shown represent averages +/- standard deviation (N=4). A t test showed that the differences between the experimental groups were not statistically significant. B. Protein was extracted from the whole contra lateral tibiae and Sox9 and Aggrecan levels were measured by Western blot and normalized to β-actin. Images were quantified with ImageJ software. There was no difference in Sox9 (p=0.9) and a trend towards increased Aggrecan levels (p=0.6) in the A2BAR KO mice relative to WT. Values represent averages +/- standard deviation (N=4).
Figure 13

A

\begin{figure}
\centering
\begin{subfigure}{0.4\textwidth}
  \centering
  \includegraphics[width=\textwidth]{sox9_expr.png}
  \caption{Sox9 expression.}
\end{subfigure}
\hfill
\begin{subfigure}{0.4\textwidth}
  \centering
  \includegraphics[width=\textwidth]{aggrecan_expr.png}
  \caption{Aggrecan expression.}
\end{subfigure}
\hfill
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  \includegraphics[width=\textwidth]{collagen10a1_expr.png}
  \caption{Collagen 10a1 expression.}
\end{subfigure}
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B

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  \includegraphics[width=\textwidth]{sox9_western.png}
  \caption{Sox9 Western blot.}
\end{subfigure}
\hfill
\begin{subfigure}{0.8\textwidth}
  \centering
  \includegraphics[width=\textwidth]{aggrecan_western.png}
  \caption{Aggrecan Western blot.}
\end{subfigure}
\end{figure}
Figure 14: Skeletons of 1 day-old WT and A2BAR KO mice. One day-old mice were removed of soft tissue and skeletons were stained with alizarin red (red: to depict bone) and alcian blue (blue: to depict cartilage). Lower limbs and skulls were removed prior to staining. A. Representative images of WT and A2BAR KO skeletons. No differences in the gross morphology of the spinal column, ribs or forelimbs of A2BAR KO mice were observed. B. Lateral view of the skulls of WT and A2BAR KO mice. The parietal and interparietal bones (depicted with arrows and outlined in black) may be smaller and less ossified in the A2BAR KO mice. C. Superior view of the skulls of WT and A2BAR KO mice. Again, parietal and interparietal bones (depicted with arrows and outlined in black) may be smaller and less ossified in the A2BAR KO mice. 1x magnification.
Figure 14

A

WT

A2BAR KO

B

WT

A2BAR KO

C

WT

A2BAR KO
Aim 3. The A2BAR affects the differentiation of mouse embryonic fibroblasts to chondrocytes

Mouse embryonic fibroblasts (MEFs) from A2BAR KO mice have increased expression of Sox9

In order to determine how lack of the A2BAR impacts chondrocyte differentiation \textit{in vitro}, MEFs were isolated from WT and A2BAR KO mice at age e11.5. MEFs were differentiated to chondrocytes by plating in micro-mass cultures and treating with 100 nM BMP2 as well as ascorbic acid and beta-glycerol phosphate (see methods). We found that prior to chondro-induction, A2BAR KO MEFs had increased expression of Sox9 relative to WT (Fig. 15). 24 hours post-induction, Sox9 was increased in the WT and there was no difference between WT and A2BAR KO samples (Fig. 15). As Sox9 expression increases with differentiation initiation, and then subsequently decreases [141], it is possible that Sox9 expression did increase with chondro-induction but then returned to baseline levels by 24 hours post-induction. It is also possible that Sox9 expression in A2BAR KO samples was relatively high at chondro-induction and did not increase further. The consequence of these differences in Sox9 expression on chondrocyte terminal differentiation remain to be examined.
Figure 15: MEFs from A2BAR KO mice have increased expression of Sox9. Mouse embryonic fibroblasts (MEFs) were isolated from WT and A2BAR KO mice at age e11.5. MEFs were differentiated to chondrocytes by plating in micro-mass cultures and treating with chondro-induction reagents (see methods). RNA expression was measured by qRT-PCR and normalized to GAPDH mRNA expression. A2BAR KO MEFs had significantly higher expression of Sox9 at baseline, and there was no difference in expression 24 hours post-induction between WT and A2BAR KO samples. Values represent averages +/- standard deviation (N=3). *p<0.05
Figure 15

