Ectopic Mineralization of Spinal Tissues in Mice Lacking Equilibrative Nucleoside Transporter 1

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Graduate Program in Physiology
A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science
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ECTOPIC MINERALIZATION OF SPINAL TISSUES IN MICE LACKING EQUILIBRATIVE NUCLEOSIDE TRANSPORTER 1

(Thesis format: Integrated Article)

by

Sumeeta Warraich

Graduate Program in Physiology

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

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ABSTRACT

Equilibrative nucleoside transporter 1 (ENT1) regulates the bi-directional transfer of hydrophilic nucleosides, such as adenosine, across the plasma membrane. Mice lacking ENT1 developed calcified lesions resembling diffuse idiopathic skeletal hyperostosis (DISH) in humans. By 12 months of age, ENT1−/− mice exhibit spine stiffness, hind limb dysfunction, and paralysis. Histological examination of ENT1−/− mice revealed irregular accumulations of eosinophilic material in paraspinal ligaments and entheses, intervertebral discs, and sternocostal articulations. There was no evidence of mineralization in appendicular joints or blood vessels. Analysis of intervertebral disc tissues of 6 month old ENT1−/− and wild-type mice revealed reduced expression of mineralization inhibitors, including matrix gla protein (Mgp), ectonucleotide pyrophosphatase/phosphodiesterase 1 (Enpp1), progressive ankylosis (Ank), and osteopontin (Spp1). The expression of these genes was not altered in tissues which do not exhibit ectopic mineralization. Thus, a reduction of proteins that normally prevent soft tissue mineralization may induce the ectopic calcification of spinal tissues observed in ENT1−/− mice.

Key words: biomineralization, adenosine, equilibrative nucleoside transporters, intervertebral disc, soft connective tissue
Never give up on something you cannot go a day without thinking about.
ACKNOWLEDGEMENTS

Thank you to Dr. James Hammond and Dr. Cheryle Séguin for providing me with the opportunity to carry out my Master’s degree within their labs. A heartfelt thanks to Dr. Jeff Dixon, my surrogate supervisor, who always had his door open to me, patiently guiding me in the right direction. To the above supervisors and members of my advisory committee, my research moved forward in pleasantly unexpected ways – thank you for the invaluable insight and advice that was provided along the way. A special thanks to Diana Quinonez for assisting in my research and providing invaluable help (professionally and personally) – my gratitude and appreciation is endless. Thank you to Courtney Brooks for her assistance in the lab. A heartfelt thank you is owed to Dr. Malhontra, without whose weekly chats, I would have been unable to see my full potential as a student, a scientist, and as a person.

I am extremely grateful for all my lab mates (past and present) for their endless assistance, words of encouragement, and countless laughter. I came from one lab into another, which fortunately led me to interact with many intelligent scholars. Jaime Park, Eddie Chan, Priya Patel, Matthew McCann, Katherine Lee, Michael Pest, Anusha Ratneswaran, Teresa Dean, Arielle Best, Jake Bedore, and Ilma Xhaferllari – I am glad to have met you, and thank you for the support you provided me in times of happiness and struggle. I would like to thank Kim Beaucage, my friend and my roommate, a wise soul who helped me every step of the way as I completed this chapter in my life. Lauren Solomon, thanks for giving me uncensored advice – you told me what I needed to hear.

To my friends back home, V, Shahid, Asmaa, Tanuj, I am indebted to you for keeping me sane and laughing; even with the distance, I always felt you near. V – our daily chats got me through some of the hardest days, and I am forever grateful for you. Shahid – your ability to make me laugh for hours was a gift, thank you for always being there for me. Asmaa – I think I would have missed you, even if we had not met. Tanuj – knock knock, love; thanks for consistently checking up on me. To my biology crew girls, Kavita, Priya, and Nina – thanks for showing me that hard work does pay off. Your friendships, positivity, and advice have meant the world to me.

To my family – the people I missed the most – thanks for being there, I love you. To my mom and dad, thank you for the love and support. Mom – thank you for your unending love, the delicious food you made especially for me, for being my biggest supporter and always believing in me, and for encouraging me to always move forward even when it was difficult. To my siblings Suneea, Sujeeta and Jashan, thanks for everything (the jokes, the laughs, and the memories that we have shared and will continue to do so in the future).

To Nimesh, the one closest enough to my thoughts to truly know what the completion of this thesis means; know that without your continuous, unwavering love, encouragement and support, this achievement would not have been possible. This thesis is as much yours, as it is mine.
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<tr>
<td>Ada</td>
<td>adenosine deaminase</td>
</tr>
<tr>
<td>Adk</td>
<td>adenosine kinase</td>
</tr>
<tr>
<td>Adora</td>
<td>adenosine receptors</td>
</tr>
<tr>
<td>AF</td>
<td>annulus fibrosus</td>
</tr>
<tr>
<td>ALL</td>
<td>anterior longitudinal ligament</td>
</tr>
<tr>
<td>Alpl</td>
<td>alkaline phosphatase</td>
</tr>
<tr>
<td>AMP</td>
<td>adenosine monophosphate</td>
</tr>
<tr>
<td>Ank</td>
<td>progressive ankylosis</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>Bglap</td>
<td>osteocalcin</td>
</tr>
<tr>
<td>Bp</td>
<td>base pair</td>
</tr>
<tr>
<td>BSP</td>
<td>bone sialoprotein</td>
</tr>
<tr>
<td>Ca$^{2+}$</td>
<td>calcium</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic AMP</td>
</tr>
<tr>
<td>CEP</td>
<td>cartilage endplate</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CNT</td>
<td>concentrative nucleoside transporter</td>
</tr>
<tr>
<td>Col10a1</td>
<td>collagen, type X, alpha 1</td>
</tr>
<tr>
<td>COL6A1</td>
<td>collagen, type 6, alpha 1</td>
</tr>
<tr>
<td>COL11A2</td>
<td>collagen, type 11, alpha 2</td>
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<tr>
<td>CT</td>
<td>cervical-thoracic</td>
</tr>
<tr>
<td>DISH</td>
<td>diffuse idiopathic skeletal hyperostosis</td>
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</table>
DNA  deoxyribonucleic acid
ECM  extracellular matrix
Enpp1  ectonucleotide pyrophosphatase/phosphodiesterase 1
ENT  equilibrative nucleoside transporter
ENT1−/− mice  ENT1-null mice
GAGs  glycosaminoglycans
Gdf10  growth differentiation factor 10
HA  hydroxyapatite
hENT1  human ENT1
IAF  inner annulus fibrosus
Ibsp  integrin binding sialoprotein
IVD  intervertebral disc
mENT1  mouse ENT1
Mgp  matrix gla protein
MV  matrix vesicle
MVEC  microvascular endothelial cells
NBMPR  nitrobenzylthioinosine, nitrobenzylmercaptopurine riboside
NP  nucleus pulposus
Nt5e  ecto-5′-nucleotidase
OAF  outer annulus fibrosus
OPLL  ossification of posterior longitudinal ligament
PGs  proteoglycans
P_i or PO_4^{3−}  inorganic phosphate
<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>PLL</td>
<td>posterior longitudinal ligament</td>
</tr>
<tr>
<td>PMAT</td>
<td>plasma membrane monoamine transporter</td>
</tr>
<tr>
<td>Pnp</td>
<td>purine nucleoside phosphorylase</td>
</tr>
<tr>
<td>PP_i</td>
<td>inorganic pyrophosphate</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>Runx2</td>
<td>runt related transcription factor 2</td>
</tr>
<tr>
<td>Spp1</td>
<td>secreted phosphoprotein 1, osteopontin</td>
</tr>
<tr>
<td>VB</td>
<td>vertebral body</td>
</tr>
<tr>
<td>WT</td>
<td>wild-type</td>
</tr>
<tr>
<td>Xdh</td>
<td>xanthine dehydrogenase</td>
</tr>
<tr>
<td>µCT</td>
<td>micro-computed tomography</td>
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</table>
Chapter 1

Introduction
1.1 Nucleobases, Nucleosides, and Nucleotides

Nucleobases, also known as nitrogenous bases, are nitrogen-containing heterocyclic compounds present in DNA, RNA, nucleotides and nucleosides. Based on their structure, nucleobases can be classified into one of two groups. Purine bases contain two carbon-nitrogen rings, whereas pyrimidine bases contain one carbon-nitrogen ring (1). There are five nucleobases: adenine, guanine, cytosine, thymine (DNA only) and uracil (RNA only); adenine and guanine are purine bases and cytosine, thymine and uracil are pyrimidine bases.

Nucleosides (adenosine, guanosine, cytidine, thymidine, uridine and inosine) are pyrimidine or purine nitrogenous bases attached to a ribose or 2-deoxyribose ring of a pentose sugar via a β-glycosidic bond (2) (Fig. 1.1). Nucleotides are nucleosides bound to one or more phosphate groups that covalently attach to the ribose or 2-deoxyribose ring of the pentose sugar (3).

Nucleosides are important biological molecules. These molecules form the structural subunit of nucleic acids (DNA and RNA) and nucleotides (such as adenosine triphosphate, ATP) that are important in biological processes like cellular replication and energy supply (4). Nucleosides and nucleotides have been shown to serve as neurotransmitters and neuromodulators (5,6), and play important roles in cell signaling through cyclic adenosine monophosphate (cAMP) and adenosine (discussed in section 1.2).

Cytotoxic nucleoside analogues and nucleobases are also used as antiviral, antiparasitic and anticancer agents, and induce toxicity by interacting with a large number
Figure 1.1: Chemical structures of physiological nucleosides. (modified from Young et al., 2008)
Adenosine, inosine, and guanosine are purine nucleosides while cytidine, uridine, and thymidine are pyrimidine nucleosides.
of intracellular targets (7-9). Cytotoxic nucleoside analogues compete with physiological nucleosides as these molecules share common characteristics, including transport mediated by membrane transporters, activation by intracellular processes that retain the residues in the cell, and formation of the active derivatives (7). These agents can exert cytotoxic activity by direct incorporation into nucleic acids; by interfering with various enzymes involved in the synthesis of nucleic acids; or by modifying the metabolism of physiological nucleosides (7,8).

1.2 Adenosine

Adenosine is a purine nucleoside which plays a critical role in many different processes. Adenosine has a well-established role as the building block for the important energy molecule ATP, and is also required for nucleic acid synthesis. Free adenosine is an important signaling molecule (10). It is recognized as a local regulator of tissue function, especially when the available energy supply is unable to meet cellular energy demands (11). In cases like this, adenosine is produced by the tissue under stress which increases the energy supply (by vasodilation) and decreases the energy demand by acting as a negative feedback regulator.

There has been an increasing interest in the role of purinergic signaling, specifically that of adenosine, in the physiology and pathophysiology of musculoskeletal tissues (12). Recent studies point to a critical role for purine metabolism in the regulation of biomineralization in diseases associated with either insufficient or ectopic mineralization (Fig. 1.2) (12-15). A study showed that mutations in the gene encoding
Figure 1.2: Schematic of pathways influencing biomineralization.
Adenosine can regulate cell behaviour and gene expression through cell surface receptors. ATP is released into the extracellular fluid and subsequently metabolized by cell-surface enzymes. Ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1) metabolizes ATP, and releases inorganic pyrophosphate (PP\textsubscript{i}), which inhibits mineralization. Extracellular PP\textsubscript{i} levels are also regulated by progressive ankylosis protein (ANK), a putative transporter that mediates PP\textsubscript{i} efflux. PP\textsubscript{i} is metabolized by another cell surface enzyme, alkaline phosphatase (ALPL), which produces inorganic phosphate (P\textsubscript{i}), promoting mineralization. P\textsubscript{i} and Ca\textsuperscript{2+} accumulate in matrix vesicles or on extracellular matrix components resulting in the crystallization of calcium phosphate, leading to formation of hydroxyapatite and thus, matrix calcification. In addition to the regulation of biomineralization through changes in PP\textsubscript{i} levels or PP\textsubscript{i}:P\textsubscript{i} ratio, cells express and secrete proteins that can promote or inhibit calcification of the extracellular matrix. These proteins include the mineralization inhibitors matrix Gla protein (MGP) and osteopontin (SPP1).
ENPP1 have been associated with a hypermineralization disorder known as idiopathic infantile arterial calcification (13). ENPP1 is an ecto-enzyme responsible for the hydrolysis of ATP to adenosine monophosphate (AMP), generating inorganic pyrophosphate \( \text{PP}_i \), which inhibits soft tissue calcification. Through characterization of mice lacking ecto-5'-nucleotidase (\( \text{Nt}5e \)), Takedachi and colleagues demonstrated that adenosine promotes osteoblast differentiation (12). The \( \text{Nt}5e \) gene encodes the CD73 cell-surface enzyme, which converts AMP to adenosine. Furthermore, it was found that CD73\(^{-}\) mice had significantly lower bone mineral content, and as a result reduced bone volume and thickness, compared to wild-type controls (12). As demonstrated by St. Hilarie et al., mutations in \( \text{NT}5E \) show a genetic association with symptomatic arterial and joint calcifications in humans (14). All reported mutations in \( \text{NT}5E \) resulted in a non-functional CD73 enzyme. In addition, cultured fibroblasts from affected individuals showed reduced expression of the \( \text{NT}5E \) mRNA and the CD73 protein, increased tissue-nonspecific alkaline phosphatase (\( \text{ALPL} \)) enzyme activity, and consequently the accumulation of calcium phosphate crystals (14). \( \text{ALPL} \) is a cell-surface enzyme responsible for hydrolyzing \( \text{PP}_i \) to inorganic phosphate (\( \text{P}_i \), which promotes calcification). These studies suggest a possible link between adenosine metabolism and the regulation of biomineralization.

1.2.1 Adenosine Bioactivity

Adenosine, released locally, mediates its physiological and pharmacological actions via interactions with G protein-coupled receptors. Extracellular adenosine acts as a ligand for purinergic cell surface adenosine receptors; there are four subtypes of these receptors – \( \text{A}_1, \text{A}_2a, \text{A}_2b, \text{A}_3 \) – with specific tissue distribution, pharmacology, and
signaling mechanisms (16,17). The A_1 and A_3 receptors signal through an inhibitory G protein (G_i), which inhibits cAMP production by adenylate cyclase. In contrast, the A_{2a} and A_{2b} receptors signal through a stimulatory G protein (G_s), which increases cAMP production (16,18). cAMP has been shown to induce abnormal calcification of vascular smooth muscle cells via a mechanism involving reduction in extracellular PP_i accumulation (19).

Bone is a dynamic organ that undergoes continuous remodeling through a balance of bone formation and bone resorption. Osteoblasts, which are bone forming cells, synthesize new bone, while osteoclasts resorb bone (20,21). Recent studies have shown that adenosine receptors are involved in osteoblast and osteoclast differentiation and function. Studies demonstrated the presence of all four adenosine receptors in murine bone marrow cell osteoclast precursors, and showed that receptors were up-regulated during osteoclast differentiation (22). In the A_1 receptor-knockout mouse, there was a significant increase in bone density and volume due to compromised osteoclast-mediated resorption ability (23). Furthermore, the presence of A_1 receptor antagonists have been shown to inhibit osteoclast formation (24). In contrast to the A_1 receptor, activation of the A_{2a} receptor inhibits osteoclastogenesis; mice deficient in the A_{2a} receptor show a reduction in bone density (25). It has also been reported that the A_{2a} receptor plays a role in promoting the proliferation of mouse bone marrow-derived mesenchymal stem cells and is up-regulated at later osteoblast differentiation stages (26,27), however the A_{2b} receptor is reported as the functionally dominant receptor involved in osteoblast differentiation (28). Little is known about the role of the A_3 receptor in musculoskeletal
tissues, however the receptor is expressed on the surfaces of osteoblasts (29), and its selective agonist increases primary osteoblast proliferation (30).

1.3 Nucleoside Transporters

As hydrophilic molecules, nucleosides and their analogues require specialized glycoprotein transporters to cross the hydrophobic cellular membrane (4,8). Given the importance of these molecules in intracellular nucleotide generation, as well as extracellular signaling, the ability of cells to accumulate these molecules depends on their efficient movement across membranes. In addition, cells incapable of de novo nucleoside synthesis, such as enterocytes, bone marrow cells, and certain brain cells require transporters to salvage nucleosides from the extracellular environment (4). In mammals, nucleoside transport is carried out by seven transporters which are classified into two distinct families based on their transport mechanism: the concentrative nucleoside transporters (CNTs 1-3, also known as SLC28) and the equilibrative nucleoside transporters (ENTs 1-4, also known as SLC29) (4,8,31).

1.3.1 Concentrative Nucleoside Transporters (CNTs)

CNTs are Na\(^+\)-dependent symporters that move nucleosides and nucleobases against a concentration gradient. The CNT family consists of three subtypes (CNT1, CNT2, and CNT3), all of which differ in their substrate specificities (32). CNT1 prefers pyrimidine nucleosides and adenosine, CNT2 prefers purine nucleosides and uridine, and CNT3 transports both pyrimidine and purine nucleosides. CNTs are primarily found in barrier cell types such as renal and intestinal epithelia, the blood-brain barrier and blood-testes barrier, and thus play a role in absorption, distribution and elimination of nucleosides and their analogues (33).
**1.3.2 Equilibrative Nucleoside Transporters (ENTs)**

ENTs are Na\(^+\)-independent, facilitative diffusion transporters which transport nucleosides bi-directionally down a concentration gradient. ENTs are widely distributed among cell types (4,33). The ENT family consists of four members (ENT1, ENT2, ENT3, and ENT4) which share an ability to transport adenosine but differ in their abilities to transport other nucleosides and molecules (31). For example, ENT2 has a higher affinity for inosine than ENT1, and is the only transporter that transports nucleobases. In addition, many studies reveal that ENT3 has a similar substrate specificity as ENT2, but a lower affinity (31). The distribution of these transporters is not homogenous among tissues, and expression is regulated in a cell-type specific manner. ENT orthologues have been identified in many different eukaryotic species; over the course of evolutionary time, the protein has been found to be highly conserved, highlighting the physiological significance of ENTs (34). It should be noted that mice have the same nucleoside transporter proteins as humans and share similar characteristics (Table 1.1); although studies to date have suggested that certain ENTs are ubiquitous, expression has yet to be investigated in all tissues (35).

