ETV5 AND MMP2 IN HUMAN CHONDROSARCOMA

ETV5 AS A REGULATOR OF MATRIX METALLOPROTEINASE 2 IN HUMAN CHONDROSARCOMA

By:

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ABSTRACT

Chondrosarcoma is a unique type of bone cancer in that it does not respond to chemotherapy or radiation therapy, and therefore many affected patients die from metastatic disease. Metastasis has been correlated to upregulation of the matrix metalloproteinase (MMP) family of proteases, which can degrade a wide range of extracellular components. MMP2 is an enzyme secreted from the cell and degrades extracellular gelatin, as denatured collagen. ETV5 is a transcription factor which has shown to be overexpressed in multiple types of invasive tumours. The regulatory relationship between ETV5 and MMP2 has not been studied in chondrosarcoma. We hypothesized that ETV5 regulates MMP2 in human chondrosarcoma with the protease acting as a downstream effector. We have determined that both ETV5 and MMP2 are upregulated in the KC human chondrosarcoma cell line. Gene knockdown of ETV5 in KC cells resulted in a reduction in MMP2 mRNA expression as well as decreased protein production, and significantly decreased MMP2 gelatinase activity. When ETV5 is overexpressed, MMP2 expression is upregulated at the RNA and protein levels. Data from our bone resorption studies revealed that when a matrix metalloproteinase 2 inhibitor is added to the KC cell growth media, collagen released from bone chips decreased significantly. Upon histological examination, bone chips incubated with KC cells and the MMP2 inhibitor showed less bone resorption. Analyses of patient cell lines show similar expression profiles of ETV5 and MMP2 as the KC cell line. ETV5 knockdown (KD) in the patient cell

lines also showed a decrease in MMP2 expression, similar to the KC cells, suggesting that the ETV5-MMP2 pathway has clinical importance. This data advocates that ETV5 has a significant role in regulating MMP2 expression and activity, and bone resorption in human chondrosarcoma, and thus may be a targetable effector of the metastatic cascade in this cancer.

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Dedicated to my Mom, my #1 Fan

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LIST OF ABBREVIATIONS

APMA: 4-aminophenylmercuric acid
BSA: Bovine Serum Albumin
cDNA: Complimentary DNA
CT: Chemotherapy
DBD: DNA-Binding Domain
D-MEM: Dulbecco's Modified Eagle Medium
ECM: Extracellular Matrix
ELISA: Enzyme-Linked Immunosorbent Assay
ETS: E Twenty Six
ETV5: ETS Translocation Variant Gene 5 / ETS Variant 5
GAPDH: Glyceraldehyde 3-Phosphate Dehydrogenase
GDNF: Glial Cell Line-Derived Neurotrophic Factor
GF: Growth Factor
hFOB: Human Fetal Osteoblastic
FBS: Fetal Bovine Serum
FGF: Fibroblast Growth Factor
KD: Knockdown
KO: Knock-out
MEM-α: Minimum Essential Media-α
MMP: Matrix Metalloproteinase
MMP2: Matrix Metalloprotease 2
MMI: Matrix Metalloproteinase Inhibitor
MT-MMP: Membrane-Type MMP
OE: Overexpression
PBS: Phosphate Buffered Saline
PCR: Polymerase Chain Reaction
PEA3: Polyomavirus Enhancer-Binding Activator
RANKL: Receptor Activator of NF-κβ Ligand
ROS: Reactive Oxygen Species
RT: Radiation Therapy
SDS: Sodium Dodecyl Sulfate
siRNA: Small Interfering Ribonucleic Acid
SSC: Spermatogonial Stem Cell
TBS-T: Tris Buffered Saline-Tween
TF: Transcription Factor
TGF: Transforming Growth Factor
TIMP: Tissue Inhibitor of Metalloproteinases
qRT-PCR: Quantitative Real-Time Polymerase Chain Reaction
WT: Wild-Type

CHAPTER ONE: INTRODUCTION

1.1 Bone

Anatomy

Bone tissue is composed of multiple layers providing strength and protection to the body (**Figure 1.1**). The periosteum is a layer of fibrous connective tissue which encompasses the bone. Compact and trabecular bones form the main portions of the bone; compact bone comprises the outer portion, while trabecular bone is found closest to the bone cavity. Osteons within compact bone are made up of osteocytes and arranged as circular columns running the length of the bone, containing Haversian canaliculi. These tunnels allow for the vasculature, lymphatic system and nerves to run along the bone and across to neighboring osteons.