Sox9

<p>| | |</p>
<table>
<thead>
<tr>
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</thead>
<tbody>
<tr>
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<td>A2BAR KO</td>
</tr>
<tr>
<td>baseline</td>
<td>24 hrs post-induction</td>
</tr>
</tbody>
</table>

relative expression

0 0.5 1 1.5 2 2.5

* *
A2BAR activation inhibits chondrocyte differentiation in MEFs

In addition to examining the effect of A2BAR KO, we also tested whether activation of the receptor with a specific agonist (BAY 60-6583; denoted as Bay) would modulate chondrocyte differentiation. We utilized commercially available WT C57Bl/6 MEFs and induced differentiation as described above and in methods. Concurrent with chondro-induction, we treated cultures with 1 μM Bay and found it to reduce Sox9 expression, relative to treatment with vehicle, at 1 and 7 hours post-induction (Fig 16A). To test whether the effect of Bay is via an increase in cAMP, we also treated cultures with 1 μM of the direct adenylyl cyclase activator Forskolin. Interestingly, there was no effect of Forskolin until 7 hours post-chondro-induction (Fig. 16A). This suggests the effect of A2BAR activation on Sox9 expression is due to additional signaling mechanisms or the kinetics of the cAMP signaling upon Bay treatment are needed for the early Sox9 decrease. Further experiments will elucidate the mechanism of Bay effect on Sox9 expression.

To test whether the changes in Sox9 expression translate into changes in chondrocyte differentiation, we analyzed cultures 6 days post-chondro-induction. The expression of the cartilage proteins Col 2a1, Aggrecan and Col 10a1 increased significantly with chondro-induction, demonstrating that we are indeed inducing chondrocyte differentiation in vitro (Fig. 16B). Treatment with Bay (1 μM) did not significantly alter mRNA expression of Col 2a1 or Aggrecan but Col 10a1 mRNA expression was significantly reduced with Bay treatment (Fig 16B). Proteoglycan accumulation, as evidenced by alcian blue staining was not different between vehicle and
Bay treated cultures (Fig. 16C). A larger sample size is needed to account for the amount of variability in these measures, however the Col 10a1 results suggest that A2BAR activation impairs chondrocyte differentiation.
Figure 16: A2BAR activation inhibits chondrocyte differentiation in MEFs. Commercially available WT MEFs were plated in micro-mass and treated with chondro-induction reagents (see methods). Concurrent with chondro-induction, cells were treated with 1 μM Bay, 1 μM Forskolin (an adenylyl cyclase activator) or vehicle. A. RNA was isolated 1 and 7 hours post-chondro-induction and Sox9 expression was measured by qRT-PCR and normalized to GAPDH mRNA expression. Bay-treated cultures had significantly lower expression of Sox9 at 1 and 7 hours post-chondro-induction, compared to vehicle-treated ones (*p<0.05). The expression of Sox9 in Forskolin-treated cultures was significantly lower at 7 hours post-chondro-induction, compared to vehicle-treated ones (*p<0.05). B. RNA was also collected 6 days post-chondro-induction. The expression of Col 2a1, Aggrecan and Col 10a1 was measured by qRT-PCR and normalized to GAPDH mRNA expression. For Aggrecan and Col 2a1, the differences between the experimental groups were not statistically significant (p>0.05) (N=5). There was a significant decrease in Col 10a1 mRNA expression in Bay treated samples (p<0.05) (N=5). C. Proteoglycan accumulation was detected by alcian blue staining of cultures 6 days post-chondro-induction. 1, 2 and 3 represent each biological replicate. Staining was quantified by measuring density using ImageJ software and values are graphed relative to vehicle treatment (N=3). Values represent averages +/- standard deviation.
Figure 16

A

![Graph showing relative expression of Sox9 across different conditions and time points.]

B

![Graphs showing relative expression of Collagen 2a1, Collagen 10a1, and Aggrecan.]

C

![Image showing relative density of vehicle and Bay conditions.]