ENT1 and ENT2 have been the most extensively characterized transporters and were distinguished based on their sensitivity to inhibition by nitrobenzylthioinosine (nitrobenzylmercapturine riboside; NBMPR). ENT1 is *sensitive* to inhibition by NBMPR at nMolar concentrations and is referred to as the *es* transporter, or equilibrative-sensitive. ENT2 is *resistant* to inhibition by NBMPR, requiring \(10^4\) Molar concentrations and is referred to as the *ei* transporter, or equilibrative-insensitive (Fig. 1.3) (8).
Table 1.1: Properties of the mammalian nucleoside transporters (modified from Young et al., 2008)

<table>
<thead>
<tr>
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<th>Number of residues</th>
<th>Permeant selectivity</th>
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<td>465</td>
<td>Purine and pyrimidine nucleosides and nucleobases</td>
<td>Ubiquitous</td>
</tr>
<tr>
<td>hENT2</td>
<td>465</td>
<td>Purine and pyrimidine nucleosides and nucleobases</td>
<td>Ubiquitous</td>
</tr>
<tr>
<td>hENT3</td>
<td>475</td>
<td>Purine and pyrimidine nucleosides and adenine (at pH 5.5)</td>
<td>Ubiquitous</td>
</tr>
<tr>
<td>hENT4</td>
<td>530</td>
<td>Adenosine (at pH 5.5) and organic cations, including serotonin</td>
<td>Heart, liver, brain</td>
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<tr>
<td>mENT1.1</td>
<td>460</td>
<td>Purine and pyrimidine nucleosides</td>
<td>Ubiquitous</td>
</tr>
<tr>
<td>mENT1.2</td>
<td>458</td>
<td>Purine and pyrimidine nucleosides</td>
<td>Ubiquitous</td>
</tr>
<tr>
<td>mENT2</td>
<td>456</td>
<td>Purine and pyrimidine nucleosides and adenine</td>
<td>Ubiquitous</td>
</tr>
<tr>
<td>mENT3</td>
<td>475</td>
<td>Purine and pyrimidine nucleosides and nucleobases</td>
<td>Ubiquitous</td>
</tr>
<tr>
<td>mENT4</td>
<td>528</td>
<td>Adenosine and adenine (at pH 5.5), organic cations not tested</td>
<td>Heart, liver, brain</td>
</tr>
</tbody>
</table>
Figure 1.3: Mammalian nucleoside transport systems of the equilibrative nucleoside transporter (ENT) and concentrative nucleoside transporter (CNT) protein families. (modified from Young et al., 2008)

ENTs are Na\(^+\)-independent, facilitative diffusion transporters which transport nucleosides and nucleobases bi-directionally down a concentration gradient. ENT1 and ENT2 are distinguished based on their sensitivity to inhibition by nitrobenzylthioinosine (NBMPR). ENT1 is sensitive to inhibition by NBMPR at nanomolar concentrations. ENT2 is resistant to inhibition by NBMPR. CNTs are Na\(^+\)-dependent symporters that move nucleosides and nucleobases against a concentration gradient.
ENT1 is ubiquitously distributed in human and rodent tissues, but abundance varies from tissue to tissue. For example, human ENT1 (hENT1) is most abundant in the frontal and parietal lobes of the cortex, thalamus and basal ganglia (36). hENT1 expression has also been noted in the brain, heart, liver, placenta, adrenal gland, erythrocytes, stomach and vascular endothelium (37). With respect to mouse ENT1 (he), expression has been found in similar tissues such as the heart, brain, and liver, as well as the colon, spleen, and kidney (35). Although ENT1 is primarily found on the surface of cells, functional hENT1 has also been associated with the membranes of the endoplasmic reticulum and nuclear envelope, suggesting that this transporter may play a role in the passage of nucleosides between these compartments and the cytosol (38). A stark difference that should be noted is that hENT1 has also been found functionally expressed in the mitochondria, whereas mENT1 has no association with the mitochondria (39,40).

ENT2 differs from ENT1 in its ability to transport nucleobases such as hypoxanthine, in addition to nucleosides (31,41). mENT2 mRNA has been identified in a variety of tissues such as the small intestine, kidney, spleen, heart, and liver, but is particularly abundant in skeletal muscle (35).

ENT3 and ENT4 are less characterized but appear to have a more specialized role in the cell (8,42). The ENT3 transcript and protein is abundant in many human and rodent tissues but is highly expressed in placental tissue (42). Unlike ENT1 and ENT2, ENT3 is not found at the plasma membrane, but rather is located on intracellular membranes (42). ENT3 contains an endosomal/lysosomal characteristic motif, which along with its ability to optimally function at a pH of 5.5, suggests that the location of the transporter is in an acidic, intracellular compartment such as the lysosome (8,42,43). ENT4, present at the
cell surface, was originally identified as a monoamine/organic transporter and was named plasma membrane monoamine transporter (PMAT), since it transports organic cations such as MPP+, dopamine and serotonin (44-46). It was later discovered that ENT4 was able to efficiently transport adenosine at an acidic pH of 5.5 (47), and hypothesized to contribute in the regulation of extracellular adenosine concentrations under acidic conditions associated with ischemia (8). ENT4 is expressed primarily in the heart, liver and brain (8).

It is important to note that the reason and degree of functional redundancy between EN Ts is not known; it is unclear why so many cells and tissues express multiple nucleoside transporters with similar or overlapping substrate selectivity (35).

1.4 ENT1-null mice (ENT1⁻/⁻)

The ENT1⁻/⁻ mouse was created by Dr. Doo-Sup Choi at the Mayo Clinic College of Medicine. The ENT1⁻/⁻ mouse was generated on a C57BL/6J x 129X1/SyJ background using the targeting vector pKSloxPNT-mod to delete coding exons 2 to 4 (Fig. 1.4). This targeting strategy eliminated 425 of the 1380 base pairs in the protein coding-region (48) and produced a non-functional protein.

Initial studies demonstrated that at 3 months of age, ENT1⁻/⁻ mice had <10% lower body weight, but showed no differences in open-field locomotor activity, and had no evidence of gross anatomical abnormalities in the brain compared to their wild-type (WT) littermate controls (48). There were also no significant differences reported in spontaneous mortality rates up to 6 months of age.
Figure 1.4: Generation of ENT1-null mice. (modified from Choi et al., 2004)
Figure shows the organization of the gene encoding mouse ENT1, the targeting construct, and the allele resulting from homologous recombination. Boxes represent the targeted exons (2–4), which span 425 base pairs. Gray arrowheads represent $loxp$ sequences, the recognition sites for Cre recombination, and deleted coding exons 2–4.
These mice were developed to investigate the role of adenosine transport in the central nervous system pathways regulating alcohol consumption (48,49). In these studies, $ENT1^{-/-}$ mice consumed significantly higher amounts of ethanol, and had a greater preference for ethanol than water. Despite the increase in ethanol consumption, these mice demonstrated a decreased ataxic effect of ethanol. This behavior was associated with a reduced stimulation of $A_1$ receptors due to a decrease in endogenous adenosine tone and not due to the loss of $A_1$ receptors or a decrease in $A_1$ receptor affinity (48). $ENT1^{-/-}$ mice have also been shown to exhibit reduced anxiety-like behaviors, specifically mediated by $A_1$ receptors (49).

Previous studies in the Hammond lab established that microvascular endothelial cells (MVECs) isolated from $ENT1^{-/-}$ mice demonstrate compromised nucleoside transport compared to cells isolated from WT controls. There was no compensation reported for the loss of $ENT1$ by other transporters (50). In contrast, MVECs isolated from $ENT1^{-/-}$ mice show an increase in the $A_{2a}$ receptor and adenosine deaminase ($Ada$) mRNA transcript and protein compared to WT cells (50). $Ada$ is a metabolic enzyme that converts adenosine to a related nucleoside, known as inosine. At 2-3 months of age, $ENT1^{-/-}$ mice were also found to have a significantly lower blood pressure, possibly due to the 2.8-fold increase reported in circulating adenosine concentrations. Furthermore, others have demonstrated that cardiomyocytes from $ENT1^{-/-}$ mice appear to be protected from ischemic insult and that $ENT1^{-/-}$ mice show decreased myocardial infarction in response to ischemia and hypoxia (10).
1.5 Connective Tissues

During previous studies in the Hammond lab using the \( ENTI^{+/−} \) mice, it was observed that these mice develop a mineralized soft connective tissue phenotype. Connective tissue is the most diverse tissue type with a wide variety of functions; it can be considered the “glue” that holds the body together. All forms of connective tissue are made up of: (i) extracellular matrix molecules, (ii) amorphous matrix called ground substance, and (iii) stationary and migrating cells (51). Depending on the structural requirements, the proportions of these three components vary from one part of the body to another. The extracellular matrix (ECM) consists of collagenous, elastic, and reticular fibers. The amorphous matrix surrounds the cellular and fibrous components and is made up of glycosaminoglycans (GAGs), proteoglycans (PGs) and glycoproteins. Fibroblasts are the principle cells of connective tissue that synthesize all of the ECM components (collagen, elastic and reticular fibers, and the complex carbohydrates of the ground substance) (52). Specialized connective tissue includes cartilage, blood, bone, ligaments, and intervertebral discs.

1.5.1 Cartilage

Cartilage is an avascular and aneural tissue composed of two distinct components, chondrocytes and the ECM (53). Chondrocytes are specialized cells of mesenchymal origin that produce and, to some degree, maintain the cartilage tissue. More than 95% of the cartilage volume consists of the ECM, which is the functional element of this tissue (53,54). Despite the low fraction of chondrocytes in the cartilage, these cells are important as they produce the matrix that provides mechanical stability to the tissue (52,54). The composition of the ECM is important to the survival of chondrocytes; the
large ratio of GAGs to type II collagen fibers in the matrix allow for the diffusion of nutrients from blood vessels in surrounding tissue to the chondrocytes (52).

Cartilage is a key tissue in the development of the fetal skeleton and growing bones (52). In healthy cartilage, physiological remodeling of the cartilage is accomplished through a balance between matrix synthesis and matrix degradation (55). Based on the characteristics of their matrix, there are three types of cartilage that differ in appearance and properties: hyaline cartilage, elastic cartilage and fibrocartilage.

1.5.2 Hyaline cartilage, elastic cartilage and fibrocartilage

*Hyaline* cartilage consists predominantly of type II collagen fibers, GAGs, PGs, and glycoproteins and is found on the articular surfaces of synovial joints, costal cartilages of the rib cage, the nasal cavity and the trachea. In early fetal development, hyaline cartilage forms the template for the skeleton, acting as the precursor of bones that develop by endochondral ossification (56).

*Elastic* cartilage is characterized by the presence of elastic fibers within an ECM with a composition otherwise similar to hyaline cartilage; the elastin fibers and collagen are arranged in a wavy pattern that makes it flexible. It is found in the outer ear and the epiglottis of the larynx. In contrast to hyaline cartilage, elastic cartilage does not calcify with age (52).

*Fibrocartilage* is rich in type I collagen fibers in addition to the ECM composition of hyaline cartilage; it is a combination of dense regular connective tissue and hyaline cartilage (52). It is the only type of cartilage that contains type I collagen in addition to the normal type II. Fibrocartilage is a tough form of cartilage that consists of rows of chondrocytes in lacunae between parallel bundles of collagenous fibers, which
allow the tissue to be strong and flexible. Fibrocartilage lacks a perichondrium, which is a layer of dense irregular connective tissue that surrounds the cartilage of developing bone, and can be found around hyaline and elastic cartilage. Fibrocartilage is present in the annulus fibrosus of intervertebral discs (IVDs), menisci of the knee joint, articular discs of the sternoclavicular and temporomandibular joints, the pubic symphysis and certain insertion points of tendons (52). Fibrocartilage is a transitional tissue where soft connective tissues anchor into bone or cartilage, especially at joints. Fibrocartilage at these locations serves as a shock absorber, and provides resistance to both compressive and shearing forces (52). The tissue provides support and rigidity to attached and surrounding structures, and is the strongest of the three types of cartilage.

1.6 Fibrocartilaginous Tissues of the Axial Skeleton

1.6.1 The Intervertebral Disc

The vertebral column makes up a part of the axial skeleton, and supports the head and encloses the spinal cord. It consists of alternating vertebral body (VB) bones and soft connective tissue IVDs. The mouse vertebral column can be divided into five distinct regions: cervical (7 vertebrae), thoracic (13 vertebrae), lumbar (6 vertebrae), sacral (4 fused vertebrae), and caudal (28 vertebrae) (57,58).

The IVD is a specialized connective tissue that: protects against mechanical loading delivered by the body mass; acts as the spine’s shock absorbing system; and provides limited vertebral motion, such as extension and flexion (59,60). The IVD is composed of three distinct yet interdependent tissues: 1) nucleus pulposus (NP);
2) annulus fibrosus (AF) (inner annulus fibrosus, IAF; outer annulus fibrosus, OAF); and
3) cartilage endplates (CEP) (Fig. 1.5).

The NP is a highly-hydrated gelatinous tissue in the center of the IVD, formed from the embryonic notochord (61). The NP is rich in proteoglycans and water and is held together by a network of type II collagen and elastin fibers. It contains two distinct cell types, the larger notochordal cells and their smaller mature derivatives known as chondrocyte-like nucleus pulposus cells (61,62). The major proteoglycan of the NP is aggrecan, which is a high molecular weight proteoglycan, that consists of 100-150 sulfated GAG chains (such as chondroitin sulfate and keratin sulfate) attached to a protein core (63). The negatively charged GAG chains of aggrecan draw in water (64), and allows the NP to distribute hydraulic pressure in all directions, enabling the IVD to resist compression and maintain its height and turgor (65-67). The amount of water in the NP varies throughout the day, depending on the activity (60).

The fibrocartilaginous AF makes up the peripheral portion of the disc, which serves to contain and protect the NP. The cells of the OAF have been described as fibroblast-like, while the cells in the IAF are more rounded. The IAF is rich in proteoglycans and type II collagen, where GAGs are intertwined between bundles of collagen (68,69). The IAF has the appearance of lamellae and is a specialized structure as it serves as the transition zone between the gelatinous NP and the ligamentous-like outer part of the annulus fibrosus, the OAF (61,70). The composition and organization of the OAF is more ligamentous and is formed by concentric lamellae, consisting mainly of type I collagen. The type 1 collagen fibers of the AF run in oblique sheets, and serve to
Figure 1.5: Schematic overview of an intervertebral disc (IVD). (Modified from Kurtz S, Edidin A. Spine Technology Handbook. 1 edn. Elsevier Academic Press, 2006)
Figure shows the gelatinous nucleus pulposus in the centre, surrounded by the fibrous annulus fibrosus, and the cartilaginous endplates adjacent to the vertebral body. The lamellar structure of the annulus fibrosus is also evident. Orientation of the IVD is denoted as axial, radial and circumferential.
anchor into the adjacent vertebral endplate (70,71). The direction of these fibers in successive layers alternate, with one layer crossing the other at angles of 30-60 degrees; this property provides tensile strength to the annulus fibrosus, allowing it to be stretched or extended.

The endplates are distinct regions found above and below the IVD between adjacent vertebrae. The CEP consists of a thin layer of hyaline cartilage between the disc and vertebral bodies, and similarly, is rich in type II collagen and proteoglycans (72,73). Through diffusion, the CEP supplies nutrients from blood vessels in the vertebral body to the inner two-thirds of the AF and the entire NP (74).

1.6.2 Paraspinal Ligaments

Ligaments are a type of dense connective tissue, where fiber bundles run in a parallel arrangement to provide tensile strength. Paraspinal ligaments are fibrous bands of dense connective tissue; and serve to provide structural flexibility, strength and stability to the vertebral column (Fig. 1.6). There are two main ligaments termed the anterior longitudinal ligament (ALL) and the posterior longitudinal ligament (PLL). The ALL is a thick, broad fibrous band that lies on the anterior surface of the VBs and IVDs and prevents hyperextension of the spine. The PLL runs on the posterior surface of the VBs and IVDs inside the spinal canal and prevents hyperflexion of the spine; the ligament is narrower and weaker than the ALL (75). The ALL and PPL merge with the AF of the IVD and are well innervated (76). There are also additional ligaments associated with the spine that provide further support and strength. The ligamentum flavum is a thick ligament which connects laminae of adjacent vertebrae and is part of the posterior wall of the vertebral column. There are also two ligaments that connect neighboring spinous
Figure 1.6: Schematic overview of paraspinal ligaments associated with the vertebral column. (Reproduced from: Save Your Aching Back and Neck, A Patient's Guide by Eidelson, 2002)

Figure shows the organization of the paraspinal ligaments that provide structural flexibility, strength and stability to the vertebral column. The paraspinal ligaments consist of the anterior longitudinal ligament, posterior longitudinal ligament, ligamentum flavum, interspinous ligament, supraspinous ligament, intertransverse ligament, and the facet capsular ligament.
processes, portions of the vertebrae that extend towards the back. The *interspinous ligament* runs between the top and bottom of the processes, whereas the *supraspinous ligament* is a stronger ligament found along the posterior surface (75). These ligaments insert into the bone, through fibrocartilaginous intermediate regions.

### 1.6.3 Sternum

The sternum is an elongated, flattened bone which forms the middle anterior portion of the thorax, and its margins connect with the cartilages of the first seven pairs of ribs (75). In humans, the sternum consists of three parts: the manubrium, the body, and the xiphoid process (77). It is important to note that before puberty, the body of the sternum consists of four sternebrae which eventually fuse to form one structure; however this is not the case in mice (77,78). The sternum in mice is made up of six components: the manubrium (rostral), followed by three separate sternebrae, the xiphisternum, and the xiphoid cartilage (58). In humans, the sternomanubrial joint (between the sternum and manubrium) is considered a cartilaginous joint, where the articulating ends are covered by hyaline cartilage and linked by fibrocartilage and ligaments (79).

### 1.7 Extracellular Matrix Calcification

Biological mineralization is a cell-regulated event that, under normal physiological conditions, is restricted to the extracellular matrices of bone, dentin, cementum, enamel and cartilage. Mineral deposition is an extremely balanced and tightly controlled process (80). There appears to be two fundamental models for how mineral deposition in bone is initiated: the cell-mediated model and the matrix-regulated model. In the cell-mediated model, authors suggest that osteoblasts, which are bone forming
cells, secrete matrix vesicles (MVs) which control the initial site of mineral deposition (52). In a mineralization-inducing environment, MVs are proposed to bud off of the outer cell plasma membrane in a polarized manner towards newly formed osteoid (81). Several studies suggest that as MVs initially bud off, the vesicles do not contain crystals, but rather that the first crystalline mineral appears after the MV has been immobilized in the collagen matrix (81). These vesicles contain enzymes, and accumulate calcium (Ca$^{2+}$) and inorganic phosphate (P$_i$ or PO$_4^{3-}$). These components allow for the crystallization of calcium phosphate, leading to the formation of initial hydroxyapatite (HA, Ca$_{10}$(PO$_4$)$_6$(OH)$_$_$_2$) crystals within the lumen of MVs (82). Once the mineral has reached a certain size, the MV ruptures, and initial crystals of HA grow rapidly to join crystals produced around other matrix vesicles, contributing to extracellular matrix calcification (81,83). It should be noted that the mechanism by which MVs accumulate Ca$^{2+}$ and P$_i$, and the mechanism by which crystals penetrate through the membrane are not yet fully understood.

On the other hand, it has been proposed that the extracellular matrix mineralizes directly, without the involvement of matrix vesicles (84,85). This matrix-regulated model states that all components necessary for the initiation and deposition of calcification are an inherent property of the extracellular matrix once they have been secreted. It has been suggested that nucleation occurs within gaps of the collagen bundles and crystals subsequently grow, mediated by certain non-collagenous proteins, which associate with the extracellular matrix (84,85).

In bone, there are several events that occur to enable mineralization (52). First, alkaline phosphatase (ALPL) increases the P$_i$ concentration, and hydrolyzes the
mineralization inhibitor, PP$_i$. It is known that PP$_i$ antagonizes the formation of HA crystals, hence the importance of having a tight balance between the levels of extracellular P$_i$ and PP$_i$ for normal mineral deposition (86). Bone sialoprotein (BSP), which is a major protein in the mineralized matrix of bone has been proposed to nucleate crystals, and initiate the deposition of calcium phosphate (87,88). BSP and several other bone proteins play a critical role in the growth of crystals, and extracellular matrix calcification.

Many genes involved in regulating mineral deposition have been identified (Fig. 1.2). ATP is released by as yet unresolved pathway into the extracellular fluid, and metabolized by cell-surface enzymes, such as ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1), which releases PP$_i$ (13). Extracellular PP$_i$ levels can also be regulated by progressive ankylosis (ANK), a transmembrane protein postulated to mediate PP$_i$ efflux [84]. PP$_i$ is metabolized by the cell-surface enzyme ALPL, to P$_i$, which promotes mineralization. The *Nt5e* gene encodes ecto-5'-nucleotidase (CD73), an enzyme that converts AMP to adenosine and P$_i$. Matrix gla protein (MGP) (89) and osteopontin (SPP1) (90) are matrix proteins that inhibit mineralization.