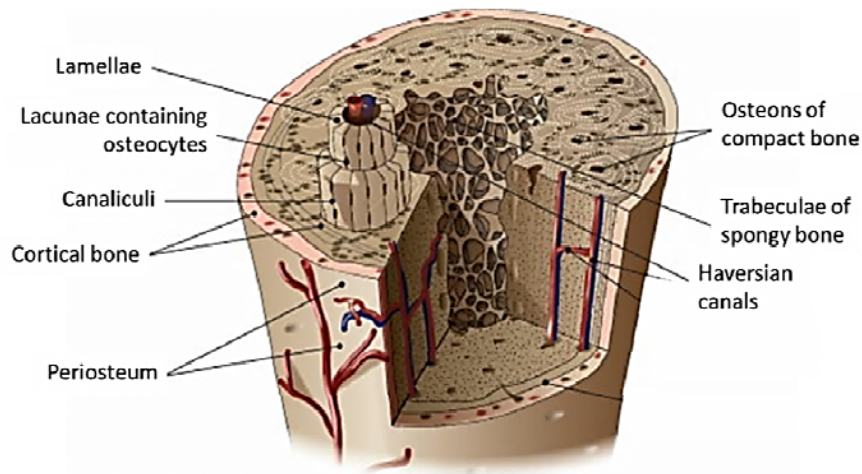


Figure 1.1: Anatomy of the layers of bone, taken from Apatech [1].

Collagen

Ninety percent of organic bone matrix is built of type I collagen [2, 3]. This gives bone its tensile strength to act as a scaffold for the tissues [4]. Collagen is formed from three helical α chain forming homotrimeric or heterotrimeric peptides wrapped together to create a microfibril. These combine with others to form a collagen fibril. Many of these fibrils together aggregate to make a bundle (**Figure 1.2**).

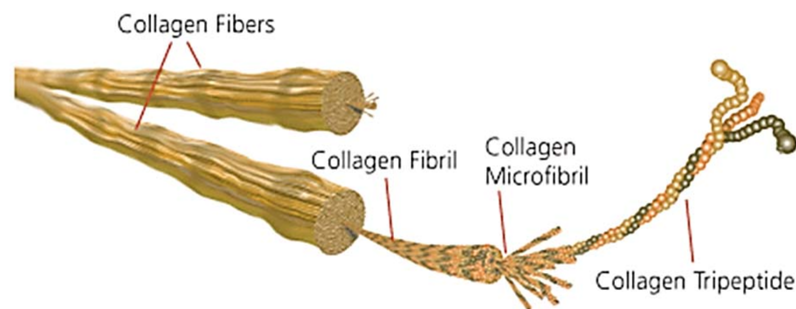


Figure 1.2: Formation of collagen bundles, taken from Sigma-Aldrich [5].

There are over 40 types of α collagen peptides which aggregate in different combinations to create over 25 types of collagen [4]. These types are clustered depending on their triple helix structure, homology and function [4] (See **Supplemental Table A** for the grouping of collagen forms). Type I collagen is the most abundant protein in the body and the majority is found in the bone, skin and tendons [6]. Collagen synthesized and secreted by chondrocytes in the cartilage include types II and IX [4, 7], whilst type IV collagen is produced by fibroblasts as part of the basement membrane in a "chicken wire" two-dimensional framework [8]. This provides the network for other extracellular matrix (ECM) components to build upon to strengthen the basement membrane, endowing it with more functional diversity.