1 hr post-induction

7 hr post-induction
A2BAR activation increases C/EBP-β expression in MEFs during chondrogenesis

Previously we have demonstrated that treatment of marrow-derived MSCs with Bay increases Runx2 expression [54]. Additionally, it has been shown that Runx2 inhibits specific stages of chondrocyte differentiation and maturation [142]. Here we found that in MEFs, Bay treatment had no effect on Runx2 expression at 1 and 7 hours post chondro-induction (Fig. 17). Therefore, the inhibitory effect of A2BAR activation on chondrocyte differentiation is not dependent on increased Runx2 mRNA expression. This, of course does not rule out an effect of A2BAR agonism on Runx2 protein level or activation.

CCAAT/enhancer binding protein-β (C/EBP-β) is another factor known to regulate MSC differentiation [143]. Here we found that treatment with Bay significantly increased C/EBP-β mRNA expression, relative to vehicle treatment, 1 hour post-induction (Fig. 18). Forskolin also tended to increase C/EBP-β expression, though this increase was not statistically significant (Fig 18). To test whether the inhibitory action of Bay on Sox9 expression is due to its stimulatory effect on C/EBP-β expression, we obtained C/EBP-β KO MEFs and differentiated these cells to chondrocytes with and without Bay treatment (data not shown). In this single pilot experiment (carried out in triplicates), Sox9 mRNA expression was higher in C/EBP-β KO at baseline (by 1 fold), relative to WT controls. Though Bay treatment decreased Sox9 expression in the WT cells by half a fold, it had no effect in the C/EBP-β KO cells. Additionally, C/EBP-β KO cells failed to differentiate to chondrocytes, as evidenced by alcian blue staining. In the same experiment, WT MEFs upregulated Sox9 mRNA expression upon chondro-induction and were positive for alcian blue staining, indicating chondrocyte differentiation. Further experiments are needed to
test the significance of increased C/EBP-β expression on chondrocyte differentiation and whether this is a mechanism of Bay’s inhibitory action.
Figure 17: Runx2 expression during chondrocyte differentiation with Bay treatment. Commercially available WT MEFs were plated in micro-mass and treated with chondro-inductive reagents (see methods). Concurrent with chondro-induction, cells were treated with 1 μM Bay, 1 μM Forskolin or vehicle. RNA was collected 1 and 7 hours post-chondro-induction and Runx2 expression was measured by qRT-PCR and normalized to GAPDH mRNA expression. Treatment with Bay or Forskolin had no effect on Runx2 expression at these time points. Values represent averages +/- standard deviation (N=3); p>0.05 (comparing each treatment to baseline).
Figure 17

Runx2

relative expression

baseline  vehicle  Bay  Forskolin  vehicle  Bay  Forskolin

1 hr post-induction  7 hr post-induction
Figure 18: C/EBP-β expression during chondrocyte differentiation with Bay treatment. Commercially available WT MEFs were plated in micro-mass and treated with chondro-inductive reagents (see methods). Concurrent with chondro-induction, cells were treated with 1 μM Bay, 1 μM Forskolin or vehicle. RNA was collected 1 hour post-chondro-induction and C/EBP-β expression was measured by qRT-PCR and normalized to GAPDH mRNA expression. Treatment with Bay significantly increased C/EBP-β expression, relative to vehicle treatment (*p<0.05). Forskolin also tended to increase C/EBP-β expression (p=0.05). Values represent averages +/- standard deviation (N=3).
Figure 18

C/EBP-β

relative expression

baseline  vehicle  Bay  Forskolin

1 hr post-induction
Aim 1: The role of the A2BAR in bone repair and in the differentiation of bone marrow mesenchymal stem cells to osteoblasts

MSC differentiation to osteoblasts plays a necessary role in the development and postnatal growth and homeostasis (reviewed in [144]) as well as skeletal tissue repair after injuries such as fracture [145]. Though the origin of the osteoblasts involved in fracture healing has not been demonstrated unequivocally, experimental evidence is accumulating to support the hypothesis that some of the MSCs that contribute to repair are harbored in the bone perivascularure [146], and upon receiving a signal (one of which being stromal derived factor-1 (SDF-1) [145]) travel, through the marrow capillaries [144] or neovasculature [147], to the site of injury or stress.