**1.8 Disorders of Tissue Mineralization**

Abnormal calcification of the ECM of soft connective tissues is associated with a number of pathologies including vascular disease, chronic kidney disease, and skeletal disorders (91-94). Soft tissues do not mineralize under normal conditions but, under certain pathological conditions, tissues such as articular cartilage and cardiovascular tissues are prone to mineralization (95,96). Mutations in many of the genes (mentioned in
section 1.7, and Fig. 1.2) affect the rate of generation, transport and degradation of PP, leading to an unbalanced level, which ultimately leads to ectopic deposition of mineral (12-15,86). For example, a study showed that mutations in the gene encoding ENPP1 have been associated with a hypermineralization disorder known as idiopathic infantile arterial calcification (13). In mice, mutations of *Ank* lead to arthritis, ectopic crystal formation and joint fusion throughout the body (97); in humans, dominant mutations were associated with craniometaphyseal dysplasia (98). A study by Yadav et al. reports that a disruption in *Alpl* prevents skeletal mineralization (15). MGP deficiency in mice resulted in premature calcification, and aberrant mineralization in normal cartilage including the growth plate, which eventually lead to short stature, osteopenia and fractures, and severe arterial calcification, which lead to premature death (99).

1.8.1 Ossification of the Posterior Longitudinal Ligament and Diffuse Idiopathic Skeletal Hyperostosis

Ossification of paraspinal ligaments is identified in the middle-aged and elderly, and can be diagnosed as one of two non-inflammatory conditions: (1) ossification of the posterior longitudinal ligament (OPLL), or diffuse idiopathic skeletal hyperostosis (DISH, formally known as Forestier disease (100)).

OPLL is the abnormal calcification of the posterior longitudinal ligament, and has the highest incidence in Japan (101). OPLL is commonly detected in the cervical region of the spine, where lesions can compress the spinal cord resulting in neurological complications. This condition is twice as common in woman and symptoms often present between the ages of 50 to 60 (102). Since OPLL is commonly found in Asian populations, genetic factors are believed to play a role in its development. Many genes
have been associated with OPLL, including polymorphisms in collagen 11A2 (COL11A2) (103), collagen 6A1 (COL6A1) (104) and mutations in ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1) (105).

The overall prevalence of DISH is estimated to be around 6-12% of North Americans (106). As reported by The Arthritis Society, DISH is the second most common form of arthritis, after osteoarthritis. DISH involves the pathological calcification of the anterolateral spinal ligaments, at ligamentous attachments known as entheses, and pararticular soft tissues (107-109). DISH is a progressive condition in which calcifications accumulate over many years forming flowing calcified material along sides of the spine vertebrae (Fig. 1.7). DISH is often underdiagnosed and widely misdiagnosed (110). It is predominantly diagnosed though radiographs of the thoracic spine or chest, which reveal distinct calcified outgrowths along the vertebrae (111). There are three radiographic criteria for DISH: 1) flowing calcifications along anterolateral aspect of at least four continuous vertebral bodies; 2) preservation of disc height in involved areas (in contrast to degenerative disc disease); and 3) absence of bony ankylosis of facet joints and absence of sacroiliac erosion, sclerosis or fusion (in contrast to ankylosing spondylitis) (112). DISH often presents as back pain and stiffness, and is associated with increased vertebral fractures in the elderly (113) and in severe cases, dysphagia (114) and compression of the spinal cord and nerve roots (114-116). To date, no single gene defect has been associated with DISH; however single nucleotide polymorphisms in the COL6A1 and FGF2 genes have been shown to confer genetic susceptibility to DISH (117,118). The etiology of DISH is unknown; however numerous studies have associated obesity, hypertension, diabetes mellitus, hyperinsulinemia, dyslipidemia, elevated growth
Figure 1.7: Diffuse idiopathic skeletal hyperostosis (DISH) in 55-year-old man. (Reproduced from: *Journal of Clinical Neuroscience* 17: 336-1338, 2010)

Computed tomography scan in the sagittal orientation of the cervical region shows ventral calcifications at the level of C4 to C6 (white arrow). Patient presented with a history of progressive dysphagia, associated with neck stiffness.
hormone levels, elevated insulin-like growth factor-1, and hyperuricemia with DISH (119-121). Pathological findings of DISH have been reported in the anterior longitudinal ligament, paraspinal connective tissue, and annulus fibrosus of the intervertebral disc (112); lesions are thought to originate in fibrocartilaginous structures including entheses (122).

1.9 Rationale, Objectives and Hypotheses

Preliminary observations suggest that mice lacking the gene encoding ENT1 developed lesions of ectopic mineralization which resemble DISH in humans. With advanced age, ENT1<sup>−/−</sup> mice displayed a stiff back, hind limb dysfunction and eventual hind limb paralysis at 12 months of age. Preliminary micro-computed tomography (µCT) scans and histological analyses revealed hypermineralized lesions began in the cervical-thoracic region of the spine and extended towards the lumbar region with advanced age.

Since the etiology and mechanism of DISH is unknown, the ENT1<sup>−/−</sup> mouse may provide a model to investigate mechanisms underlying ectopic mineralization and evaluate therapeutics for preventing pathological calcification in DISH and related disorders. The ultimate goal of this study was to investigate how the loss of the gene encoding ENT1 leads to the aberrant skeletal phenotype demonstrated in the ENT1<sup>−/−</sup> mice. Specifically, it was hypothesized that: **loss of ENT1 leads to progressive accumulation of ectopic mineral in paraspinal tissues due to altered expression of genes that regulate biomineralization.**
We sought to explore this hypothesis with the following specific objectives:

1) Characterize pathological changes in paraspinal tissues in $ENT1^{-/-}$ mice over time pertaining specifically to hypermineralization.

2) Determine changes in the expression of genes associated with adenosine transport and metabolism, as well as, extracellular matrix mineralization in the $ENT1^{-/-}$ mice compared to wild-type controls.
1.10 References


Chapter 2

Loss of Equilibrative Nucleoside Transporter 1 (ENT1) in Mice Leads to Progressive Ectopic Mineralization of Spinal Tissues Resembling Diffuse Idiopathic Skeletal Hyperostosis (DISH) in Humans

Co-Authorship Statement

Chapter 2: Adapted from: Warraich S, Bone DBJ, Quinonez D, Li H, Choi DS, Holdsworth DW, Drangova M, Dixon SJ, Séguin CA, and Hammond JR. Loss of Equilibrative Nucleoside Transporter 1 (ENT1) in Mice Leads to Progressive Ectopic Mineralization of Spinal Tissues Resembling Diffuse Idiopathic Skeletal Hyperostosis (DISH) in Humans. J Bone Miner Res. 2013 May;28(5):1135-49. doi:10.1002/jbmr.1826. DBJB: Figs. 2.2 and 2.6. DWH and MD: Figs. 2.3, 2.4, and 2.5. SW: Figs. 2.1, 2.7, 2.8, 2.9, 2.10, and 2.11. Experiments for Fig. 2.1 were carried out by DQ and HI, and analyzed by SW. SW, DBJB, DWH, MD, SJD, JRH and CAS contributed to the writing of the manuscript. All authors read and approved the submitted version of the manuscript.
2.1 Chapter Summary

Diffuse idiopathic skeletal hyperostosis (DISH) is a non-inflammatory spondyloarthropathy, characterized by ectopic calcification of spinal tissues. Symptoms include spine pain and stiffness, and in severe cases dysphagia and spinal cord compression. The etiology of DISH is unknown and there are no specific treatments. Recent studies have suggested a role for purine metabolism in the regulation of biomineralization. Equilibrative nucleoside transporter 1 (ENT1) transfers hydrophilic nucleosides, such as adenosine, across the plasma membrane. In mice lacking ENT1, we observed the development of calcified lesions resembling DISH. By 12 months of age, ENT1<sup>−/−</sup> mice exhibited signs of spine stiffness, hind limb dysfunction, and paralysis. Micro-CT revealed ectopic mineralization of paraspinal tissues in the cervical-thoracic region at 2 months of age, which extended to the lumbar and caudal regions with advancing age. Energy-dispersive X-ray microanalysis of lesions revealed a high content of calcium and phosphorus with a ratio similar to that of cortical bone. At 12 months of age, histological examination of ENT1<sup>−/−</sup> mice revealed large, irregular accumulations of eosinophilic material in paraspinal ligaments and entheses, intervertebral discs and sternocostal articulations. There was no evidence of mineralization in appendicular joints or blood vessels, indicating specificity for the axial skeleton. Plasma adenosine levels were significantly greater in ENT1<sup>−/−</sup> mice than in wild-type, consistent with loss of ENT1 – a primary adenosine uptake pathway. There was a significant reduction in the expression of Enpp1, Ank, and Alpl in intervertebral discs from ENT1<sup>−/−</sup> mice compared to wild-type mice. Elevated plasma levels of inorganic pyrophosphate in ENT1<sup>−/−</sup> mice indicated generalized disruption of pyrophosphate homeostasis. This is the first report of
a role for ENT1 in regulating the calcification of soft tissues. Moreover, ENT1–/– mice may be a useful model for investigating pathogenesis and evaluating therapeutics for the prevention of mineralization in DISH and related disorders.

2.2 Introduction

Abnormal calcification of the extracellular matrix of soft connective tissues is associated with a number of pathologies including vascular disease, chronic kidney disease, and multiple skeletal disorders (1-4). Ossification of paraspinal ligaments is often detected in middle-aged and elderly patients, presenting as one of two non-inflammatory spondyloarthopathies: (1) diffuse idiopathic skeletal hyperostosis (DISH, formerly referred to as Forestier disease (5)), which involves calcification of spinal ligaments and extra-axial structures including entheses (4); and (2) ossification of the posterior longitudinal ligament (OPLL), which is common in the Japanese population (6). Although often misdiagnosed, DISH and OPLL are distinct from degenerative disc disease, osteoarthritis, and ankylosing spondylitis (7).

DISH is typically diagnosed using radiographs of the thoracic spine or chest, which demonstrate: (1) flowing calcifications along the anterolateral aspect of at least four contiguous vertebral bodies; (2) preservation of intervertebral disc height (in contrast to degenerative disc disease); and (3) absence of bony ankylosis of facet joints and absence of sacroiliac erosion, sclerosis or fusion (in contrast to ankylosing spondylitis) (8). DISH often manifests as back pain associated with limited range of spinal motion, but can progress to the extent that lesions interfere with neighbouring structures, including compression of the spinal cord and nerve roots (9,10). Lesions in DISH can
also cause dysphagia (11), and DISH is associated with increased susceptibility to spinal fractures (12) and postsurgical heterotopic ossifications (13). Correlative studies have associated obesity, hypertension, diabetes mellitus, hyperinsulinemia, dyslipidemia, elevated growth hormone levels, elevated insulin-like growth factor-1, and hyperuricemia with DISH (14-16). It has been proposed that DISH lesions originate in fibrocartilaginous structures including entheses (17); however, due to the lack of suitable animal models, the underlying pathogenesis remains obscure.

Several factors including genetic background have been postulated to be involved in the etiology of DISH; however to date no single gene defect has been associated with the disease. In a subset of DISH patients of Asian descent, single-nucleotide polymorphisms in the COL6A1 and FGF2 genes have recently been shown to confer genetic susceptibility to DISH (18,19). In contrast to DISH, OPLL has been studied extensively in the Japanese population, leading to identification of a number of associated genes, including NPSS (20,21), COL11A (22), COL6A1(23), BMP2 (24), TGFβ (25), and FGFR1 (19).

Recent advances point to a critical role for purine metabolism in the regulation of biomineralization in diseases associated with either insufficient or ectopic mineralization (26-31). For example, mutations in the gene encoding ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1) have been associated with hypermineralization disorders (26,27). Moreover, a recent study has linked ectopic arterial and joint calcifications with loss of ecto-5'-nucleotidase (NT5E) function leading to decreased levels of extracellular adenosine (28). To further explore the role of purine metabolism in the regulation of biomineralization, we examined the phenotype of the mouse lacking the gene encoding
the nucleoside transporter ENT1. ENT1 (equilibrative nucleoside transporter 1 or solute carrier family 29 member 1, encoded by the Slc29a1 locus) is the predominant nucleoside transporter expressed in mammalian cells (32). This sodium-independent, facilitative diffusion carrier is responsible for the movement of hydrophilic nucleosides, such as adenosine, across biological membranes. Loss of ENT1 activity would be expected to modify extracellular adenosine levels, thus altering overall purine metabolism and signaling through adenosine receptors. The present study reports the novel skeletal phenotype of the ENT1/− mouse that resembles DISH in humans.

2.3 Materials and Methods

2.3.1 Animals

ENT1/− mice were generated through targeted deletion of exons 2 to 4 of the gene encoding ENT1 by a cre-loxP targeting strategy (33). ENT1/− mice were backcrossed with C57BL/6 mice. The mouse colony was maintained through the breeding of heterozygous animals (ENT1+/-) to obtain wild-type (ENT1+/+) and knockout (ENT1/−) littermates. Mice were housed in standard cages and maintained on a 12-hour light/dark cycle, with rodent chow and water available ad libitum. Genotyping was performed as described (34). Given the increased reported prevalence of DISH in males (25% of men versus 15% of women over 50 years of age) (35), male mice were used for all experiments. Mice were euthanized at the following ages: 1 month (4-4.5 weeks), 2 months (8-11 weeks), 4 months (16-18 weeks), 6 months (26-30 weeks) and 12+ months (12-17 months). Experimental results were derived from groups of at least 3 wild-type and 3 ENT1/− mice. All aspects of this study were conducted in accordance with the
policies and guidelines set forth by the Canadian Council on Animal Care and were approved by the Animal Use Subcommittee of the University of Western Ontario, London, ON, Canada.

2.3.2 Micro-computed tomography imaging

Micro-computed tomography (µCT) scanning, reconstruction and analysis were performed based on reported protocols (36) with the following modifications. Formalin-fixed or snap-frozen whole mice were imaged at Robarts Research Institute (London, ON, Canada) using a dedicated laboratory µCT scanner (eXplore speCZT; GE Healthcare Biosciences). Data were acquired with an X-ray tube voltage of 90 kV and a current of 40 mA. In one continuous rotation, 900 views were obtained at an angular increment of 0.4° and an exposure interval of 16 ms per view. The total scan time was 5 minutes per animal. A calibrating phantom, consisting of a vial of water, air and a synthetic bone-mimicking epoxy (SB3; Gammex Inc. Middleton, WI, USA), was imaged together with the specimens. Images were acquired at isotropic voxel size of 100 µm and reconstructed into 3D images, using a modified cone-beam algorithm (37). The reconstructed data were expressed in Hounsfield units by calibrating the gray-level values against those of water and air. Mineralized tissue density within the volume of interest was expressed as hydroxyapatite (HA) equivalent density (mg HA cm⁻³), based on the calibration provided by the SB3 bone-mimicking material.

Images acquired for each animal were scored for severity of ectopic mineralization, based on the percentage of affected sites within the spine (sites defined as an intervertebral disc and/or associated paraspinal ligaments and entheses) in each
anatomical region (cervical, thoracic, lumbar and caudal). Values ranged from 0 to 4, with a score of 0 reflecting no detectable mineralized lesions, 1 indicating lesions involving 0 to 30% of sites within the anatomical region, 2 indicating lesions involving 30 to 60% of sites, 3 indicating lesions involving 60 to 90% of sites and 4 indicating lesions involving 90 to 100% of sites.

2.3.3 Measurement of mineralized tissue density

Using data acquired by µCT, quantification of the density and volume of mineralized tissues was performed in a region of interest restricted to the spine (C<sub>1</sub> to sacrum), rib cage, and sternum. For this purpose, a 3D volume of interest was defined by an operator within each volume image, using manually drawn contours lofted to create a volume that included only the anatomy of interest (MicroView 2.2, GE Healthcare Biosciences). Three measurements were obtained within this volume of interest: the volume of hypermineralized tissue, the volume of tissue with density equivalent to that of normal cortical bone, and the maximum density. Hypermineralized tissue was defined as material that exceeded the maximum density of cortical bone in the spine of wild-type mice within a specified age group. In the present study, we defined threshold values of 610, 630 and 710 mg HA cm<sup>-3</sup> for maximum spinal bone density in animals of ages 2, 4 and 6 (or greater) months, respectively. Previous studies have reported a similar increase in murine cortical bone density during the first 24 weeks of postnatal development (38). Normal density tissue was defined as material that exceeded a minimum threshold (126 mg HA cm<sup>-3</sup>), but fell below the maximum thresholds defined above. Volume measurements are reported in cubic millimeters (mm<sup>3</sup>), representing the summation of all
the volume elements that fell within the defined range of mineral density. Additionally, the maximum mineralized tissue density within the volume of interest was reported. Nonlinear least squares fits were obtained using GraphPad Prism; data for volume of hypermineralized lesions were fit with an exponential growth equation, and data for the volume of normal bone and maximum density values were fit with one-phase association equations.

2.3.4 Scanning electron microscopy and energy-dispersive X-ray spectroscopy

Scanning electron microscopy (SEM) imaging and energy-dispersive X-ray spectroscopy (EDX) microanalyses were performed based on previous reports (39) using a LEO 1540XB FIB/SEM instrument (Carl Zeiss, Oberkochen, Germany) and X-ray analysis system (Oxford Instruments, Oxford, UK) at the Western Nanofabrication Facility (The University of Western Ontario). Prior to SEM imaging, dried samples were coated with 5 nm of osmium using a plasma coater (OPC-80T; Filgen Inc.). EDX spectra were collected from bone and mineralized lesions identified by electron backscatter imaging at 20 keV beam energy. Elemental analysis of the EDX spectra was performed using INCA software (Oxford instruments), including background correction and fitting of all peaks.

2.3.5 Histology

Formalin-fixed tissue samples were decalcified with Shandon TBD-2 Decalcifier (Thermo Scientific, Nepean, ON Canada) at a ratio of 10:1 (fluid:tissue) for 5 days with gentle rocking. Following standard histological processing, decalcified samples were
embedded in paraffin and 5 µm-thick serial sections were cut. Tissues were sectioned in the coronal plane, mounted on glass slides and baked for 48 hours at 45°C. Slides were stained with haematoxylin and eosin (H&E) and images were acquired using a Leica DM1000 microscope.

For visualization of cell nuclei, sections described above were dewaxed in xylene and rehydrated by successive immersion in descending concentrations of ethanol. Mounting was performed with VECTASHIELD Mounting Medium with 4',6-diamidino-2-phenylindole (DAPI) (Burlingame, CA) and images were captured using a Leica DMI6000B microscope.

2.3.6 Blood chemistry

High-performance liquid chromatography (HPLC) was used to analyze plasma for levels of adenosine and adenosine metabolites to determine the functional consequence of loss of ENT1. At 2 months of age, mice were anesthetized with pentobarbital. Blood was collected by cardiac puncture into a syringe containing NaCl (118 mM), KCl (5 mM), EDTA (13.2 mM), 5-iodotubercidin (10 µM) to inhibit adenosine kinase, erythro-9-(2-hydroxy-3-nonyl)adenine hydrochloride (EHNA, 100 µM) to inhibit adenosine deaminase and dilazep (10 µM) to inhibit adenosine transport (40). Plasma was isolated by sedimentation at 3,000g for 10 minutes at 4°C. Plasma was applied to a 10-kDa cutoff ultra-filtration column and sedimented at 14,000g for 15 minutes at 4°C. Filtered plasma was analyzed on an Onyx monolithic C18 column as described (41), using a Hewlett Packard 1090 LC with UV detector. Adenosine was detected at 260 nm and adenosine metabolites at 250 nm.
To screen for systemic changes resulting from loss of ENT1 function, serum chemistry was performed using established panels of clinical chemistry parameters. At 2 months of age, mice were anesthetized with pentobarbital and blood was collected by cardiac puncture. Blood was allowed to coagulate at room temperature for 30 minutes. Samples were then sedimented at 3,000 g for 10 minutes at 4°C and the serum supernatant was transferred to a fresh tube and frozen at -80°C. Chemical and biochemical analyses were performed by the Centre for Modeling Human Disease at the Toronto Centre for Phenogenomics (Toronto, Canada).