Cartilage

Cartilage is a dynamic connective tissue composed of two main substances, type II collagen, giving cartilage its strength, and hydrophilic proteoglycans which draw water into the tissue to retain moisture and provide resistance against compression forces [9-11]. Cartilage serves many roles in the body; it provides a template for developing bones, gives flexibility to joints, repairs damaged bones and offers structural support [7]. As cartilage is a poorly vascularized tissue, it largely depends on mechanical forces for signal transduction [12] as well as signals passed through the matrix and across Haversian canaliculi within the bone [13].

Chondrocytes are the only cells that reside within the cartilage [9, 14]. They are slow growing cells which secrete matrix around themselves which form the cartilage [12]. The secreted matrix causes separation from adjacent cells; therefore any cell-cell contact is mediated through changes in the secreted matrix and diffused signals [12]. Chondrocytes can undergo neoplastic changes and cause chondrosarcoma [12].

1.2 Chondrosarcoma

Physiology

Chondrosarcoma is characterized as a hyaline cartilage matrix-producing tumour [12, 15-18]. Chondrosarcoma, a type of malignant cartilage, represents approximately 25% of all primary bone cancers [18, 19], and succeeds osteosarcoma as the second most common malignancy of the skeletal system [16, 20-23]. Most chondrosarcomas (80-90%) are primary (conventional) chondrosarcomas, which occur in the medullar cavity (central chondrosarcoma) (**Figure 1.3**) or at the bone surface (peripheral chondrosarcoma) [16, 17].

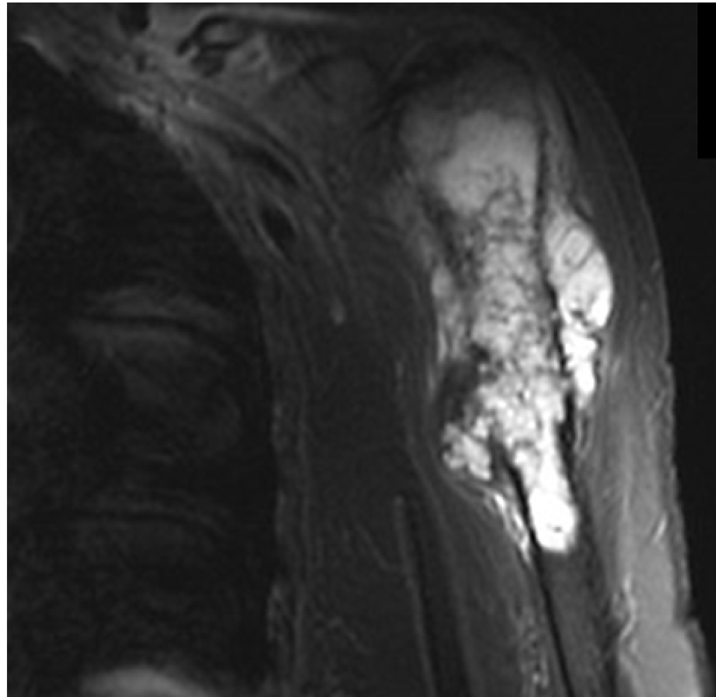


Figure 1.3: Magnetic Resonance Imaging image showing central chondrosarcoma of the proximal humerus. Infiltration of the marrow and invasion of the adjacent soft tissue is clearly seen.

Chondrosarcoma is divided into six categories; primary, dedifferentiated, mesenchymal, clear cell, secondary and periosteal, based on the site of the tumour as well as the histological presentation [13]. The incidence and differences in these subtypes are outlined below in **Table 1.1**.