Here, we used genetic ablation to study the role of the A2BAR in bone homeostasis and repair in vivo, and examined the potential mechanisms that were involved using in vitro studies of MSC differentiation. We present direct evidence that the A2BAR plays a role in osteoblast differentiation. We have also demonstrated that activation of this receptor with a pharmacological agonist can enhance osteoblast differentiation. This effect is likely due to the modulation of levels of the transcription factors essential for osteoblast differentiation, Runx2 and Osterix. This claim is supported by previous data demonstrating increased osteoblast differentiation with the overexpression of either Runx2 [148] or Osterix [149]. Of note, however, the effect of A2BAR activation on osteoblast differentiation is much milder than the effect of this receptor ablation. This raises the possibility that the receptor influences these processes
independent of its activation, e.g., via its potential association with other regulatory pathways or proteins yet to be explored, which would be lost upon receptor deletion.

Genetic deletion of the receptor impacted bone fracture callus development and likely delayed normal fracture physiology. It had long been accepted that the low affinity A2BAR plays an important role in tissue injury, as extracellular adenosine levels increase after various types of stress/injury and in various organs (reviewed in [76]). Many studies of bone have examined extracellular concentrations of ATP and ADP, and the expression of their receptors, P2X and P2Y [150]. Extracellular ATP concentrations increase after bone injury and, when exposed to hypoxic conditions, osteoblasts secrete ATP in the high nM to μM range [151]. This ATP is catabolized by ectonucleotidases, also expressed on osteoblasts [133]. Of note, it was recently demonstrated that genetic ablation of the ectonucleotidase CD73, an enzyme upstream of A2BAR signaling (by converting AMP to adenosine), results in osteopenia and decreased osteoblast differentiation [152]. Although we too demonstrate diminished osteoblast differentiation ex vivo in A2BAR KO samples, it is likely that this explains only part of the reduced bone density in the fracture callus. Fracture healing is complex, and the A2BAR may have a role in other stages of the process, such as the inflammatory response. This possibility should be considered in the context of fracture healing since inflammatory cytokines, particularly TNF-α, have been shown to downregulate the expression of the osteoblast differentiation genes Runx2 [90] and Osterix [95]. Also, A2BAR KO mice tend to display a mild increase in the levels of inflammatory cytokines at baseline, and
more so post-vascular injury, partially attributed to cAMP modulation of macrophage expression of cytokines [65, 104].

Our current study also points to a potential role for A2BAR signaling in the regulation of callus cartilage density post-injury. Others have documented A2BAR expression in chondrocytes [80], the source of cartilage, as well as the role of cAMP signaling in preventing [153] as well as promoting [71] their differentiation. This differential effect of cAMP might be attributed to how chondrocyte differentiation is being measured, i.e. differentiation versus maturation of chondrocytes. In Aim 2 of this thesis research we initiated studies to explore the potential effect of A2BAR expression on the development of embryonic and adult chondrocytes.

Finally, our study is the first to describe a role for the A2BAR in bone development and homeostasis. Intriguingly, the bone phenotype of the A2BAR KO mouse produces a similar but milder phenotype comparable to what is observed for the PTH receptor (PPR) KO mouse [42], suggesting a possible synergy between the two receptors during bone development. We found that genetic ablation of the A2BAR causes a significant decrease in the early (4-week old) development of trabecular bone however, the differences normalize by 8 weeks of age. The genesis of trabecular bone depends on the differentiation of cells of multiple lineages, including osteoblasts and chondrocytes, and the coordinated action of these cells [154]. Impairment in osteoblast differentiation in the A2BAR KO mouse may impact the development of the perichondrium and, at least partially, explain the reduced trabecular bone formation in the A2BAR KO. The lack of difference in trabecular bone in older mice (8 and 15-weeks old) may be due to the
diminished contribution of stem cell differentiation towards trabecular bone homeostasis at these ages, since in earlier periods of growth many more cells contribute towards juvenile tissue development. In addition, our finding that the A2BAR KO femurs are shorter, with a trend towards increased height of the hypertrophic zone and entire growth plate, are consistent with the effects of removing the perichondrium during endochondral bone development [154]. Therefore, A2BAR deletion may be delaying periosteal development with subsequent consequences on endochondral ossification and growth plate regulation. Future investigation of skeletal development in the A2BAR KO mouse could test these hypotheses.

Taken together, our study identified the A2BAR as an important regulator of osteoblast transcription factor expression and of MSC differentiation to osteoblasts, as well as a regulator of bone homeostasis and fracture physiology.