To assay plasma levels of inorganic pyrophosphate (PP$_i$), mice were anesthetized with pentobarbital and blood was collected by cardiac puncture. Samples were transferred to microfuge tubes containing heparin (5 USP units/mL of blood) and plasma was isolated by sedimentation at 3,000 g for 10 minutes at 4°C. Plasma was applied to 10-kDa cutoff ultra-filtration spin columns and sedimented at 14,000 g for 20 minutes at 4°C. A fluorometric PP$_i$ assay kit (ab112155; Abcam, Cambridge, MA, USA) was first validated using plasma samples from wild-type mice, plasma supplemented with a saturating concentration of PP$_i$ (30 µM) in the presence or absence of inorganic pyrophosphatase (0.0012 units/µL sample; Sigma) (Fig. 2.1). Plasma samples were isolated from 2-month-old wild-type and ENT1$^{-/-}$ mice (n=6 mice for each genotype) and analyzed according to the manufacturer’s protocol with an incubation time of 20 minutes. Fluorescence was measured at excitation and emission wavelength of 316 and 456 nm, respectively, using a SpectraMax M5 microplate reader (Molecular Devices) with Softmax Pro v5 software. Values were interpolated using linear regression. Background levels of plasma autofluorescence were negligible.
Figure 2.1: Validation of inorganic pyrophosphate assay.
The fluorometric Pyrophosphate Assay Kit was validated using pooled plasma obtained from wild-type mice. To reduce protein concentration, plasma was applied to a 10 kDa cutoff ultra-filtration columns. Pyrophosphate was measured in: i) filtered plasma; ii) filtered plasma supplemented with a saturating concentration of exogenous pyrophosphate (PP\(_i\), 30 µM); and iii) filtered plasma supplemented with PP\(_i\) (30 µM) and pyrophosphatase (PPase, 0.0012 units/µL sample). Samples were analyzed according to the manufacturer’s protocol with an incubation time of 20 min. Exogenous pyrophosphate caused a significant increase in the signal (to maximal detectable levels). Moreover, incubation with pyrophosphatase significantly diminished the pyrophosphate-induced increase in signal intensity. Taken together, these data establish that the kit effectively detects pyrophosphate in ultra-filtered plasma. Assay was performed using 4 replicate samples for each condition. Data are presented as means ± SEM, n=4. Bars labeled with the same lower case letter are not significantly different, based on one-way analysis of variance followed by a Tukey multiple comparisons test.
2.3.7 Quantitative real-time RT-PCR

At 6 months of age, littermate-paired wild-type and ENTI\textsuperscript{−/−} mice (n=8 for each genotype) were dissected to isolate intervertebral discs (IVDs) inclusive of annulus fibrosus, nucleus pulposus, and cartilage endplates. IVDs from each animal were separated according to anatomical location as cervical-thoracic, lumbar, or caudal. Tissues were placed directly in 1 mL of TRIzol Reagent (Life Technologies, Wilmington, DE) and homogenized using a PRO250 Polytron benchtop homogenizer (PRO Scientific, Oxford, CT). Total RNA was extracted according to the manufacturer’s protocol and quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Mississauga, ON). For each sample, 1 µg RNA was reverse transcribed into cDNA using Superscript II Reverse Transcriptase (Life Technologies). Gene expression patterns were determined by real-time PCR using the Bio-Rad CFX384 system. PCR reactions were run in triplicate, using 25 µM forward and reverse primers (primer sequences in Table 2.1) with 2X SsoFast EvaGreen Supermix (Bio-Rad, Mississauga, ON). The PCR program consisted of the following: initial 3 min at 95°C for denaturing; 95°C for 10 s denaturing; 30 s annealing/elongation (temperatures provided in Table 2.1), for a total of 40 cycles. Gene transcript levels were determined relative to a six-point calibration curve made from cDNA generated from heart, brain, kidney, muscle, intervertebral disc, and calvarial RNA (from wild-type mice). The starting concentration of the cDNA standard (83 – 136 ng/µl) was inputted into the Bio-Rad CFX Manager 2.0 software to generate the standard curve (1/5 serial dilution). Values for gene transcript levels are expressed relative to the calibration curve in ng/µl. Standard curves were generated to control for primer efficiency and specificity of primers was determined by melt curve analysis (0.5°C/5 s).
Table 2.1: Real-time PCR Primer Sequences

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Primer Sequence (5’ to 3’)</th>
<th>Optimal Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adora3</td>
<td>Fwd – ACTTCTATGCCTGCCCTTTTCATGT Rev – AACCGTTCTATATCTGACTGTCAGCTT</td>
<td>56</td>
</tr>
<tr>
<td>Ank</td>
<td>Fwd – CCCTGATAGCCTACAGTGAAGTTAGG Rev – GAAGGCAGCGAGATACAGGAA</td>
<td>56</td>
</tr>
<tr>
<td>Alpl</td>
<td>Fwd - CCAACTCTTTTTGTCAGAGCTAGGA Rev – GGCTACATTGGGTGTTGAGCTTTTT</td>
<td>60</td>
</tr>
<tr>
<td>Enpp1</td>
<td>Fwd - CAAAACCCCAATGACTGCTTGCCAGAG Rev – CTGGACACTCCGGGTATCG</td>
<td>56</td>
</tr>
<tr>
<td>ENT1</td>
<td>Fwd - CAAGTATTTTCAACCAACGCCTGGAC Rev – GAAACGAGTTGGAGCCAGGTGAAGAC</td>
<td>56</td>
</tr>
</tbody>
</table>
2.4 Results

2.4.1 Ectopic mineralization in ENT1/−/− mice

Previous studies reported that ENT1/−/− mice are phenotypically normal, with only a modest decrease (~10%) in body weight compared with that of wild-type littermates (33). At young ages, open-field locomotor activity in ENT1/−/− and wild-type mice does not differ. Moreover, there is no significant difference in spontaneous mortality rates up to 6 months of age. To date, there have been no reports of phenotypic changes in skeletal tissues related to mineralization (33,34,42,43); however, all reported studies were conducted on mice less than 4 months of age.

In the present study, we noted that the spines of 6-month-old ENT1/−/− mice were extremely rigid. At 8 months, ENT1/−/− mice demonstrated decreased hind limb mobility that progressed to hind limb paralysis by 12 to 17 months of age. Postmortem analysis of a 17-month-old ENT1/−/− mouse revealed hard, chalky white lesions protruding ventrally from the thoracic spine (Fig. 2.2A). µCT images revealed large radio-opaque lesions at distinct foci in the cervical and upper thoracic regions of ENT1/−/− mice, localized to paraspinal and intervertebral tissues and protruding in some cases into the spinal canal (Fig. 2.2B). Histological examination identified large accumulations of amorphous material (Fig. 2.2C) localized to the intervertebral discs (upper panel) and paraspinal tissues associated with the spinous processes (lower panel). The presence of calcified lesions within the spinal canal, impinging on the spinal cord, was consistent with the decreased mobility and paralysis observed in ENT1/−/− mice.
Figure 2.2: Ectopic mineralization of spinal tissues in $ENT1^{-/-}$ mice.
(A) Gross appearance of ventral aspect of the thoracic spine from 17-month-old wild-type (WT) and $ENT1^{-/-}$ mice. Mineralized lesions protrude from intervertebral spaces, associated with rib entheses. Lesions demonstrated hard, chalky consistency and were whitish in colour. (B) Maximum intensity projection of $\mu$CT images showing the lateral aspect of the cervical and thoracic spine of 17-month-old WT and $ENT1^{-/-}$ mice. Ectopic radio-opaque material is evident in paraspinal and intervertebral regions of the spine (arrows) extending into the spinal canal (SC). (C) Histological sections of decalcified spinal tissues from 17-month-old $ENT1^{-/-}$ mouse (spinal column and spinal cord) stained with H&E. Mineralized lesion (outlined in black) can be seen protruding into the spinal canal and impinging on the spinal cord. Images in B and C are oriented with ventral to the left. Scale bars represent 200 $\mu$m.
2.4.2 Time course of phenotype development

µCT imaging was employed to assess the development and extent of ectopic mineralization in \(\text{ENT1}^{-/-}\) mice over time (Fig. 2.3A). Images were scored for severity, based on the percentage of affected sites within the spine (intervertebral discs, and associated paraspinal ligaments and entheses) in each anatomical region (Fig. 2.3B). Ectopic mineralization of spinal tissues was not evident in wild-type animals at any time point (Fig. 2.3A), or in \(\text{ENT1}^{-/-}\) mice at 1 month of age (not shown).

Ectopic mineralization in \(\text{ENT1}^{-/-}\) mice proceeded temporally in a consistent anatomical pattern. At 2 months of age, maximum intensity projection (MIP) images revealed radio-opaque material in the paraspinal tissues of the cervical spine and the rib entheses of the upper thoracic spine (Fig. 2.3, top panels). At this age, there was no detectable involvement of intervertebral discs.

With advancing age, accumulation of ectopic mineral progressed caudally, with involvement of the lumbar spine at 6 months (Fig. 2.3, middle panels). In addition to paraspinal tissues and rib entheses, lesions were observed within the intervertebral spaces at 6 months in both the thoracic and lumbar spine. At this time point, lesions involved 60 to 90% of the cervical spine and 30 to 60% of the thoracic spine. Interestingly, lesions did not progress caudally in a uniform manner. Following involvement of the cervical and upper thoracic spine, lesion formation initially bypassed the mid-region of the thoracic spine and first appeared specifically in thoracic vertebrae T11-T12 (Fig. 2.3A, asterisk). At greater ages, lesions developed in the intervening mid-region of the thoracic spine.

By 12 months of age, ectopic mineralization was detected in >60% of the cervical, thoracic and lumbar regions of the spine, as well as >30% of intervertebral discs.
Figure 2.3: Temporal progression of ectopic mineralization in ENT1<sup>−/−</sup> mice.

(A) Lateral and antero-posterior maximum intensity projections of micro-CT images of wild-type (WT) and ENT1<sup>−/−</sup> mice at 2, 6 or 12+ months of age. Ectopic radio-opaque material was observed in paraspinal tissues of the cervical spine at 2 months of age (top panel, white arrows). By 6 months of age, lesions were detected in the cervical (white arrows), thoracic and lumbar spine in paraspinal ligaments, rib entheses and intervertebral discs (middle panel). At 6 months, lesions involved the upper thoracic spine, but appeared to bypass the mid-region of the thoracic spine to specifically affect sites in the vicinity of thoracic vertebrae T<sub>11</sub>-T<sub>12</sub> (indicated by white asterisk). By 12 months of age, lesions involved virtually the entire cervical, thoracic and lumbar spine, and were detectable in the intervertebral discs of the caudal spine (lower panel). Note that the focal hyperintensities observed in the abdomen result from high-density material in the animal chow; these bright spots do not represent ectopic calcifications. (B) The percentage of sites affected by mineralization in each anatomical region of the spine was scored in ENT1<sup>−/−</sup> mice at 2, 6 or 12+ months of age. A severity score of 0 reflects no detectable mineralized lesions, 1 indicates lesions involving 0 to 30% of sites within the anatomical region, 2 indicates lesions involving 30 to 60% of sites, 3 indicates lesions involving 60 to 90% of sites, and a score of 4 indicates lesions involving 90 to 100% of sites. The symbols represent scores for individual mice and bars represent the mean.
of the caudal spine (Fig. 2.3, lower panel). In addition, there was notable decrease in kyphosis between 6 and 12 months of age in \( \text{ENT1}^{-/-} \) mice compared to wild-type (Fig. 2.3A, lower panel). No evidence of ectopic mineralization was detected in the vasculature of \( \text{ENT1}^{-/-} \) mice at any time point examined by \( \mu \)CT.

To examine the consequence of \( \text{ENT1} \) haploinsufficiency, 6-month-old heterozygous \( \text{ENT1}^{+/-} \) mice were examined by \( \mu \)CT (Fig. 2.4). In contrast to \( \text{ENT1}^{-/-} \) mice, heterozygous animals did not demonstrate ectopic mineral formation in paraspinal tissues, rib entheses, or intervertebral discs. Moreover, the loss of kyphosis observed in knockout animals was not apparent; \( \text{ENT1} \) heterozygous animals were indistinguishable from wild-type controls.

### 2.4.3 Density and elemental composition of ectopic lesions

Mineralized tissue density of wild-type and \( \text{ENT1}^{-/-} \) mice at different ages was quantified from \( \mu \)CT data. The region of interest for these analyses consisted of the cervical to lumbar spine, rib cage, and sternum. Hypermineralized tissue was detected in paraspinal and intervertebral tissues of the spine, as well as the sternocostal articulations (highlighted in red in Fig. 2.5A). Quantification revealed that the volume of hypermineralized tissue grew exponentially with age in the \( \text{ENT1}^{-/-} \) mice, whereas there was little hypermineralized tissue detectable in wild-type mice at any age (Fig. 2.5B). We also quantified the volume of mineralized tissue with density equivalent to that of normal cortical bone (Fig. 2.5C). Whereas the wild-type animals appeared to reach a plateau of approximately 355 mm\(^3\) by day 200 (reflecting bone growth), \( \text{ENT1}^{-/-} \) mice reached a plateau of 552 mm\(^3\) by day 400 (reflecting both bone growth and accumulation...
Figure 2.4: Micro-CT images of wild-type, heterozygous, and $ENTI^+$ mice. Images are lateral (top panel) and antero-posterior (bottom panel) maximum intensity projections of wild-type (WT), heterozygous ($ENTI^{+/}$), and $ENTI^{-/-}$ mice at 6 months of age. No ectopic mineral was observed in the WT or heterozygous mice. Ectopic radio-opaque material was observed in $ENTI^{-/-}$ mice; lesions were detected in the paraspinal ligaments, rib entheses and intervertebral discs of the cervical, thoracic, and lumbar regions (white arrows). Images are representative of 3 animals of each genotype.
Figure 2.5: Quantification of the volume and density of mineralized material in wild-type and \textit{ENT1}^{-/-} mice over time.

(A) Lateral maximum intensity projections of the \textmu CT images of a wild-type (WT) and an \textit{ENT1}^{-/-} mouse at 17 months of age. The region of interest for these analyses was restricted to the cervical, thoracic and lumbar spine, rib cage and sternum. The regions highlighted in red represent hypermineralized tissue within the region of interest. (B) The volume of hypermineralized tissue grew exponentially with age in the \textit{ENT1}^{-/-} mice. (C) \textit{ENT1}^{-/-} mice also showed increased volume of mineralized tissue with normal bone density (consisting of both bone and ectopic calcifications). Whereas the wild-type animals appeared to reach a plateau (of approximately 355 mm$^3$ by day 200), the \textit{ENT1}^{-/-} mice reached a plateau (552 mm$^3$) by day 400. (D) Likewise, the maximum mineral density of the wild-type mice reached a plateau before an age of 200 days, whereas that of the \textit{ENT1}^{-/-} mice continued to increase past day 400. For B-D, the symbols represent \textmu CT-derived parameters for individual mice and the lines represent the non-linear fits.
of ectopic mineral). Likewise, the maximum mineral density of the wild-type mice reached a plateau before an age of 200 days, whereas that of the $ENT1^{+/−}$ mice continued to increase past day 400 (Fig. 2.5D), reflecting hypermineralization of the ectopic lesions.

A summary and statistical analysis of these data is presented in Table 2.2. From 2 to 17 months of age, the volume of bone in wild-type mice increased from 259 to 356 mm$^3$ with only about 0.5 mm$^3$ appearing hypermineralized (regardless of age) according to the thresholds used. The mineralized tissue volume of $ENT1^{+/−}$ mice was similar to that of wild-type controls up to 6 months of age. In contrast, 12-to 17-month-old $ENT1^{+/−}$ mice had a significantly greater volume of mineralized tissue than the equivalent age of wild-type mice (527 versus 356 mm$^3$). In addition, the $ENT1^{+/−}$ mice had a significantly greater volume of hypermineralized tissue than wild-type mice between 12 to 17 months old (16 versus 0.5 mm$^3$), and the maximum mineral density was significantly greater in $ENT1^{+/−}$ mice than wild-type controls at both 6 and 12 month of age. Note that, whereas the maximum mineral density observed in $ENT1^{+/−}$ mice (1044 mg HA cm$^3$) does not exceed the typical value for fully mineralized cortical bone (approximately 1050 mg HA cm$^3$), it is significantly greater than the mineral density of cortical bone observed in the spine of wild-type littermates (744 mg HA cm$^3$).

Samples from wild-type and $ENT1^{+/−}$ mice at 6 months of age were analyzed by EDX to determine the elemental content of both cortical bone and mineralized lesions. Mineralized lesions appeared by SEM as a disordered amorphous material (Fig. 2.6A, B) and EDX revealed a high content of calcium and phosphorus (Fig. 2.6C). The mean elemental content of bone and lesions is displayed as percent atomic ratio in Table 2.3. The vertebral bone from $ENT1^{+/−}$ mice was not significantly different from that of wild-
Table 2.2: Mineralized Tissue Density in \textit{ENT1}^{-/-} and Wild-type Mice

\begin{table}
\centering
\begin{tabular}{|c|c|c|c|c|c|}
\hline
Age & \multicolumn{2}{|c|}{\textit{ENT1}^{-/-}} & \multicolumn{2}{|c|}{Wild-type} \\
(months) & Volume (mm\(^3\)) & Max Density (mg HA cm\(^3\)) & Volume (mm\(^3\)) & Max Density (mg HA cm\(^3\)) \\
& Hypermineralized & Normal & Hypermineralized & Normal \\
\hline
12+ & 16.4 ± 3.2** & 527 ± 48* & 1044 ± 77** & 0.5 ± 0.4 & 356 ± 35 & 744 ± 10 \\
(n = 4) & & & & (n = 3) & & \\
6 & 3.5 ± 1.1 & 381 ± 55 & 921 ± 38* & 0.6 ± 0.5 & 351 ± 92 & 746 ± 54 \\
(n = 3) & & & & (n = 3) & & \\
4 & 1.9 ± 1.5 & 341 ± 16 & 763 ± 110 & 0.5 ± 0.6 & 318 ± 79 & 639 ± 65 \\
(n = 3) & & & & (n = 3) & & \\
2 & 0.4 ± 0.5 & 256 ± 52 & 652 ± 84 & 0.5 ± 0.5 & 259 ± 32 & 651 ± 58 \\
(n = 4) & & & & (n = 4) & & \\
\hline
\end{tabular}
\caption{\textbf{ENT1}^{-/-} and Wild-type Mice}
\end{table}

\(\mu\)CT-derived values for the volume of normal and hypermineralized tissue and the maximum density in \textit{ENT1}^{-/-} and wild-type mice. Data are means ± SD. Differences were evaluated by two-way analysis of variance (ANOVA) followed by a Bonferroni multiple comparison test.