INCIDENCE RATES OF THE SUBTYPES OF CHONDROSARCOMA

Subtype	Incidence	Description	Age of Onset (years)
Primary	80-90% (Roseman *et al.*, 2006)	Arise de novo (Nishida *et al.*, 2008; Rizzo *et al.*, 2000; Soderstrom *et al.*, 2001).	40-60 (Kim *et al.*, 2011)
Dedifferentiated	10% (Roseman *et al.*, 2006)	Variety of chondrosarcoma in which a well-differentiated low-grade cartilage tumour is juxtaposed to a high-grade non-cartilaginous sarcoma. These are aggressive lesions with poor prognosis. (Jamil *et al.*, 2010)	50-60 (Kim *et al.*, 2011)
Mesenchymal	3-10% (Roseman *et al.*, 2006)	Mainly composed of two characteristic tumor components: highly cellular and poorly differentiated and the other showing cartilage formation. (Muller *et al.*, 2005)	10-20 (Kim *et al.*, 2011)
Clear Cell	2% (Roseman *et al.*, 2006)	Characterized by the clear cytoplasm of the tumor cells (Kim *et al.* ,2011).	20-30 (Kim *et al.*, 2011)
Secondary	1-5% (Bovée *et al.*, 2010)	Secondary CS occurs when the cancerous cells arise from other neoplasias including osteochondromas and enchondromas (Bovée *et al.*, 2010; Capps *et al.*, 2008).	Range
Periosteal (Juxtacortical)	1% (Bovée *et al.*, 2010)	Rare tumor arising on the surface of the bone (Kim *et al.*, 2011)	20-30 (Kim *et al.*, 2011)

Table 1.1: Incidence and descriptions of the chondrosarcoma subtypes

Chondrosarcoma normally arises *de novo* from the cartilage of healthy bone (the primary subtype) in adulthood [11, 23, 24]. Chondrosarcoma most often presents in the long bones such as the femur [19, 23, 25-27] but has also been found in the ribs, spine [22], pelvis and scapula [25]. Chondrosarcoma mostly affects individuals in their fourth to fifth decades of life [20, 24, 25],

however in rare cases, patients younger than 20 years of age have been reported [17, 28]. The most common symptoms reported by patients with chondrosarcoma are on-going local bone pain, swelling at the tumour site [25] as well as pathologic fracture [26, 29], and can present years before the diagnosis is made [25]. Chondrosarcoma incidence shows a male prevalence [17] but no preference over ethnicity [13].

Cellular Biology

As chondrosarcoma can originate through different means, the cellular changes observed in chondrosarcoma are quite diverse. Benign cartilage masses of enchondroma and osteochondroma can undergo malignant transformation to become central or peripheral chondrosarcoma, respectively [30]. Enchondroma is the most common type of benign cartilage tumour [30] and is usually located at one bone site [31]. It characteristically shows hyaline cartilage tissue and occurs most often in the medullary of the bone [31]. Surgical resection is the common method of removal and local recurrences or transformations into a low grade chondrosarcoma are rare events [31]. Osteochondromas develop into malignant chondrosarcoma in 1-5% of cases [30]. These tumours normally found at the metaphyseal region of long bones [30] act as a growth plate that grows off an angle to the original bone during puberty before the cartilage cap has closed. Both of these benign bone tumours are caused by irregular development of chondrocytes adjacent to the growth plate

[30]. Both enchondromas and osteochondromas are difficult to diagnose as some of their features are perceived as low grade chondrosarcoma [30]. Chondrosarcoma as a whole affects 1 out of 200,000 individuals per year, which accounts for 20-30% of all bone malignancies [26].

As with many neoplasms, the specific details of how cartilage transforms into malignant chondrosarcoma are still unknown [21]. Some hypotheses include the dysregulation of signaling pathways, as well as changes in vascularization of the tissue and to the rates of proliferation [30]. Loss of heterozygosity is a common finding among chondrosarcoma tumours, caused either by deletion or uniparental disomy [21, 32], which may explain their susceptibility to carcinogenesis. Chondrosarcoma shows various genetic changes from single nucleotide polymorphisms, to larger deletions, which can influence the neoplastic nature of the cells [21]. One group determined that of the 138 altered chondrosarcoma tumour karyotypes included losses and gains of chromosomal material, diploidy, hyperhaploidy as well as hyperhexaploidy [32]. The commonly affect genes being p53, pRB and other tumour suppressor genes [21]. Less than 46 chromosomes were found in 49 tumours and 89 tumours showed more than 46 chromosomes 27 of these having translocations [32].