**Aim 2: The impact of A2BAR deletion on skeletal development**

We analyzed femurs of adult WT and A2BAR KO mice at baseline (i.e. in the absence of stress or injury) and found A2BAR KO mice to have slightly shorter femurs and a decrease in the number of trabeculae in the distal metaphysis. Additionally, we found that these differences in the A2BAR KO mice diminished with age. For this aim of research, we chose to expand the investigation of the baseline bone phenotype of the A2BAR KO mice by studying the skeleton, particularly the growth plate, in growing mice. Longitudinal growth of the femur is dependent on specific aspects of the growth plate, specifically chondrocyte proliferation, extracellular matrix secretion and hypertrophy [155]. Additionally, the formation of trabecular bone is the result of
mineralization of the vertical cartilage septa, remaining after chondrocyte hypertrophy and physiological death, by osteoblasts [155]. Therefore, we hypothesized that A2BAR KO mice would possess morphological differences in the growth plate, compared WT mice.

The development and maturation of the growth plate is a dynamic process. In WT mice, the height of the growth plate increases between 1 and 2 weeks and then decreases between 2 and 4 weeks. We found that at 2 weeks of age A2BAR KO mice have a significantly shorter growth plate. However at 4 weeks of age the height of the growth plate of A2BAR KO mice is significantly taller. Histological measurement of the growth plate is the historical method for analyzing bone growth. New technology, using micro-CT analysis of bones stained with a cartilage specific dye, will allow us to measure the growth plate more precisely and should be sensitive enough to detect small changes in growth plate morphology.

In addition to histology, we also analyzed the mRNA expression of chondrocyte genes, as a decrease in the number of chondrocytes may result in a decrease in the amount of mRNA. At 2 weeks of age there was a tendency towards decreased mRNA expression of Sox9, Col 2a1, Aggrecan and Col 10a1, however the variability between mice was large and the values were not statistically significant. The mRNA expression of Sox9, Aggrecan and Col 10a1 at 1 week of age also tended to be lower in the A2BAR KO mice, compared to WT. Conversely, the protein level of Aggrecan tended to be higher in the A2BAR KO at 1 week of age. Therefore, mRNA expression does not necessarily correlate with protein level, and whether there is a difference between WT
and KO growth plates at 1 week of age remains to be determined. Biochemical analysis of total glycosaminoglycan content of WT and A2BAR KO bones will be a better indicator of total cartilage, and will be used in the future to validate histology and micro-CT data.

Based on this data we can conclude that the dynamics of the growth plate are different in the A2BAR KO mice, compared to WT mice. This may be attributed to a delay in the initiation of endochondral ossification in the A2BAR KO, thereby shifting the curve of growth plate expansion and diminution. In essence, the growth plate of the A2BAR KO may be “lagging behind” the normal progression of the WT growth plate. Analysis of the initiation of chondrocyte differentiation, the formation of the cartilage analgen and of the initiation of the primary ossification center in embryos are needed to test this supposition.

Often, growth plate alterations are due to differences in the proliferation or maturation (hypertrophy and cell death) of chondrocytes. Typically, these differences persist during bone growth and are reflected in the relative heights of the zones of the growth plate (e.g. [156]). However, we found the growth plate of the A2BAR KO mice to be shorter at 2 weeks of age and taller at 4 weeks of age, compared to WT, with no differences in the ratios of proliferating or hypertrophic zones. This suggests that if there are differences in chondrocyte proliferation or maturation, they are not persistent. Additionally, any effect on one process would have to be compensated for by a similar action on the other process, so that the relative ratios remain equal. An additional, and more likely explanation for the observed growth plate aberrations in the A2BAR KO is a
delay in the initiation of growth plate formation. This would result in a lag in normal growth plate changes, relative to the WT. Further analysis of the formation of the growth plate during embryogenesis and of proliferation and cell death, via immunohistochemical staining, are needed to understand the mechanisms involved.