\textit{ENT1} = equilibrative nucleoside transporter 1; HA = hydroxyapatite.

Significantly different from wild type: * \(p < 0.05\), ** \(p < 0.001\).
Figure 2.6: Scanning electron microscopy (SEM) and elemental analysis of mineralized lesions by energy dispersive X-ray spectroscopy (EDX).

(A, B) SEM of mineralized lesion in ENTI^{−/−} mouse at 6 months of age. Mineralized lesions appeared as a disordered amorphous material. Representative areas (2 or 3 per sample) were selected and probed by EDX for calcium (Ca), phosphorous (P), carbon (C) and oxygen (O). Magnesium (Mg) and sodium (Na) were detected in some, but not all, samples and hence were not included in the quantitative analysis shown in Table 2.3.

(C) A representative EDX spectrum (from the region highlighted in Panel A) is shown, revealing a high content of calcium and phosphorus. Images and spectrum are representative of lesion samples from 4 mice.
Table 2.3: Energy-Dispersive X-ray Spectroscopy

<table>
<thead>
<tr>
<th>Location</th>
<th>Genotype</th>
<th>Carbon</th>
<th>Oxygen</th>
<th>Phosphorous</th>
<th>Calcium</th>
<th>Ca/P Ratio</th>
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<tr>
<td>Vertebra</td>
<td>WT</td>
<td>45.1 ± 4.8</td>
<td>34.0 ± 1.8</td>
<td>7.4 ± 0.7</td>
<td>12.7 ± 2.3</td>
<td>1.7 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>ENT1⁻/⁻</td>
<td>45.6 ± 2.2</td>
<td>37.5 ± 0.9</td>
<td>6.8 ± 0.5</td>
<td>10.5 ± 0.6</td>
<td>1.6 ± 0.1</td>
</tr>
<tr>
<td>Lesion</td>
<td>ENT1⁻/⁻</td>
<td>37.6 ± 3.5</td>
<td>41.6 ± 2.3</td>
<td>8.7 ± 0.4*</td>
<td>13.3 ± 1.1*</td>
<td>1.5 ± 0.1</td>
</tr>
</tbody>
</table>

Scanning electron microscopy was used to identify regions of vertebral cortical bone and ectopic mineralization (lesion) in samples isolated from 6-month-old WT and ENT1⁻/⁻ mice. Energy-dispersive X-ray spectroscopy was then applied to these selected regions to obtain the % atomic ratios of the indicated elements. Data are mean ± SEM, n = 4. WT = wild-type; ENT1 = equilibrative nucleoside transporter 1. * Significant difference between lesion and ENT1⁻/⁻ vertebra (p < 0.05; Student’s t-test).
type mice in terms of elemental ratios. In contrast, ectopic mineralized lesions in $ENTI^{−/−}$ mice displayed higher levels of phosphorus and calcium relative to vertebral bone, consistent with the greater mineralized tissue density observed using µCT. The calcium/phosphorus ratio was similar in all samples.

2.4.4 Histological appearance of mineralized lesions

We first assessed the intervertebral disc regions of wild-type and $ENTI^{−/−}$ mice between 12 and 17 months of age. Decalcified sections stained with H&E revealed the presence in $ENTI^{−/−}$ mice of large accumulations of amorphous, eosinophilic acellular material suggestive of niduses of mineralization in the cervical, thoracic, lumbar and caudal regions (Fig. 2.7). Interestingly, within the intervertebral disc, lesions appeared to be localized within the annulus fibrosus, leading to lateral compression of the nucleus pulposus and extensive bulging of the annulus fibrosus out of the intervertebral space (Fig. 2.7, right panels). There was no evidence that lesions were associated with inflammation or increased vascularization. Since the sacral intervertebral discs undergo progressive fusion with the onset of skeletal maturity in mice, this region was not examined for ectopic mineralization.

Lesions were also detected in the sternocostal region of $ENTI^{−/−}$ mice between 12 and 17 months of age, by both µCT and histology (Figs. 2.3, 2.5A and Fig. 2.8A). In this region, extensive ectopic mineral was detected within the connective tissue of the sternocostal articulations, leading to deformation of the sternum. In contrast, examination of appendicular joints revealed no aberrant morphology or evidence of ectopic mineralization (Fig. 2.8B-E) establishing that lesions are specifically associated with the
Vertebral structures are indicated in panel A: the intervertebral disc (IVD) is formed by the annulus fibrosus outlined in yellow and nucleus pulposus outlined in red, which are located between adjacent vertebral bones (VB). The spines of ENTI−/− mice show irregular lesions within the annulus fibrosus of the intervertebral disc. Lesions are encapsulated by layers of fibrocartilaginous cells. Lesions are outlined with black dashed lines and are shown at higher magnification in the panels to the right. Samples were sectioned in the coronal plane and stained with haematoxylin and eosin (H&E). Images are oriented with rostral at the top and are representative of 3 animals of each genotype 12-17 months of age. Scale bars represent 200 μm.
Figure 2.8: Histological appearance of sternocostal (A), humeroulnar (B), tibiofemoral (C), radiocarpal (D) and talocrural (E) joints in 12+ month-old wild-type (WT) and ENT1−/− mice (arrows indicate joint spaces). In ENT1−/− mice, sternocostal joints displayed lesions in fibrocartilaginous connective tissues, similar to those seen in the spine. In contrast, the appendicular joints appeared to be unaffected. Lesion in panel A is outlined with black dashed line and tissues are shown at higher magnification in the panel to the right. Samples were sectioned in the coronal plane and stained with haematoxylin and eosin (H&E). Images are representative of 3 animals of each genotype 12-17 months of age. Scale bars represent 200 μm.
Comparison of histological sections of spinal tissues from wild-type and \textit{ENT1}^{−/−} mice at 6 months of age revealed further insights into the development of lesions. Within the cervical spine of 6-month-old \textit{ENT1}^{−/−} mice, large lesions were associated with the paraspinal ligaments, but no distinct lesions were detected within the intervertebral discs in this region (Fig. 2.9A). However, the outer annulus fibrosus of the intervertebral discs did appear altered, with regions of metaplasia and disruption of normal tissue architecture (Fig. 2.9A, arrowhead). Within the thoracic spine of 6-month-old \textit{ENT1}^{−/−} mice, extensive lesions were localized within: (1) the annulus fibrosus; (2) the fibrocartilaginous tissue of the posterior paraspinal ligaments; and (3) the fibrocartilaginous tissue of the rib entheses (Fig. 2.9B). In the lumbar spine of 6-month-old \textit{ENT1}^{−/−} mice, small lesions were detected within the annulus fibrosus, leading to disruption of intervertebral disc structure and displacement of the nucleus pulposus (Fig. 2.9C). At this time point, no changes were detected within the caudal spine.

At 2 months of age, when ectopic mineral was first detected in \textit{ENT1}^{−/−} mice by \(\mu\)CT, histological examination also demonstrated lesions within the paraspinal ligaments of the cervical spine (Fig. 2.10A). Interestingly, these lesions were associated with large regions of metaplasia, with a disruption of normal tissue architecture and increased cellularity (Fig. 2.10A, arrowheads). In the upper thoracic spine, the mineralized lesions detected by \(\mu\)CT in \textit{ENT1}^{−/−} mice were localized to the fibrocartilaginous structures of the paraspinal ligaments and rib entheses, within larger regions demonstrating evidence of hyperplasia and desmoplasia (Fig. 2.10B, arrowheads). At this time point, no lesions were detectable in intervertebral discs within any region of the spine. Early lesions evident at 2
Figure 2.9: Histological appearance of spinal tissues in cervical (A), thoracic (B) and lumbar (C) spine segments from 6-month-old wild-type (WT) and $ENTI^{+/−}$ mice. Representative images demonstrate the location of lesions consisting of irregular accumulations of eosinophilic material in the intervertebral disc (IVD), paraspinal ligaments, and rib entheses in $ENTI^{+/−}$ mice. Lesions are outlined with black dashed lines and tissues are shown at higher magnification in the panels to the right. Arrowhead in (A) indicates a nonmineralized region of metaplasia accompanied by disruption of normal tissue architecture in the annulus fibrosus of the IVD. Images are oriented with rostral at the top and are representative of 3 animals of each genotype. Scale bars represent 200 μm.
Figure 2.10: Histological appearance of the intervertebral disc (IVD), paraspinal ligaments, and rib entheses in cervical (A) and thoracic (B) spine segments from 2-month-old wild-type (WT) and ENT1−/− mice. Lesions are outlined with black dashed lines and tissues are shown at higher magnification in the panels to the right. Arrowheads indicate regions demonstrating tissue metaplasia. (C) Serial sections of representative lesion localized to the paraspinal tissue of the thoracic spine of a 2-month-old ENT1−/− mouse stained with H&E (top panel) and DAPI (bottom panel). Cells with eosinophilic nuclei are detected in the transition zone (TZ) between normal fibrocartilaginous tissues and lesion. DAPI staining demonstrates that these cells retain an intact nuclear structure (arrows indicate individual cells). Images are oriented with rostral at the top and are representative of at least 3 ENT1−/− animals. Scale bars represent 200 μm.
months of age were surrounded by a clearly demarcated transition zone (TZ; Fig. 2.10C). The periphery of this zone consisted of typical fibrocartilaginous tissue and, as expected, cells with basophilic nuclei that stained positively with DAPI (Fig. 2.10C, lower panels). However, the more central region of the transition zone was characterized by metaplasia, with cells displaying eosinophilic nuclei that stained positively with DAPI, revealing an intact nuclear structure. The TZ surrounded the presumptive mineralized lesion, which contained large accumulations of amorphous matrix and necrotic cell debris. There was no evidence of apoptotic nuclei within the lesion or TZ.

2.4.5. Differences in blood chemistry and gene expression associated with the

*ENT1*<sup>−/−</sup> phenotype

Blood plasma or serum from 2-month-old wild-type and *ENT1*<sup>−/−</sup> mice was analyzed for components related to mineralization as well as adenosine and adenosine metabolites (Table 2.4). HPLC analysis of plasma showed 2.8-fold greater adenosine levels in *ENT1*<sup>−/−</sup> than wild-type mice. In contrast, there were no significant differences in levels of the adenosine metabolites xanthine and uric acid, arguing against the possibility that ectopic lesions involved deposition of monosodium urate crystals. Importantly, serum levels of alkaline phosphatase, calcium, phosphate, and magnesium were not significantly different in *ENT1*<sup>−/−</sup> and wild-type mice. On the other hand, quantification of plasma PP<sub>i</sub> levels revealed 3.4 ± 1.0-fold greater levels in *ENT1*<sup>−/−</sup> mice than in wild-type controls (n=6 two-month-old mice for each genotype, p < 0.05).

Given this alteration in PP<sub>i</sub> levels, we interrogated the expression of genes associated with pyrophosphate metabolism in intervertebral disc tissues isolated from
Table 2.4: Blood Chemistry

<table>
<thead>
<tr>
<th></th>
<th>Wild-type ($n = 4$)</th>
<th>ENT1⁻/⁻ ($n = 5$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenosine (µM)</td>
<td>2.2 ± 0.4</td>
<td>6.2 ± 1.4*</td>
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<tr>
<td>Xanthine (µM)</td>
<td>4.3 ± 1.5</td>
<td>5.2 ± 2.4</td>
</tr>
<tr>
<td>Uric acid (µM)</td>
<td>163 ± 23</td>
<td>176 ± 14</td>
</tr>
<tr>
<td>ALP (IU/L)</td>
<td>97 ± 9</td>
<td>109 ± 10</td>
</tr>
<tr>
<td>Ca (mM)</td>
<td>2.30 ± 0.03</td>
<td>2.31 ± 0.02</td>
</tr>
<tr>
<td>Mg (mM)</td>
<td>1.26 ± 0.04</td>
<td>1.30 ± 0.08</td>
</tr>
<tr>
<td>P$_i$ (mM)</td>
<td>3.1 ± 0.3</td>
<td>3.7 ± 0.4</td>
</tr>
</tbody>
</table>

Data are means ± SEM. Serum was obtained from wild-type and ENT1⁻/⁻ mice at two months of age.

ALP = total alkaline phosphatase; ENT1 = equilibrative nucleoside transporter 1; P$_i$ = inorganic phosphate.

* Significantly different from wild-type ($p < 0.05$, Student’s $t$-test).
Real-time PCR analysis of genes associated with PP\(_i\) metabolism and tissue mineralization revealed significant reductions in the expression of (A) *Enpp1*, (B) *Ank*, and (C) *Alpl* in *ENT1*\(^{-/-}\) mice compared to wild-type controls. (D) In contrast, there was no significant difference in the expression of the adenosine A\(_3\) receptor (*Adora3*), indicating specificity of changes in gene expression. (E) Real-time PCR analysis of *ENT1* expression revealed no significant differences between anatomical regions of the spine in wild-type mice. **Significantly different from wild-type (*p* < 0.01, Student’s *t*-test).
ENT1−/− and wild-type littermate control mice at 6 months of age. Real-time PCR analysis demonstrated a significant decrease in the expression of ectonucleotide pyrophosphatase/phosphodiesterase 1 (Enpp1; Fig. 2.11A), Ank (a putative PPi transporter) (Fig. 2.11B), and tissue nonspecific alkaline phosphatase (Alpl; Fig. 2.11C) in ENT1−/− mice. In contrast, no significant difference was detected in the expression of unrelated genes including the adenosine A3 receptor (Adora3; Fig. 2.11D) in ENT1−/− and wild-type mice, indicating specificity.

To determine if the specific anatomical pattern of ectopic mineralization was influenced by endogenous levels of ENT1, expression was assessed in 6-month-old wild-type mice. No differences were detected in ENT1 transcript levels in intervertebral disc tissues isolated from cervical-thoracic, lumbar, or caudal regions of the spinal column (Fig. 2.11E).

2.5 Discussion

This is the first report of a role for ENT1 in regulating biomineralization. We discovered that ENT1−/− mice develop ectopic mineralization with distribution restricted to the fibrous connective tissues of the spine and sternum. In the spine, pathological mineralization begins in the paraspinal fibrocartilaginous tissues and progresses to involve the annulus fibrosus of intervertebral discs. Aberrant mineralization is first observed in ENT1−/− mice between 6 and 8 weeks of age in the paraspinal connective tissues of the cervical vertebrae. With advancing age, lesions increase in severity and progress to other regions of the spine, with ectopic mineralization eventually involving the thoracic, lumbar and caudal spine, as well as the sternocostal articulations.
EN1−/− mice were first used to investigate the role of adenosine transport in the central nervous system pathways regulating alcohol consumption (33). In that study, male mice were examined at approximately 10 weeks of age and appeared normal in their anatomy, physiology, mortality rates and consumption of water. However, EN1−/− mice consumed twice as much alcohol compared to wild-type controls. This behavior was associated with a decrease in endogenous adenosine tone, which was not due to loss of A1 receptors or decreases in A1 receptor affinity. EN1−/− mice have also been shown to exhibit reduced anxiety-like behaviors (44). Levels of endogenous extracellular adenosine were not reported in these studies.

More recently, EN1-null mice have been used to investigate the role of this transporter in the cardiovascular system. Microvascular endothelial cells isolated from EN1−/− mice have enhanced expression of the A2A adenosine receptor and adenosine deaminase (34). Cardiomyocytes and microvascular endothelial cells from EN1−/− mice are relatively resistant to ischemic insult and EN1−/− mice show decreased heart damage in response to ischemia and hypoxia (45). Furthermore, loss of EN1 protects against ischemic acute kidney injury through control of postischemic renal perfusion (46). Overall, these studies point to a cardioprotective role of EN1. However, it is unlikely that changes in the nervous and cardiovascular systems give rise to ectopic mineralization in EN1−/− mice, especially of the inner annulus fibrosus, which is considered an avascular and aneural tissue (47,48).

In the present study, we found that plasma adenosine concentrations are significantly elevated in the EN1−/− mice compared to wild-type littermates. This finding is in keeping with differences reported (42). The greater plasma concentration of
adenosine in \textit{ENT1}\textsuperscript{−/−} mice likely reflects extracellular accumulation of adenosine due to lack of uptake by cells that normally express ENT1, one of the primary uptake pathways for adenosine (32, 49). The role of elevated extracellular adenosine in the formation of abnormal mineral deposits in fibrocartilaginous tissues remains to be explored, but may involve local changes in adenosine receptor signaling.

In this regard, recent advances point to a critical role for purine metabolism and signaling in the regulation of biomineralization and in diseases associated with either insufficient or ectopic mineralization (26-31). Once released by the cell, extracellular adenosine triphosphate (ATP) is sequentially metabolised by cell-surface enzymes, leading first to the production of PP\textsubscript{i} and adenosine monophosphate (AMP), which in turn are converted to inorganic phosphate and adenosine. Ectonucleotide pyrophosphatase/phosphodiesterase 1 (Enpp1) is responsible for the first step in this process, and the release of PP\textsubscript{i} has been shown to inhibit ectopic calcification in soft tissues (50). Interestingly, we show that \textit{ENT1}-null mice demonstrate significant downregulation of \textit{Enpp1} expression in intervertebral disc tissues relative to wild-type littermate controls. These findings are in keeping with the reported association between mutations in the gene encoding Enpp1 and hypermineralization disorders such as idiopathic infantile calcification (26) and OPLL (27). Furthermore, \textit{Enpp1}\textsuperscript{−/−} mice display soft tissue mineralization in the Achilles tendon, paraspinal ligaments and intervertebral discs, as well as hyperostosis of peripheral joints and calcification of articular cartilage (51). However, elements of the appendicular skeleton are not affected in \textit{ENT1}\textsuperscript{−/−} mice; changes are limited to fibrocartilaginous tissues of the axial skeleton.

We also observed a significant decrease in \textit{Ank} expression in the intervertebral
discs of \( \textit{ENT}1^- \) mice. Ank is a transmembrane protein that is thought to mediate \( \text{PP}_i \) transport. In mice, loss-of-function mutations of \( \text{Ank} \) lead to arthritis, ectopic crystal formation and generalized joint fusion (52), whereas, in humans, dominant mutations are associated with craniometaphyseal dysplasia (53,54) and familial chondrocalcinosis (55,56). Thus, like \( \text{Enpp1} \), decreased \( \text{Ank} \) expression in the spinal tissues of \( \text{ENT}1^- \) mice would be expected to further suppress extracellular \( \text{PP}_i \) levels, permitting the formation of ectopic mineral deposits.

Unexpectedly, expression of \( \text{Alpl} \) was also found to be decreased in the intervertebral discs of ENT1-null mice. \( \text{Alpl} \) encodes tissue-nonspecific alkaline phosphatase, which is responsible for the hydrolysis \( \text{PP}_i \) (51), and \( \text{Alpl} \) expression has been shown previously to be regulated by extracellular adenosine (28). In humans, disruption of \( \text{ALPL} \) causes hypophosphatasia, characterized by skeletal hypomineralization (57) and presumably a result of excessive accumulation of \( \text{PP}_i \). Thus, decreased \( \text{Alpl} \) expression in the spinal tissues of \( \text{ENT}1^- \) mice would be expected to increase extracellular \( \text{PP}_i \) levels, counteracting the decrease in \( \text{PP}_i \) arising from changes in \( \text{Enpp1} \) and \( \text{Ank} \) expression. Moreover, it is conceivable that a generalized decrease in \( \text{Alpl} \) expression could account for the increase in plasma \( \text{PP}_i \) that we observed in \( \text{ENT}1^- \) mice.