**Aim 3: The A2BAR affects the differentiation of mouse embryonic fibroblasts to chondrocytes**

Analysis of the fracture callus of A2BAR KO mice (Aim 1; [54]) demonstrated an increased volume of cartilage at 14 days post-fracture, relative to WT. This caused us to question whether the A2BAR is involved in the regulation of chondrocyte differentiation. To this end, we chose to study chondrocyte differentiation *in vitro*, utilizing mouse embryonic fibroblasts (MEFs). Though these cells are a heterogeneous mixture of embryonic cells, they have been shown to differentiate in culture similarly to MSCs [132]. Initially, we found that A2BAR KO MEFs have a higher baseline expression of the chondrocyte differentiation transcription factor Sox9, relative to WT. Additionally, this expression did not increase after 24 hours post-chondrocyte-induction, as it did in the WT cells. These results are difficult to interpret as the MEFs isolated from A2BAR KO embryos are likely different than those isolated from WT mice in numerous ways, and therefore may respond differently to culturing and differentiation conditions. A more powerful experiment will be to knockdown A2BAR expression in WT MEFs prior to chondro-induction. These results could reveal a more direct role of the A2BAR on chondrocyte differentiation.
In addition to testing the effect of absence of the A2BAR on chondrocyte differentiation, we also examined whether receptor activation would effect chondrocyte differentiation. For these experiments, we treated cultures with the specific A2BAR agonist BAY 60-6583 (Bay). We found that A2BAR activation caused a decrease in the expression of Sox9 and tended to decrease chondrocyte differentiation, though a larger sample size is needed. We explored a number of different mechanisms for this decreased expression. We have previously shown that A2BAR activation causes the upregulation of Runx2 mRNA expression in bone marrow-derived MSCs [54]. As Runx2 is thought to promote osteoblasts versus chondrocyte differentiation of MSCs [9], we tested whether A2BAR activation is causing an increase in Runx2 mRNA, which is subsequently downregulating Sox9 mRNA expression. We found that in these cultures, A2BAR activation had no effect on Runx2 mRNA levels at time points when Sox9 is already decreased. Therefore, it does not appear that Bay treatment decreases Sox9 mRNA expression through increases in Runx2 transcription. However it is still possible that Runx2 is being regulated at the protein or activation level.

Another mechanism we explored is inhibition of chondrocyte differentiation through increased C/EBPβ expression. C/EBPβ is a transcription factor well established to be important for adipocyte differentiation, but also involved in various aspects of cell physiology, including apoptosis and metabolism, as well as differentiation to various lineages, including keratinocytes and mammary epithelial cells (reviewed in [157]). We found that A2BAR activation caused an increase in C/EBPβ mRNA expression 1 hour after chondro-induction. We have furthered this line of experimentation by treating
C/EBPβ KO MEFs with the A2BAR specific agonist Bay, during chondro-induction. We hypothesized that the inhibitory action of Bay would be diminished or abolished in the absence of C/EBPβ, if this factor is responsible for mediating the effect of Bay on Sox9. A single pilot study demonstrated that Bay treatment did not reduce Sox9 mRNA levels in C/EBPβ cells. However, C/EBPβ KO cultures had elevated Sox9 mRNA expression at baseline, relative to WT, and therefore, Sox9 expression may have been insensitive to the effect of Bay. In future experiments we will use siRNA to knockdown C/EBPβ in WT MEFs, prior to Bay treatment and chondro-induction.

**Thesis summary and future directions**

In summary, we have established a role for the A2BAR in the differentiation of osteoblasts and we were the first to utilize cells from A2BAR KO mice for osteoblast differentiation. In addition, ours is the first to demonstrate the importance of the A2BAR KO in bone repair *in vivo*. Additionally, these data support involvement of the A2BAR in the regulation of some aspects of skeletal development, such as the growth plate and some bones of the skull. Finally, our data point to the A2BAR as regulator of chondrocyte differentiation. These findings have been summarized in Schematic 4.

The A2BAR has been thought of as important modulator of inflammatory processes during physiological stress or injury, when extracellular adenosine levels are elevated [158]. Our research demonstrates a novel role of the A2BAR in MSC differentiation to osteoblasts, likely resulting in less total bone during normal and injured physiological circumstances, and possibly implications for the differentiation to the chondrocyte lineage as well. Currently, there is no evidence that extracellular adenosine
reaches concentrations necessary for A2BAR activation during development. This conjures the possibility that MSCs are secreting a sufficient amount of adenosine, or ATP, to activate A2BAR in a paracrine manner. Interestingly, Pannexin 3 is a hemichannel that has been shown to be responsible for ATP release from cells and to promote chondrocyte differentiation [153] and osteoblast differentiation [159]. Pannexin 3, in combination with extracellular nucleotidases, is one possible mechanism for increased extracellular adenosine during differentiation. The relationship between Pannexin 3 and A2BAR signaling in MSC differentiation could be an interesting area of future research.

Another possible explanation for A2BAR activation during development is that the receptor has functions that are ligand independent, possibly through interactions with unknown protein or receptors. LRP5/6 are co-receptors involved in canonical Wnt signaling by interacting with Frizzled, a G-protein coupled receptor (GPCR) [27]. Recent studies have also shown LRP5/6 to be essential to signaling of other GPCRs including the PTH receptor [160]. Of particular interest, cAMP levels failed to increase in response to adenosine in bronchial smooth muscle cells with LRP6 and LRP5/6 knocked down by siRNA [160]. The particular adenosine receptor was not identified in this study and further experiments with specific adenosine receptor agonists should be performed. Another recent publication has demonstrated binding of β-catenin to the C-terminal region of the PTH receptor, which modulates PTH signaling and cAMP levels [161]. Initial analysis of the amino acid sequence of the C-terminal region of the mouse A2BAR (residues 292-332) showed zero identity with the C-terminal region of the mouse PTH
receptor 1 that was found to interact with β-catenin (residues 583-591). If the A2BAR is found to interact with Wnt signaling, either directly or indirectly, one might expect lack of the A2BAR to modulate Wnt-dependent cell differentiation. Future experiments are needed to explore these possibilities.

Finally, A2BAR KO mice have a mild inflammatory phenotype [104], and inflammation, in particular TNFα, impairs the differentiation of osteoblasts and chondrocytes [88, 162]. Our lab has identified p105, an inhibitor of NFκB, as being stabilized by binding to the A2BAR and subsequently inhibiting NFκB signaling by sequestering in the cytoplasm [106]. Hence, in A2BAR KO cells, NFκB is more active. This may be inhibiting osteoblast and chondrocyte differentiation and thereby altering the development of the skeleton and the repair of the bone after fracture. Our in vitro data showing A2BAR activation to increase osteoblast differentiation and decrease chondrocyte differentiation might seem inconsistent with this explanation, as it was demonstrated that agonism of the receptor did not impact the interaction with p105 [106] and, there is not indication that A2BAR activation and signaling has an inhibitory effect on NFκB activation.
Schematic 4: Summary of findings regarding the activation of the A2BAR during osteoblast and chondrocyte differentiation, with ultimate effects on bone formation. Unknown mechanisms for future experiments are depicted in blue and represented by (X) and unknown effects of the A2BAR, also yet to be investigated, are depicted in blue and represented by (?). Lines depicted in green illustrate processes identified during this PhD thesis research.
We have found that A2BAR activation in MEFs, during chondro-induction, leads to an early upregulation of C/EBPβ mRNA expression. The majority of the data on C/EBPβ role in MSC differentiation is in the adipocyte lineage, though there are some studies in osteoblasts and chondrocytes. C/EBPβ KO mice exhibit a dwarfed phenotype at age e16.5 [163, 164]. Interestingly, after birth the skeletons of the C/EBPβ KO mice begin to “catch up” to their WT littermates and there is no difference by 1 week of age [163]. This may be explained by compensation by other C/EBP family members [163]. Utilizing primary cells from the C/EBPβ KO mice, as well as overexpression of C/EBPβ in an MSC cell line, C/EBPβ was found to transactivate p57Kip2, thereby halting proliferation and promoting cell hypertrophy, in differentiated chondrocytes [163]. The effect of C/EBPβ during the initial differentiation of chondrocytes was not reported in this study. We hypothesize, and are further testing, that the decrease in Sox9 mRNA expression with A2BAR activation is a result of the upregulation of C/EBPβ. A direct effect of C/EBPβ on Sox9 expression has not been described. Analysis of the mouse Sox9 gene promoter revealed two putative C/EBPβ binding sites at -20Kb (AGGTTGCGACAGTC and AGATTGAGAAACGT) and C/EBPα binding sites at -20Kb (AGGTTGCGACAGTC) and +2Kb (AGGTGCACTCCCC) from the transcriptional start site. A CHIP assay of the Sox9 gene promoter during chondrocyte differentiation, in the presence of A2BAR activation, would be informative in regard to negative regulators of the Sox9 promoter. Microarray analyses of C/EBPβ KO endometrial stromal cells [165] and mammary epithelial cells [166] did not reveal any
changes in Sox9 mRNA expression, however Sox9 is likely not expressed significantly in these cell types. A microarray analysis of C3H10T1/2 cells, an MSC cell line, overexpressing C/EBPβ did reveal a 1.3 fold decrease in Sox9 expression, supporting the notion that C/EBPβ is a negative regulator of Sox9 [163]. Whether this effect is direct or indirect remains to be determined. Of note, there was no change in A2BAR expression in these C/EBPβ overexpressing cells, according to the microarray data [163]. However, because these cells were analyzed 1 week into chondrocyte differentiation, based on our own observations, we would expect A2BAR expression to already be downregulated. Taken together, an additional line of future experiment could focus on identifying a potential connection between A2BAR signaling, C/EBPβ expression and a consequential control of Sox9 and chondrocyte differentiation.
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Bibliography