Adenosine and ATP can regulate cell behaviour and gene expression through cell surface receptors – the adenosine receptor family (58) and the P2 family of nucleotide receptors (59,60), respectively. Adenosine increases intracellular cyclic AMP (cAMP) levels via its \( A_{2A} \) and \( A_{2B} \) receptor subtypes. cAMP has been shown to induce abnormal calcification of vascular smooth muscle cells via a mechanism involving reduction in
extracellular PP$_i$ accumulation (61,62). In addition, activation of the P2X7 subtype of ATP receptors promotes bone formation and mineralization (27,63).

Taken together, our data suggest that disruption of adenosine signaling and PP$_i$ metabolism in ENT$^{−/−}$ mice is associated with disease onset and progression. Although there are no reported associations between mutations in the gene encoding ENT1 in humans and DISH, the high incidence of this disease in the human population argues against an underlying single gene defect. However, disruption of adenosine or PP$_i$ metabolism could result from alterations in the function of one or more regulatory or metabolic proteins – either through genetic defects or as a consequence of cell aging.

In ENT$^{−/−}$ mice, the only location outside of the spine that exhibited ectopic mineralization was the sternocostal region. This distinct anatomical pattern of mineral deposition in ENT$^{−/−}$ mice suggests some common element in spinal and sternal fibrocartilaginous tissues makes them susceptible to ectopic mineralization in the absence of ENT1. Relatively scant vascular perfusion of the affected fibrocartilaginous tissues could lessen the clearance of extracellular adenosine, giving rise to sustained adenosine signaling which may in turn lead to deregulation of PP$_i$ metabolism.

The spatial and temporal pattern of ectopic mineralization observed in the ENT$^{−/−}$ mice, along with the absence of inflammation in affected tissues, resemble characteristics of diffuse idiopathic skeletal hyperostosis (DISH) in humans. DISH affects about 20% of the male population over the age of 55, with a slightly lower prevalence in women (35). Similarly, in ENT$^{−/−}$ mice, lesions developed gradually over time with functional impairments noticeable by 8 months of age. Similar to DISH in humans, which is often first diagnosed in the cervical or thoracic spine (64), lesions in ENT$^{−/−}$ mice began in the
cervicothoracic spine and spread caudally. Moreover, sternal involvement is also noted in DISH patients (65). On the other hand, extra-axial calcifications are present in some DISH patients, but were not detected by µCT or histology in $ENT1^{-/-}$ mice. Interestingly, the reproducible pattern of mineral deposition observed in $ENT1^{-/-}$ mice was not related to differences in endogenous ENT1 expression levels in the affected tissues. The factors contributing to the timing of mineral deposition at these sites remains an intriguing question for ongoing investigation. The radiographic appearance of DISH in humans bears striking resemblance to that of ectopic mineralization in $ENT1^{-/-}$ mice. DISH is characterized by tortuous paravertebral calcifications generally anterior to the vertebral bodies (66). On gross examination the appearance is likened to that of candle wax dripping down the spine. This appearance is remarkably similar to that observed in the present study by µCT analysis of $ENT1^{-/-}$ mice.

In summary, this is the first report of a role for the primary membrane transporter for adenosine, ENT1, in regulating the calcification of soft tissues in mice. Disruption of purine homeostasis by removal of ENT1 leads to the ectopic mineralization of paraspinal ligaments and intervertebral discs in mice, resembling lesions seen in the human condition, DISH. Pathogenesis appears to be associated with both local and systemic changes in $P_{i}$ homeostasis. The $ENT1^{-/-}$ mouse may prove useful as a model for investigating the mechanisms underlying ectopic mineralization associated with DISH and for the evaluation of therapies for the prevention and reversal of DISH and associated pathologies.
2. 6 References


Chapter 3

Disruption of Biomineralization Pathways in Spinal Tissues of Mice Lacking Equilibrative Nucleoside Transporter 1 (ENT1)
Co-Authorship Statement

Chapter 3: SW: Figs. 3.1-3.8. Tissue collection for Figs. 3.1-3.8 was carried out by both DQ and SW.
3.1 Chapter Summary

Recent studies have suggested a role for adenosine metabolism in the regulation of biomineralization. Equilibrative nucleoside transporter 1 (ENT1) is a membrane protein involved in the bi-directional transport of adenosine across the plasma membrane. In mice lacking ENT1 (ENT1−/−), we reported the development of ectopic mineralization of paraspinal tissues in the cervical-thoracic region at 2 months of age, which extended to the lumbar and caudal regions with advancing age. Histological examination revealed large, irregular accumulations of eosinophilic material in spinal fibrocartilaginous tissues including ligaments, entheses and intervertebral discs. Plasma adenosine levels were greater in ENT1−/− mice than in wild-type controls and elevated plasma levels of inorganic pyrophosphate (PPi) in ENT1−/− mice indicated generalized disruption of pyrophosphate homeostasis. This skeletal phenotype closely resembles a human disorder known as diffuse idiopathic skeletal hyperostosis (DISH). DISH is a relatively common non-inflammatory spondyloarthropathy, characterized by ectopic calcification of spinal tissues. Its etiology is unknown and there are no specific treatments. The objectives of the present study were i) to characterize expression of adenosine transporters and receptors in spinal tissues; and ii) to investigate whether loss of ENT1 alters the expression of genes that regulate biomineralization, and/or genes involved in adenosine metabolism and signaling. Using quantitative RT-PCR, we compared gene expression in intervertebral disc tissues obtained from 6-month-old male ENT1−/− and wild-type mice. No differences were observed in genes encoding nucleoside transporters, adenosine receptors or enzymes involved in adenosine metabolism. In contrast, intervertebral discs from ENT1−/− mice exhibited reduced expression of genes involved in suppressing biomineralization,
including: matrix gla protein \((Mgp)\) – an inhibitor of soft tissue mineralization; ectonucleotide pyrophosphatase/phosphodiesterase 1 \((Enpp1)\) – responsible for the production of \(\text{PP}_i\), which inhibits mineralization; progressive ankylosis protein \((Ank)\) – a putative \(\text{PP}_i\) transporter; and osteopontin \((Spp1)\) – an inhibitor of mineralization. Unexpectedly, expression of alkaline phosphatase \((Alpl)\) – responsible for the hydrolysis of \(\text{PP}_i\) to \(\text{P}_i\) – was also reduced in \(\text{ENT1}^{-/-}\) discs. Of note, no differences were observed in the expression of these genes in tissues, including knee and liver, which do not exhibit ectopic mineralization in \(\text{ENT1}^{-/-}\) mice. These findings suggest that alterations in regulatory proteins that normally prevent soft tissue mineralization contribute to the ectopic calcification of spinal tissues in \(\text{ENT1}^{-/-}\) mice. \(\text{ENT1}^{-/-}\) mice may be a useful model to investigate the etiology of and therapies for DISH and related disorders of mineralization in humans.

\[3.2 \text{Introduction}\]

Aberrant calcification of soft connective tissues has been associated with a number of skeletal disorders, such as diffuse idiopathic skeletal hyperostosis (DISH) (1). DISH is a common non-inflammatory condition of the spine (2). It involves the pathological calcification of the anterolateral spinal ligaments, entheses, and fibrocartilaginous tissues present in the intervertebral discs (1,3,4). DISH often presents as back pain and stiffness and is associated with increased vertebral fractures in the elderly (5) and in severe cases, dysphagia (6) and compression of the spinal cord and nerve roots (6-8). DISH is typically diagnosed by radiographic examination (9), and is associated with the detection of distinct calcified outgrowths along the vertebrae of the
thoracic spine or chest (2). The specific radiographic diagnostic criteria for DISH are 1) flowing calcifications along anterolateral aspect of at least four continuous vertebral bodies; 2) preservation of disc height in involved areas; and 3) absence of bony ankylosis of facet joints and absence of sacroiliac erosion, sclerosis or fusion (10). There have been no reported single gene defects associated with DISH, although single nucleotide polymorphisms in the COL6A1 and FGF2 genes have been shown to confer genetic susceptibility to DISH (11,12). Furthermore, several metabolic disorders are associated with DISH including obesity, hypertension, diabetes mellitus, dyslipidemia, and hyperuricemia (13-15).

Recent studies have begun to investigate the role of purinergic signaling, specifically that of adenosine, in the physiology and pathophysiology of mineralized tissues (16). Through characterization of mice lacking ecto-5'-nucleotidase (Nt5e), Takedachi and colleagues demonstrated that adenosine promotes osteoblast differentiation via A2b adenosine receptor signaling (17). The Nt5e gene encodes the CD73 cell-surface enzyme, which converts adenosine monophosphate (AMP) to adenosine. It was found that CD73−/− mice had significantly lower bone mineral content than wild-type controls; these mice also showed a significant decrease in serum levels of osteocalcin, a metabolic marker of in vivo bone formation (17). Furthermore, real-time PCR demonstrated a decreased expression of osteoblast markers in CD73−/− mice (17). Building on these studies, St. Hilarie et al. demonstrated that mutations in NT5E resulting in a non-functional CD73 enzyme show genetic associations with symptomatic arterial and joint calcifications in humans (18). In addition, cultured fibroblasts from affected individuals showed reduced expression of NT5E mRNA and the CD73 protein, increased
tissue-nonspecific alkaline phosphatase (*ALPL*) enzyme activity, and the accumulation of calcium phosphate crystals (18). *ALPL* is an enzyme responsible for hydrolyzing inorganic pyrophosphate (PP$_i$ – an inhibitor of mineralization) to phosphate (P$_i$ – which promotes calcification). These studies suggest a possible link between adenosine metabolism and ectopic mineralization.

Recently, in mice lacking ENT1 (*ENT1*$^{−/−}$ mice), we reported the development of ectopic mineralization in paraspinal tissues that resemble DISH (19). These mineralized lesions originate at the cervical-thoracic region as early as 2 months of age, and extend to the lumbar and caudal regions with advancing age (19). Histological examination revealed large, irregular accumulations of eosinophilic material in spinal fibrocartilaginous tissues including ligaments, entheses and intervertebral discs. Interestingly, plasma adenosine levels were found to be significantly greater in *ENT1*$^{−/−}$ mice than in wild-type controls (19).

ENT1 (equilibrative nucleoside transporter 1 or solute carrier family 29 member 1, encoded by the *Slc29a1* locus) is a membrane protein involved in the bi-directional transport of nucleosides across the plasma membrane (20). The *ENT1*$^{−/−}$ mouse was originally created by Dr. Choi and colleagues to study the role of adenosine transport in central nervous system pathways regulating alcohol consumption (21). Initial studies demonstrated that at 3 months of age, *ENT1*$^{−/−}$ mice had <10% lower body weight, but showed no differences in open-field locomotor activity, and had no evidence of gross anatomical abnormalities in the brain compared to their wild-type littermate controls (21). Studies have reported that the *ENT1*$^{−/−}$ mice consumed twice as much alcohol and demonstrated a decreased ataxic effect of ethanol (21). These mice were also shown to
exhibit reduced anxiety-like behaviors (22). Microvascular endothelial cells isolated from $ENT1^{-/-}$ mice have enhanced expression of the $A_{2A}$ adenosine receptor and adenosine deaminase (23). Cardiomyocytes from $ENT1^{-/-}$ mice appear to be protected from ischemic insult and $ENT1^{+/+}$ mice show decreased myocardial infarction in response to ischemia and hypoxia (24).

The present study was conducted to understand the molecular framework underlying the development of ectopic mineralization in spinal tissues of the $ENT1^{-/-}$ mouse. We specifically interrogated gene expression differences in affected tissues isolated from $ENT1^{-/-}$ mice compared to their age- and gender-matched littermate wild-type controls to delineate the pathways through which disruption of adenosine signaling contribute to the development of soft connective tissue mineralization.

### 3.3 Materials and Methods

#### 3.3.1 Animals

$ENT1^{-/-}$ mice were generated through targeted deletion of exons 2 to 4 of the gene encoding ENT1 by a cre-loxP targeting strategy (21). $ENT1^{-/-}$ mice were backcrossed with C57BL/6 mice. The mouse colony was maintained through the breeding of heterozygous animals ($ENT1^{+/+}$) to obtain wild-type ($ENT1^{+/+}$) and knockout ($ENT1^{-/-}$) littermates. Mice were housed in standard cages and maintained on a 12-hour light/dark cycle, with rodent chow and water available ad libitum. Genotyping was performed as described (23). Given the increased reported prevalence of DISH in males (25% of men versus 15% of women over 50 years of age) (25), male mice were used for all experiments. Mice were euthanized at 6 months (26-30 weeks). All aspects of this study
were conducted in accordance with the policies and guidelines set forth by the Canadian Council on Animal Care and were approved by the Animal Use Subcommittee of the University of Western Ontario, London, ON.

3.3.2 Isolation of Intervertebral Discs and RNA Extraction

At 6 months of age, littermate-paired wild-type and \textit{ENT1}^- mice (n=8 for each genotype) were dissected to isolate intact intervertebral discs (IVDs) inclusive of annulus fibrosus, nucleus pulposus and cartilage endplates. IVDs from each animal were separated according to anatomical location as cervical-thoracic, lumbar, or tail. Non-affected tissues (including knee, heart, kidney, skeletal muscle, brain and liver) were also harvested from wild-type animals (n=3). Tissues were placed directly in 1 mL of TRIzol Reagent (Life Technologies, Wilmington, DE) and homogenized using a PRO250 Polytron benchtop homogenizer (PRO Scientific, Oxford, CT). Total RNA was extracted according to the manufacturer’s protocol and quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Mississauga, ON). For each sample, 1 µg RNA was reverse transcribed into cDNA using Superscript II Reverse Transcriptase (Life Technologies).

3.3.3 Primer Design and Optimization

For SYBR-based RT-PCR, primers were designed using PrimerBank and Integrated DNA Technologies with sequences specific to the gene of interest, to produce an amplicon between 75 to 175 bp in length. Whenever possible, primers were designed to flank an intron/exon boundary in order to detect gDNA contamination of RNA
samples. For primer optimization, the primers were assessed using serial dilutions (5-fold) of cDNA synthesized from a mix of wild-type heart, brain, kidney, muscle, intervertebral disc, and calvarial RNA at a range of temperatures between 56-62°C to optimize annealing/elongation temperatures. Standard curves were generated to control for primer efficiency (70-120%) and specificity of primers were determined by melt curve analysis (0.5°C/5 s).

3.3.4 Quantitative real-time RT-PCR

Gene expression patterns were determined by real-time PCR using the Bio-Rad CFX384 system. PCR reactions were run in triplicate, using 25 µM forward and reverse primers (primer sequences provided in Table 3.1) with 2X SsoFast EvaGreen Supermix (Bio-Rad, Mississauga, ON). The PCR program consisted of the following: initial 3 min at 95°C for denaturing; 95°C for 10 s denaturing; 30 s annealing/elongation (temperatures provided in Table 3.1), for a total of 40 cycles. Gene transcript levels were determined relative to a six-point calibration curve made from cDNA generated from heart, brain, kidney, muscle, intervertebral disc, and calvarial RNA (isolated from wild-type mice). The starting concentration of the cDNA standard (83 – 136 ng/µl) was inputted into the Bio-Rad CFX Manager 2.0 software to generate the standard curve (1/5 serial dilution). Values for gene transcript levels are expressed relative to the calibration curve in ng/µl.
<table>
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</tr>
<tr>
<td>Adk</td>
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<td>Alpl</td>
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<td>Bglap</td>
<td>Fwd – CTGACCTCACAGATCCCAAGC, Rev – TGTTCTGATAGCTGCTACAGAC</td>
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3.3.5 Statistical Analyses

Differences in gene expression levels between IVD tissues from distinct anatomical spine regions (cervical-thoracic, lumbar, and tail) and non-affected tissues (knee, heart, kidney, muscle, brain, and liver) within wild-type animals were compared using a one-way ANOVA with Bonferroni’s post-hoc test in GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA). For comparison of gene expression levels between wild-type and ENT1−/− animals in IVD tissues isolated from specific anatomical regions, Student’s t-test analysis was conducted using GraphPad Prism 5. For all analysis, significance was indicated as p < 0.05.

3.4 Results

Our previous studies established that hypermineralization of the ENT1−/− mouse was restricted to paraspinal fibrocartilaginous tissues and sternocostal articulations. In contrast, examination of appendicular joints revealed no aberrant morphology or evidence of ectopic mineralization, establishing that lesions are specifically associated with the axial skeleton. Furthermore, within the spine, there was a reproducible pattern of mineralization that progressed from cervical to caudal with age (19). We wanted to interrogate whether the temporal and spatial pattern of these hypermineralized lesions was due to differences in the levels of endogenous equilibrative nucleoside transporter and adenosine receptor expressions.

Quantitative real-time PCR was performed to characterize the endogenous gene expression of equilibrative nucleoside transporters and adenosine receptors in distinct tissues of wild-type mice. The gene expression levels in IVDs from distinct anatomical
regions (cervical-thoracic, lumbar and tail) were compared to tissues not affected by hypermineralization in the \( \text{ENT}1^{+/−} \) mouse, which include the knee, heart, kidney, muscle, brain and liver. The knee sample included the entire joint, which encompassed fibrocartilaginous tissues of the meniscus and tendons, as well as articular cartilage and subchondral bone. The gene expression levels of \( \text{ENT}1 \) were found to be comparable between IVD tissues and other tissues examined (Fig 3.1A). In contrast, the expression of related transporters \( \text{ENT}2, \text{ENT}3, \) and \( \text{ENT}4 \) was significantly lower in the IVD and knee tissues compared to tissues of the heart, kidney and brain in which previous studies have reported robust ENT expression (26,27) (Fig. 3.1B-D). Plasma adenosine levels were found to be significantly greater in \( \text{ENT}1^{+/−} \) mice than in wild-type controls, and therefore it was logical to investigate whether the expression of adenosine receptors was altered in response to elevated adenosine levels. The gene expression levels of \( \text{Adora}1, \text{Adora}2b, \) and \( \text{Adora}3 \) in IVD tissues was significantly lower compared to tissues of the heart, kidney, and brain (Fig 3.2 A, C-D). However, the gene expression level of \( \text{Adora}2a \) in IVD tissues was significantly lower only when compared to tissues of the brain (Fig. 3.2 B).

Having established the endogenous distribution of adenosine transporters and receptors in IVD tissues in comparison to other tissue types, real-time PCR was then used to investigate whether loss of \( \text{ENT}1 \) alters the expression of genes associated with adenosine metabolism and signaling, or extracellular matrix mineralization in intervertebral disc tissues. For this analysis, IVD tissues were isolated from male \( \text{ENT}1^{+/−} \) and wild-type littermate control mice at 6 months of age based on anatomical location.
Figure 3.1: Gene expression analysis of equilibrative nucleoside transporters in tissues isolated from 6-month-old wild-type mice.

Real-time PCR analysis shows the gene expression of (A) ENT1, (B) ENT2, (C) ENT3, and (D) ENT4 in wild-type animals in intervertebral discs (IVD) isolated from the cervical-thoracic (CT), lumbar or tail regions, knee, heart, kidney, muscle, brain and liver. Data are presented as the mean ± SEM, n = 8 animals for IVD, and n = 3 animals for other tissues. Values for gene transcript levels are expressed relative to a calibration curve in ng/µl as described in the materials and methods. Bars labeled with the same letter are not significantly different, based on one-way ANOVA with Bonferroni’s post-hoc test.
Figure 3.2: Gene expression analysis of adenosine receptors in tissues isolated from 6-month-old wild-type mice.
Real-time PCR analysis shows the gene expression of (A) Adora1, (B) Adora2a, (C) Adora2b, and (D) Adora3 for wild-type animals in intervertebral discs (IVD) isolated from the cervical-thoracic (CT), lumbar or tail regions, knee, heart, kidney, muscle, brain and liver. Data are presented as the mean ± SEM, n = 8 animals for IVD, n = 3 animals for other tissues. Bars labeled with the same lower case letter are not significantly different, based on one-way ANOVA with Bonferroni’s post-hoc test.
Gene expression values for each transcript in heart tissue (isolated from wild-type mice) are presented as a positive control to compare relative abundance. We first interrogated the expression of genes encoding the ENT transporters to determine if there was a compensatory effect due to the loss of ENT1. No significant differences were observed in the expression of ENT2, ENT3, or ENT4 between ENT1<sup>−/−</sup> and wild-type mice within each anatomical region (Fig. 3.3). Similarly, there were no differences observed in the expression of adenosine receptors in ENT1<sup>−/−</sup> mice compared to wild-type mice within each anatomical region (Fig. 3.4).

Since ENT1 is the predominant nucleoside transporter expressed in mammalian cells, and plasma adenosine levels were found to be higher in ENT1<sup>−/−</sup> mice than wild-type (19), we next investigated whether the expression of genes associated with the adenosine metabolism pathway were altered in the knockout mice. Adenosine kinase (Adk) is an enzyme that catalyzes the transfer of a phosphate from ATP to adenosine to form AMP; adenosine deaminase (Ada) metabolizes adenosine to inosine (another nucleoside); purine nucleoside phosphorylase (Pnp) is involved in the conversion of a nucleoside to a nucleobase, such as inosine to hypoxanthine; xanthine dehydrogenase (Xdh) catalyzes hypoxanthine to xanthine, and eventually to uric acid; and ecto-5′-nucleotidase (Nt5e) generates adenosine from the breakdown of AMP. No significant differences were observed in the expression of these adenosine metabolism enzymes in IVD tissues of ENT1<sup>−/−</sup> mice compared to wild-type mice within each anatomical region (Fig. 3.5).
Figure 3.3: Gene expression analysis of equilibrative nucleoside transporters in intervertebral discs isolated from 6-month-old wild-type mice and $ENT1^{-/-}$ mice. Real-time PCR analysis revealed no significant differences in the expression of (A) $ENT2$, (B) $ENT3$ or (C) $ENT4$ between wild-type and $ENT1^{-/-}$ mice in specific spinal regions. Gene expression values for heart tissue isolated from wild-type mice only are presented as a positive control to compare relative abundance. Data are presented as the mean ± SEM, n = 8 animals. Black bar = WT and white bar = $ENT1^{-/-}$ mice.
Figure 3.4: Gene expression analysis of adenosine receptors in intervertebral discs isolated from 6-month-old wild-type mice and \(\textit{ENT1}^{-/-}\) mice. Real-time PCR analysis revealed no significant differences in the gene expression of (A) \textit{Adora1}, (B) \textit{Adora2a}, (C) \textit{Adora2b} or (D) \textit{Adora3} between wild-type and \(\textit{ENT1}^{-/-}\) mice in specific spinal regions. Gene expression values for heart tissue isolated from wild-type mice only are presented as a positive control to compare relative abundance. Data are presented as the mean ± SEM, \(n = 8\) animals. Black bar = WT and white bar = \(\textit{ENT1}^{-/-}\) mice.
Figure 3.5: Gene expression analysis of adenosine metabolism genes in intervertebral discs isolated from 6-month-old wild-type mice and \( \text{ENT1}^- \) mice. Real-time PCR analysis revealed no significant differences in the expression of (A) \( \text{Adk} \), (B) \( \text{Ada} \), (C) \( \text{Pnp} \), (D) \( \text{Xdh} \) or (E) \( \text{Nt5e} \) between wild-type and \( \text{ENT1}^- \) mice in specific spinal regions. Data are presented as the mean ± SEM, \( n = 8 \) animals. Black bar = WT and white bar = \( \text{ENT1}^- \) mice. \( \alpha \) = significantly different between wild-type (\( p < 0.05 \), one-way ANOVA with Bonferroni’s post-hoc test).
Given that $ENT1^{−/−}$ mice demonstrate progressive mineralization of paraspinal fibrocartilaginous tissues (19), we interrogated the expression of genes associated with extracellular matrix mineralization in IVD tissues. Compared to wild-type mice, we observed reduced expression of genes involved in suppressing biomineralization in $ENT1^{−/−}$ mice, including matrix gla protein ($Mgp$ – an inhibitor of soft tissue mineralization, Fig. 3.6A); ectonucleotide pyrophosphatase/phosphodiesterase 1 ($Enpp1$ – responsible for the production of pyrophosphate, $PP_i$, which inhibits mineralization, Fig. 3.6B); progressive ankylosis protein ($Ank$ – a putative $PP_i$ transporter, Fig. 3.6C); and osteopontin ($Spp1$ – another inhibitor of mineralization, Fig. 3.6D). All four genes showed significantly reduced expression in $ENT1^{−/−}$ mice compared to wild-type in IVDs isolated from the cervical-thoracic region, the anatomical region most severely affected by ectopic mineralization at the 6 month time point; $Enpp1$ also showed reduced expression in IVDs of the lumbar region of $ENT1^{−/−}$ mice compared to wild-type gene expression levels (Fig. 3.6B). Importantly, no differences were observed in the expression of these genes between wild-type and $ENT1^{−/−}$ mice in tissues of the knee or liver, which do not exhibit ectopic mineralization (Fig. 3.6).

Unexpectedly, expression of tissue nonspecific alkaline phosphatase ($Alpl$) was found to be significantly decreased in IVDs of the cervical-thoracic and lumbar regions of $ENT1^{−/−}$ mice compared to wild-type (Fig. 3.7). Tissue-nonspecific alkaline phosphatase is responsible for the hydrolysis $PP_i$ to $P_i$, which promotes mineralization (28). No differences were observed in the expression of $Alpl$ between wild-type and $ENT1^{−/−}$ mice in tissues of the knee or liver (Fig. 3.7).
Figure 3.6: Gene expression analysis of mineralization associated genes in intervertebral discs (IVD), knee, and liver isolated from 6-month-old wild-type mice and ENT1−/− mice.

Real-time PCR analysis of genes associated with tissue mineralization revealed significant reductions in the expression of (A) *Mgp* in the cervical-thoracic region, (B) *Enpp1* in the cervical-thoracic and lumbar regions, (C) *Ank* in the cervical-thoracic region, and (D) *Spp1* in the cervical thoracic region in ENT1−/− mice compared to wild-type controls. There were no significant differences in gene expression in the knee or liver of the ENT1−/− mice compared to wild-type controls. Data are presented as the mean ± SEM, n = 8 animals. Black bar = WT and white bar = ENT1−/− mice. * Significantly different from same tissue isolated from wild-type (p < 0.05, Student’s t-test).
Figure 3.7: Gene expression analysis of alkaline phosphatase in intervertebral discs (IVD) isolated from 6-month-old wild-type mice and $\text{ENT1}^{-/-}$ mice.

Real-time PCR analysis revealed a significant reduction in the expression of $\text{Alpl}$ for cervical-thoracic and lumbar regions in $\text{ENT1}^{-/-}$ mice compared to wild-type controls. There were no significant differences in gene expression in the knee or liver in $\text{ENT1}^{-/-}$ mice compared to wild-type controls. Data are presented as the mean ± SEM, $n = 8$ animals. Black bar = WT and white bar = $\text{ENT1}^{-/-}$ mice. * Significantly different from same tissue isolated from wild-type ($p < 0.05$, Student’s $t$-test).
Our previous studies demonstrated that within fibrocartilaginous tissues, areas of ectopic calcification were surrounded by a clearly defined transition zone (19). Adjacent to fibrocartilaginous tissue with a typical histological appearance, this transition zone was characterized by metaplasia, with cells displaying eosinophilic nuclei that stained positively with DAPI, revealing an intact nuclear structure. To determine if cells within this tissue were undergoing a change in cell phenotype, we assessed the expression of markers associated with annulus fibrosus cells of the IVD (integrin binding sialoprotein, \(Ibsp\), and growth differentiation factor 10, \(Gdf10\)), hypertrophic chondrocytes (runt related transcription factor 2, \(Runx2\), and collagen, type X, alpha 1, \(Col10a1\)) and bone \((Runx2\) and osteocalcin, \(Bglap\)) lineage. Real-time PCR analysis revealed no differences in the expression of \(Ibsp\) (Fig 3.8A) but a significant reduction in the expression of \(Gdf10\) (Fig. 3.8B) in IVDs from the cervical-thoracic of \(ENT1^{-/-}\) mice compared to wild-type. No significant differences were detected in the expression of \(Runx2\) or \(Col10a1\) between wild-type and \(ENT1^{-/-}\) mice (Fig. 3.8C,D). In contrast, expression of \(Bglap\) was significantly reduced in IVDs from the cervical-thoracic region of the \(ENT1^{-/-}\) mice compared to wild-type (Fig. 3.8E).

3.5 Discussion

The first report of a role for the nucleoside transporter ENT1 in regulating biomineralization was the recent description by our group of ectopic mineralization in fibrous connective tissues of the spine and sternum of \(ENT1^{-/-}\) mice (19). In these studies, \(ENT1^{-/-}\) mice were characterized at various ages (from 1 to 17 months of age) using a combination of histology and \(\mu\)CT imaging. Aberrant mineralization was first detected
Figure 3.8: Gene expression analysis of markers associated with phenotypic transition in intervertebral discs isolated from 6-month-old wild-type mice and \textit{ENT1}\textsuperscript{-/-} mice.

Real-time PCR analysis examining the expression of markers associated with the annulus fibrosus (A, B), hypertrophic chondrocyte (C, D) and osteoblast (C, E) phenotype. Quantification revealed significant reductions in the expression of (B) \textit{Gdf10} and (E) \textit{Bglap} in the cervical-thoracic region of \textit{ENT1}\textsuperscript{-/-} mice compared to wild-type controls. There were no significant changes in the expression of (A) \textit{Ibsp}, (C) \textit{Runx2}, (D) or \textit{Col10a1} in \textit{ENT1}\textsuperscript{-/-} mice compared to wild-type controls. Data are presented as the mean ± SEM, n = 8 animals. Black bar = WT and white bar = \textit{ENT1}\textsuperscript{-/-} mice. * Significantly different from same tissue isolated from wild-type (\textit{p} < 0.05, Student’s \textit{t}-test).
between 6-8 weeks of age in the paraspinal connectives tissues of the cervical vertebrae; lesions were found to increase in severity and affected the thoracic, lumbar, caudal spine and sternocostal articulations with advancing age. Atypical mineralization was initially observed in paraspinal fibrocartilaginous tissues including paraspinal ligaments and entheses, and later detected in the annulus fibrosus of intervertebral discs and sternocostal articulations.

Despite a systemic loss of ENT1 function, our previous studies establish that ectopic mineralization in $ENT1^{-/-}$ mice is limited to fibrocartilaginous paraspinal tissues and sternocostal articulations. While previous studies have characterized the relative expression of murine ENT genes in various tissue types (26,27), musculoskeletal tissues including the IVD were not included in these analyses. The present study shows that in wild-type mice, $ENT1$ mRNA levels within the IVD are similar to levels expressed in other tissue types such as the heart, kidney, muscle, brain and liver, while the mRNA levels of $ENT2$, $ENT3$ and $ENT4$ levels within the IVD are lower than tissues of the heart, kidney, and brain. Interestingly, tissues of the knee which do not demonstrate aberrant mineralization in $ENT1^{-/-}$ mice (19), appeared to have lower levels of $ENT1$ gene expression than levels expressed in IVD tissues. The tissue-specific expression pattern of mouse $ENT1$ reported in the current study is consistent with previous reports, where $ENT1$ mRNA in male mice was found to be higher in the liver and similar in the heart, kidney, muscle and brain (26). The tissue distribution of $ENT2$ and $ENT3$ levels is also similar to the previous study (26).

We further established an absence of compensatory upregulation of $ENT2$, $ENT3$, or $ENT4$ gene expression in IVD tissues of the $ENT1^{-/-}$ mice. This is consistent with
previous studies that demonstrated gene expression levels of ENT2, ENT3, and ENT4 were similar in cardiomyocytes isolated from \( \text{ENT1}^{+/--} \) mice compared with those isolated from wild-type littermates (24). Although a small but significant increase was reported in \( \text{ENT3} \) transcript levels in microvascular endothelial cells of \( \text{ENT1}^{+/--} \) mice compared to wild-type cells, no increase in ENT3 protein levels was detected (23). Taken together, these findings suggest that in tissues from \( \text{ENT1}^{+/--} \) mice, there appears to be no compensation by \( \text{ENT2}, \text{ENT3}, \) or \( \text{ENT4} \) gene expression, with the lack of \( \text{ENT1} \). Compared to other tissues which demonstrate detectable expression of multiple ENTs, we have shown the relative abundance of equilibrative nucleoside transporter levels in the IVDs, which demonstrate that the IVDs rely predominantly on ENT1. ENT1 has been established as a predominant nucleoside transporter in mammalian cells and consequently, it is then possible that with the absence of ENT1, the knockout mice develop an aberrant mineralized skeletal phenotype.

Our previous studies demonstrated that plasma adenosine levels were significantly elevated in \( \text{ENT1}^{+/--} \) mice. Given the potential impact of disrupted adenosine signaling, we first investigated the endogenous expression of adenosine receptor expression in IVD tissues. The gene expression levels of \( \text{Adora1}, \text{Adora2b}, \) and \( \text{Adora3} \) in IVD tissues was significantly lower compared to tissues such as the heart, kidney, and brain. However, the gene expression level of \( \text{Adora2a} \) in IVD tissues was significantly lower only when compared to tissues of the brain. Our findings are consistent with previous studies that reported expression of \( \text{Adora1} \) throughout the central nervous system (CNS), heart, kidney and liver (29) and \( \text{Adora2a} \) expression in the CNS (29) and heart (30). Previous studies suggest a more widespread expression of \( \text{Adora2b} \); whereas
Adora3 expression has been reported to be enriched in brain tissues and liver, detected in the heart and kidney, and not detected in skeletal muscle (29). Compared to tissues of the heart, kidney, and brain, we have observed low gene expressions of adenosine receptors in the IVDs, which suggests that the presence of adenosine receptors may not play a big role in the development of the skeletal phenotype observed in the ENT1−/− mice.

Although we have shown that there are elevated plasma adenosine levels in the ENT1−/− mice, we have yet to investigate the levels of adenosine within the spinal tissue. Similar to our findings for ENT gene expression, no significant differences in the expression of adenosine receptors were detected in ENT1−/− mice compared to wild-type mice. A lack of change in the mRNA levels of adenosine receptors suggests that protein levels of adenosine receptors may not change. However, it is likely that adenosine receptor signaling is different in ENT1−/− mice because of differences in the extracellular concentration of adenosine, or changes in intracellular signal coupling. Previous reports suggest that changes in adenosine receptor expression in ENT1−/− mice may be cell-type dependent. In cardiomyocytes isolated from ENT1−/− mice, no significant differences were reported in the expression of adenosine receptor subtypes (24), whereas enhanced expression of Adora2a was reported in microvascular endothelial cells isolated from ENT1−/− mice (23). Furthermore, suggesting that loss of ENT1 does not alter adenosine metabolism in IVD tissues, we demonstrate no significant differences in the expression of adenosine metabolism enzymes (Adk, Ada, Pnp, Xdh, Nt5e) in ENT1−/− mice compared to wild-type control. Gene expression studies suggest that at 6 months of age, adenosine signaling and metabolism pathways do not appear to be altered in IVD tissues of ENT1−/− mice, but further studies are required to assess levels of adenosine within our tissue of
interest and measure active signaling.

Previous studies have demonstrated that purine metabolism and signaling play a critical role in the regulation of biomineralization and diseases associated with aberrant mineralization (9,16,18,31-33). In the current study, we report a significant decrease in Mgp expression in IVD tissues of ENT1−/− mice isolated from anatomical regions that demonstrate ectopic mineralization at 6 months. MGP is an extracellular matrix glycoprotein that contains γ-carboxyl groups, which allow it to chelate calcium and function as an inhibitor of tissue mineralization (34). MGP deficiency in mice results in premature calcification, aberrant mineralization of growth plate cartilage, leading to short stature, osteopenia and fractures, and severe arterial calcification leading to premature death (35). The corresponding human disorder is known as Keutal syndrome, characterized by abnormal cartilage calcification, peripheral pulmonary stenosis and midfacial hypoplasia (36). In addition, the tiptoe walking mouse (ttw, a spontaneous recessive mutant), shows disruption of the annulus fibrosus arrangement in IVDs and has been suggested to be associated with the abnormal expression of MGP (37). Interestingly, SPP1 is also a putative inhibitor of mineralization (38,39) and we demonstrate that its expression is downregulated in IVD tissues of ENT1−/− mice in regions associated with ectopic mineralization at 6 months. Decreased expressions of these inhibitors of tissue mineralization contribute to the ectopic mineralization observed in the ENT1−/− mice.

When ATP is released by the cell, it is sequentially metabolized by cell-surface enzymes to produce adenosine. ENPP1 is responsible for the first step in this process and serves as a physiological regulator of calcification via the production of adenosine monophosphate (AMP) and inorganic pyrophosphate (PPi), the latter of which inhibits
ectopic calcification in soft tissues (40). Extracellular PPi is also regulated by ANK, a transmembrane protein postulated to transport PPi across the plasma membrane to the extracellular environment (41). We show a significant downregulation of Enpp1 and Ank expression in IVD tissues of ENT1−/− mice in regions of associated ectopic mineralization at 6 months of age. Enpp1−/− mice have been shown to exhibit soft tissue mineralization in paraspinal ligaments, intervertebral discs, Achilles tendon and calcification of articular cartilage, and peripheral joint hyperostosis (28). Mutations in Enpp1 have been also been linked to mineralization disorders such as ossification of the posterior longitudinal ligament (42) and generalized arterial calcification of infancy and hypophosphatemic rickets (43). In mice, mutations in Ank lead to mineral deposition in the articular cartilage and synovial fluid (41,44), and eventual generalized joint fusion (45,46). In humans, dominant ANK mutations are associated with craniometaphyseal dysplasia (47,48) and familial chondrocalcinosis (49,50). Therefore, we postulate that reduced expression of Enpp1 and Ank would lead to reduced extracellular PPi levels, and thus increased tissue mineralization.

Interestingly, the changes in gene expression associated with the regulation of mineralization were found to correlate with the temporal progression of mineralization reported in ENT1−/− mice (19). At the 6 month time point examined in these studies, mineralization was reported in >60% of the cervical and thoracic spine, 30% of the lumbar spine, and was not detected in the caudal spine. The fact that significant changes in Mgp, Enpp1, Ank, and Spp1 gene expression were restricted to IVDs of the cervical-thoracic spine of ENT1−/− mice suggests that observed changes are secondary to the initiation of mineralization. Furthermore, no significant differences were observed in the
expression of these genes in other tissues including the knee and liver, which do not exhibit ectopic mineralization in $ENT1^{-/-}$ mice. We also observed that gene expression in caudal IVDs is different from the gene expression in cervical-thoracic and lumbar IVDs in wild-type mice. Real-time PCR analysis revealed a significantly higher expression of $ENT4$ and $Adora3$ in the tail IVDs compared to the lumbar IVDs; expression of $Xdh$ was significantly lower in the tail IVDs compared to the cervical-thoracic IVDs; and $Mgp$ expression was significantly higher in tail IVDs compared to cervical-thoracic IVDs. It is possible that these differences in gene expression contribute to the fact that the tail IVDs are the last to demonstrate ectopic mineralization in the spine. Further studies are required to explore the mechanism(s) underlying this phenomenon.