CURRICULUM VITAE

Shannon H. Carroll
DOB: 1981

Lab:
Boston University
School of Medicine
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175 Centre St #314
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Education:
Ph.D. Boston University School of Medicine, Department of Biochemistry, Sept. 2008-current
  Thesis Title: The role of A2B adenosine receptor in the differentiation of mesenchymal stem cells to osteoblasts and chondrocytes; implications for bone formation and fracture repair.
  (Advisor: Dr. Katya Ravid)
  Expected graduation: May 2013

  Minor: Nutrition
  Thesis Title: The effects of conjugated linoleic acid on body composition in mouse models of sarcopenia and cachexia
  (Advisor: Dr. Lance Baumgard)

B.Sc. University of Arizona, Veterinary Medicine, May 2004
  Minors: Chemistry, Ecology and Evolutionary Biology

Research Experience:
Laboratory Manager – Boston University School of Medicine, June 2007 – August 2008
Dr. Katya Ravid’s Laboratory. Responsibilities of this position were to perform research experiments and to manage the laboratory.

Dr. Lance Baumgard’s Laboratory. Objectives of this work were to determine the effect that dietary conjugated linoleic acid has on the body composition of aged mice.

Laboratory Assistant – University of Arizona, May 2004 – July 2005
Nutritional Physiology Lab. Responsibilities of this position were to conduct multiple rodent trials requiring whole animal body composition analysis. Also included was assisting with departmental and graduate research projects.

Conservation Biology Internship Program – University of Arizona, Aug. 2002 – May 2004
Internship consisted of coursework in experimental design and the presentation of data, as well as a research project where I determined the palatability of bullfrog tadpoles to non-native fish species.
Academic Awards/Fellowships:
2009 – 2011  T32 Research Training in Hematology (HL007501-29)

Academic Involvement:
2010-2011  Biochemistry Student Organization: Chair
2010-2011  Biochemistry Student Organization: Admissions Committee
2008-2009  Biochemistry Student Organization: Student representative

Teaching Experience as Teaching Assistant:
Courses
Spring 2012  FC705 – Graduate level Physiology of Specialized Cells
Spring 2007  Plant Science 312 - Undergraduate Animal and Plant Genetics
Spring 2006  Plant Science 312 – Undergraduate Animal and Plant Genetics

Lab Responsibilities:
2008-2010  Chemical Safety Officer in Dr. Ravid’s Laboratory
2010-2011  IACUC Officer in Dr. Ravid’s Laboratory
(significant experience with mouse IACUC protocols)

Professional Associations:
2010-2011  American Society of Biochemistry and Molecular Biology
2005-2007  American Society of Nutritional Sciences

Publications:
Refereed Articles

**Carroll, S.H.,** Gerstenfeld, L.C., Ravid, K. The control of differentiation of mesenchymal stem cells to chondrocytes by the A2B adenosine receptor. *In preparation*


Reviews


Abstracts for Attended Conferences