Unexpectedly, expression of $Alpl$ was significantly reduced in IVDs of the cervical-thoracic and lumbar spine of $ENT1^{-/-}$ mice compared to control tissue. $ALPL$ promotes mineralization by catalyzing the hydrolysis of $PP_i$, thereby decreasing the local concentration of this calcification inhibitor, while increasing the levels of $P_i$ (28). $Alpl^{-/-}$ mice mimic a severe form of hypophosphatasia, characterized by rickets, spontaneous bone fractures, osteomalacia, and elevated $PP_i$ levels, and die before weaning (51). In humans, disruption of $ALPL$ causes hypophosphatasia, characterized by skeletal hypomineralization (52). It is possible that the reduction of $Alpl$ in the spinal tissues of $ENT1^{-/-}$ mice at 6 months of age (at which age lesions are present in the cervical, thoracic and lumbar regions) is a result of excessive mineral present in the extracellular matrix. As a consequence, lower levels of $Alpl$ would attempt to decrease further mineralization.

In our initial characterization of $ENT1^{-/-}$ mice, we noted that early foci of mineralization in fibrocartilaginous structures were associated with a clearly defined
transition zone that contained cells with eosinophilic nuclei and a hypertrophic appearance (19). To begin to examine whether ectopic mineralization was associated with a phenotypic conversion of the associated fibrocartilaginous cell types, we examined the expression of cell-type specific markers. To investigate the disruption of the annulus fibrous phenotype, we quantified the expression of *Ibsp* and *Gdf10*. While levels of *Ibsp* expression were unchanged, a significant reduction in the expression of *Gdf10* was detected in IVDs from the cervical-thoracic region of *ENT1*<sup><-/-</sup> mice compared to wild-type tissues, which may suggest alterations in the annulus fibrusus phenotype. No differences were detected in the expression of either *Runx2* or *Col10a1* in IVD tissues from *ENT1*<sup><-/-</sup> mice compared to wild-type, markers associated with the osteoblast (53) and hypertrophic chondrocyte lineages (54,55). In contrast, expression of osteocalcin (*Bglap*), an abundant non-collagenous bone matrix protein, which has been previously reported to be specific to osteoblasts (56,57), was significantly reduced in IVDs of the cervical-thoracic region of the *ENT1*<sup><-/-</sup> mice compared to wild-type tissues. There have been contradictory results over the role of osteocalcin in bone mineralization. Ducy et al. showed that osteocalcin-deficient mice had increased bone density without a change in bone resorption or mineralization, suggesting that osteocalcin directly inhibits osteoblastic bone formation (58). However, some studies show a positive correlation between osteocalcin concentration and mineralization (59). Furthermore, the involvement of osteocalcin in bone resorption has also been demonstrated, principally in the regulation of osteoclast formation and activity (60,61). Despite controversial data, the dual role of osteocalcin in bone can be presumed: firstly, osteocalcin regulates bone remodeling by modulating osteoblasts and osteoclast activity, secondly, it acts as a regulator of bone
mineralization (62). Taken together, these results suggest that the development of ectopic lesions within the fibrocartilaginous tissue of the IVD in ENT1⁻/⁻ mice is not associated with the phenotypic conversion of annulus fibrosus cells to either a hypertrophic chondrocyte or osteoblastic lineage. Further studies are required to understand the specific cellular changes associated with the metaplasia and initiation of tissue mineralization in ENT1⁻/⁻ mice.

In summary, the present study was conducted to begin to elucidate the molecular framework underlying the progressive mineralization of paraspinal tissues in the ENT1⁻/⁻ mouse. The pattern of ectopic mineralization observed in ENT1⁻/⁻ mice is intriguing as pathological changes are restricted to the spine and sternocostal regions, despite being a whole-body knockout of ENT1 and demonstrates a reproducible temporal pattern of development (19,21). The current data suggests that spinal tissues may be affected by ectopic mineralization due to the endogenous expression of ENTs, which shows that ENT1 is the predominant nucleoside transporter in IVDs. Furthermore, as demonstrated by real-time PCR, there appears to be no compensation by related transporters for the loss of ENT1. The data also shows significant changes in the expression of genes involved in the suppression of biomineralization that may contribute to calcification. Since there is a downregulation of mineralization associated genes, pathogenesis also appears to be associated with changes in PP₃ homeostasis. In addition, although the annulus fibrosus is affected in the IVD with lesions, changes do not appear to be correlated with the phenotypic conversion of annulus fibrosus cells to either a hypertrophic chondrocyte or osteoblast lineage. A limitation of this study was the targeted candidate gene approach used to evaluate changes in gene expression; furthermore, these changes are yet to be
verified by protein data. In order to investigate changes associated with the early
induction of tissue mineralization, we propose that identification of primary changes will
require a more global approach to analysis, including microarray or proteomic
quantification at an earlier time-point. Understanding the $ENT1^{-/-}$ mouse may be
advantageous for investigating the mechanisms of ectopic mineralization observed in
DISH and associated mineralization disorders.
3.6 References


Chapter 4

General Discussion
4.1 Summary and Conclusions

The studies presented in this thesis were undertaken based on a preliminary observation that mice lacking the gene encoding ENT1 developed progressive ectopic mineralization of spinal tissues. We hypothesized that: **loss of ENT1 leads to progressive accumulation of ectopic mineral in paraspinal tissues due to altered expression of genes that regulate biomineralization.**

We sought to explore this hypothesis with the following specific objectives:

1) Characterize pathological changes in paraspinal tissues in \( ENT1^- \) mice over time pertaining specifically to hypermineralization.

2) Determine changes in the expression of genes associated with adenosine transport and metabolism, as well as, extracellular matrix mineralization in the \( ENT1^- \) mice compared to wild-type controls.

In chapter two, we characterized the temporal and spatial pattern of the mineralized lesions present in the \( ENT1^- \) mice.

**Conclusion 2.1:** Mice lacking functional ENT1 demonstrate ectopic mineralization restricted to paraspinal, intervertebral and sternocostal fibrocartilaginous tissues. There was no evidence of mineralization in appendicular joints or blood vessels, indicating specificity of the axial skeleton.

**Conclusion 2.2:** \( ENT1^- \) mice demonstrate a reproducible temporal pattern of mineralization which originates in the cervical paraspinal ligaments at 2 months of age, and extends towards the thoracic, lumbar, and caudal region with age involving intervertebral disc (IVD) tissues and sternocostal joints.
Conclusion 2.3: Micro-computed tomography (µCT) imaging of radio-opaque mineralized lesions in the spinal tissues of \( ENT1^{-/-} \) mice bear striking resemblance to the radiographic appearance of spinal lesions characteristic of diffuse idiopathic skeletal hyperostosis (DISH) in humans.

In chapter three, we investigated the molecular framework underlying the development of ectopic mineralization of spinal tissues in the \( ENT1^{-/-} \) mouse. We first characterized the endogenous expression of nucleoside transporters and adenosine receptors in spinal tissues, and then interrogated gene expression differences in affected tissues isolated from \( ENT1^{-/-} \) mice compared to their age- and gender-matched littermate wild-type controls. Using a targeted candidate gene approach, these studies sought to delineate the pathways through which disruption of adenosine signaling contribute to the development of soft connective tissue mineralization.

Conclusion 3.1: In wild-type mice, the gene expression levels of \( ENT1 \) within the IVDs are similar to levels expressed in other tissue types such as the heart, kidney, muscle, brain and liver. In contrast, the gene expression levels of related transporters, \( ENT2 \), \( ENT3 \), and \( ENT4 \) were significantly lower in the IVD and knee tissues compared to tissues of the heart, kidney and brain.

Conclusion 3.2: Compared to wild-type, expression of genes encoding nucleoside transporters, adenosine receptors, or enzymes involved in adenosine metabolism were not altered in IVDs isolated from \( ENT1^{-/-} \) mice.

Conclusion 3.3: Gene expression findings suggest that decreased gene expression of regulatory proteins that normally prevent soft tissue mineralization (\( Mgp, Enpp1, Ank, \)
Spp1) may contribute to the ectopic calcification of spinal tissues in ENT1−/− mice. Furthermore, elevated plasma levels of inorganic PP, in ENT1−/− mice indicate generalized disruption of pyrophosphate homeostasis.

4.2 Significance of Research

Is the ENT1−/− mouse a model for diffuse idiopathic skeletal hyperostosis (DISH)?

DISH is a common human disorder which involves the calcification of the anterior longitudinal ligament, paraspinal connective tissues and annulus fibrosus (1,2). DISH is often underdiagnosed and widely misdiagnosed (3); this is due to the fact that it is primarily diagnosed through radiographs of the thoracic spine or chest, which are usually taken due to another disease or injury (4). The etiology of DISH is unknown, and currently there are no specific treatments. It has been proposed that lesions originate in fibrous and fibrocartilaginous structures including entheses, however the underlying pathogenesis remains obscure since prior to our studies, no suitable animal model had been reported to pursue mechanistic studies.

In this study, we observed that the lesions of ectopic mineralization present at the cervical and upper thoracic regions of ENT1−/− mice resemble the radiographic appearance of lesions present in patients diagnosed with DISH. The ectopic calcification observed in the ENT1−/− mice is restricted to paraspinal ligaments, entheses, intervertebral discs and sternocostal regions. However, it is important to note that although the thoracic spine is most commonly affected, appendicular joints such as metacarpophalangeal joints, elbows, and shoulders are also affected in DISH (5-9). Entheseal ossification of the heel, ribs, and pelvis are also common findings in DISH (5).
Equilibrative nucleoside transporters (ENTs) are sodium-independent, facilitative diffusion transporters which transport nucleosides bi-directionally down a concentration gradient. ENTs are found distributed among various cell types (10,11) and consist of four members (ENT1, ENT2, ENT3, ENT4) which share an ability to transport adenosine but differ in their abilities to transport other nucleosides and molecules (12). Recent advances point to a critical role for purine metabolism in the regulation of biomineralization in diseases associated with either insufficient or ectopic mineralization (13-18). Our studies (Chapter 2) are the first to report a role of the primary membrane transporter for adenosine – ENT1 – in regulating soft tissue calcification in mice.

Loss of ENT1 activity would be expected to modify extracellular adenosine levels, thus altering overall purine metabolism and signaling through adenosine receptors. In the present study, we found that plasma adenosine concentrations are significantly elevated in the ENT1−/− mice compared to wild-type littermates. The increased plasma concentration of adenosine in ENT1−/− mice likely reflects extracellular accumulation of adenosine due to the lack of uptake by cells that normally express ENT1 – one of the primary uptake pathways for adenosine (12,19). The role of elevated extracellular adenosine in the formation of abnormal mineral deposits in fibrocartilaginous tissues remains to be explored, but may involve local changes in adenosine receptor signaling, which in turn alter the expression of genes important for the regulation of biomineralization. A recent study has linked ectopic arterial and joint calcifications with loss of ecto-5'-nucleotidase (NT5E) function leading to decreased levels of extracellular adenosine (15), and therefore implicates aberrant adenosine signaling in the pathogenesis of arterial calcification.

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The gene expression data demonstrates reduced expression of genes involved in suppressing biomineralization, such as *Mgp*, *Enpp1*, *Ank*, and *Spp1*, which could explain the ectopic mineralization observed in the *ENT1*−/− mice. It is also possible that the reduction of *Alpl* in the spinal tissues of *ENT1*−/− mice is a result of excessive mineral present in the extracellular matrix. As a consequence, lower levels of *Alpl* would attempt to decrease further mineralization. This data suggests that altered adenosine levels lead to changes in pathways associated with mineralization signaling, specifically in the expression of enzymes regulating PPi and secreted proteins that regulate biomineralization (Fig. 4.1).

In conclusion, the *ENT1*−/− mouse may prove useful as a model for investigating the mechanisms underlying the onset and progression of ectopic mineralization associated with DISH and other mineralization disorders. This may lead to the identification of risk factors, and for the evaluation of therapies for the prevention and reversal of DISH and associated pathologies. The findings will represent a breakthrough in our understanding of this common but as yet under-recognized disease.

### 4.3 Limitations of the Research and Suggestions for Future Studies

The present study was the first to report the skeletal phenotype of the *ENT1*−/− mouse. As such, there are many future studies that can be undertaken using this model. In Chapter 3, we performed gene expression experiments which demonstrated a down-regulation of genes that regulate biomineralization (*Mgp*, *Enpp1*, *Ank*, *Spp1*, and *Alpl*) in *ENT1*−/− mice. However, we are unable to make any conclusive statements about a
Figure 4.1: Proposed schematic of pathways regulating biomineralization in the \textit{ENT1}\textsuperscript{−/−} mouse.

In contrast to Figure 1.2, which demonstrates the pathway for physiological biomineralization, results from these studies demonstrate disruption of these pathways in the \textit{ENT1}\textsuperscript{−/−} mice. The fibrocartilage cell appears to be the cell type affected in the \textit{ENT1}\textsuperscript{−/−} mice. Plasma adenosine levels are significantly elevated in the \textit{ENT1}\textsuperscript{−/−} mice compared to the wild-type due presumably to the loss of \textit{ENT1}-mediated transport. Under normal conditions, ATP is released extracellularly and subsequently metabolized by cell-surface enzymes. Our studies demonstrate a significant decrease in the expression of ectonucleotide pyrophosphatase/phosphodiesterase 1 (\textit{Enpp1}), an enzyme that metabolizes ATP and releases inorganic pyrophosphate (\textit{PP}_i, which inhibits mineralization). There is also a decrease in the expression of progressive ankylosis (\textit{Ank}), a putative transporter that mediates \textit{PP}_i efflux. A decrease in \textit{ENpp1} and \textit{ANK} levels would result in lower levels of the mineralization inhibitor \textit{PP}_i, thus allowing for mineralization to proceed. Our studies also show a decrease in the expression of mineralization inhibitors, matrix Gla protein (\textit{Mgp}) and osteopontin (\textit{Spp1}), which could explain the ectopic mineralization observed in the \textit{ENT1}\textsuperscript{−/−} mice. \textit{PP}_i is metabolized by alkaline phosphatase (\textit{Alpl}), which produces inorganic phosphate (\textit{P}_i), promoting mineralization. \textit{P}_i and Ca\textsuperscript{2+} accumulate in matrix vesicles or on extracellular matrix components resulting in the crystallization of calcium phosphate, leading to formation of hydroxyapatite and thus, matrix calcification. Pathogenesis appears to be associated with the dysregulation of biomineralization pathways, and may involve both local and systemic changes in \textit{PP}_i homeostasis, or \textit{PP}_i:\textit{P}_i ratio.
mechanism for ectopic mineralization, without any protein data. Therefore, it is necessary to follow-up the differences we observed in gene expression with analysis of protein data or assays of enzymatic activity. Additionally, the global differences in protein levels between the ENT1−/− mouse and age-and-gender matched wild-type controls could also be assessed using mass spectrometry-based proteomics.

In our studies, characterization of gene expression was performed on 6-month old mice, at which age lesions were present in varying degrees in the spine; mineralization was reported in 60 to 90% of the cervical spine, 30 to 60% of the thoracic spine, 0 to 30% of the lumbar spine, and not detected in the caudal spine. Detected changes in gene expression were restricted to IVD tissues in the presence of mineralized lesions and therefore it is likely that the differences we observed are due to a secondary effect (the presence of ectopic mineralization). Therefore, in order to delineate the pathway(s) responsible for the development of hypermineralized lesions, gene expression studies should be carried out at a younger time-point, such as 1-2 month-old mice when lesions are first detectable by µCT. A non-biased global analysis for gene expression of ENT1−/− mouse tissues (by microarray), may be necessary to identify causative factors for the observed ectopic mineralization. Furthermore, it should be noted that although lesions are encapsulated by the annulus fibrosus in the IVDs, gene expression studies were carried out on the entire IVDs, which included three different tissue types. Therefore, the existing research is technically limited as we were unable to micro-dissect the tissue of interest (in the lab, studies are currently undergoing in order to isolate cells of the annulus fibrosus).

In addition to genetic approaches, it would be interesting to test whether long-term administration of ENT1 blockers leads to the development of DISH in wild-type
mice. These *in vivo* pharmacological studies would help determine whether loss of ENT1 activity leads directly to abnormal mineralization, or whether it acts indirectly through a developmental defect that in turn impacts biomineralization. Administration of nitrobenzylmercaptopurine riboside (NBMPR), which is a specific inhibitor of ENT1 (20), to wild-type animals, would allow us to investigate whether animals develop mineralization of spinal tissues similar to the phenotype observed in *ENT1*−/− mice.

A critical issue arises from our findings to date: ENT1 function was compromised in all tissues and extracellular adenosine and PPi levels were systemically elevated, yet ectopic calcification was limited to paraspinal tissues. There was no calcification in blood vessels, cartilage and other soft connective tissue (including fibrocartilaginous structures of the appendicular skeleton). Gene expression data does not show compensation by other related transporters (*ENT2, ENT3, and ENT4*) for the loss of *ENT1* in spinal tissues, however, it is possible that in non-spinal tissues, there is an upregulation of other transporters or the endogenous expression of related ENT transporters compensates for the loss of ENT1. Alternatively, changes in expression of *Mgp, Enpp1, Ank, Spp1*, and *Alpl* may not occur in non-spinal tissues, because of tissue-specific differences in signaling. Further studies are required to better understand the tissue specificity of the *ENT1*−/− phenotype.

Interestingly, the Boxer dog breed exhibits an unusually high incidence of DISH (41% prevalence) (21), and has a 7-amino-acid deletion in a critical region of *ENT1* that may impact transport function (22). The high prevalence in a particular breed and the relative absence of the disease in other breeds is consistent with a genetic origin. Therefore, it is possible that ENT1 function is compromised in the Boxer dog. Tissue
samples from Boxer dogs, as well as from humans diagnosed with DISH, will provide information on whether there is a genetic association between mutations or single nucleotide polymorphisms in the ENT1 gene and DISH.

Lastly, in vitro studies provide important insights into fundamental mechanisms of biological processes. Cell and tissue culture models allow for manipulation of the system, and could be used to investigate the mechanism that underly changes in gene expression and excessive mineralization. To mimic hypermineralization in wild-type cells, ENT blockers such as NBMPR, or elevated extracellular concentration of adenosine could be used. Furthermore, in vitro experiments to attempt to “rescue” hypermineralization in \( ENT1^{−/−} \) cells would be informative, and can be done by maintaining the concentration of extracellular adenosine at physiological levels, and/or through the use of selective adenosine receptor antagonists to block adenosine signaling via \( A_1, A_{2a}, A_{2b} \) or \( A_3 \) receptors.

The experiments outlined above will provide insight into the mechanism underlying the development of ectopic mineralization in \( ENT1^{−/−} \) mice and hopefully provide a means to correlate this mechanism with biochemical and gene expression changes in human DISH patients to determine the mechanism in humans.
4.4 References


APPENDIX A

Ethics Approval of the Animal Use
AUP Number: 2009-030
AUP Title: Purine and Pyrimidine Transport in the Cardiovasculature

Approval Date: 04/24/2009

The YEARLY RENEWAL to Animal Use Protocol (AUP) 2009-030 has been approved.

1. This AUP number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this AUP number.
3. Purchases of animals other than through this system must be cleared through the ACVS office.
   Health certificates will be required.

REQUIREMENTS/COMMENTS
Please ensure that individual(s) performing procedures on live animals, as described in this protocol, are familiar with the contents of this document.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

Submitted by: Kinchlea, Will D
on behalf of the Animal Use Subcommittee
APPENDIX B

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License Number 3135730270991
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