Chondrosarcoma can be diagnosed and graded by radiographic presentation [23], although, chondrosarcoma is primarily graded based on the

histological presentation, which assists in determining the course of action for treatment, estimation of aggressiveness, [33] and prognosis [12, 18, 19, 23, 34]. The general changes that occur throughout tumourigenesis are interpreted in **Figure 1.4**. Not all chondrosarcomas follow this rubric [26] however, which presents further challenges for a proper grading of the tumour.

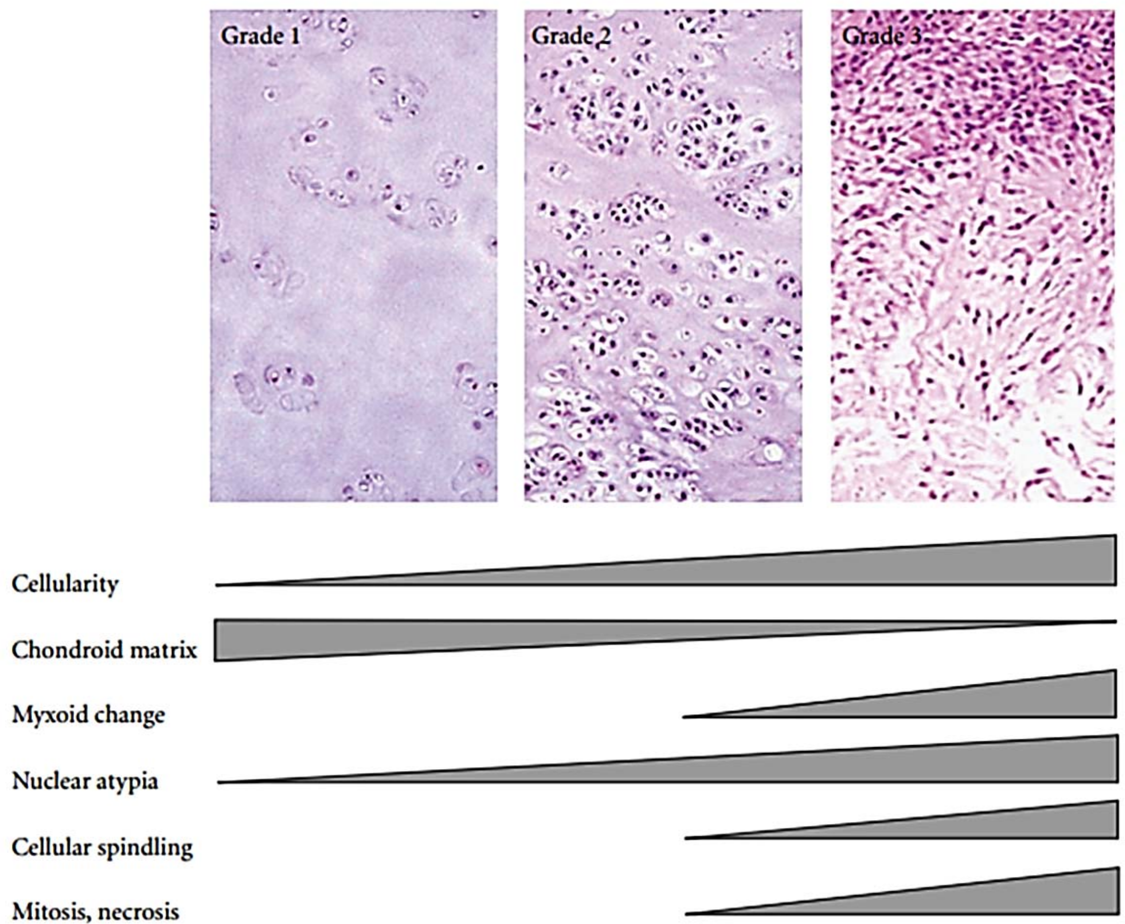


Figure 1.4: Schematic of the cellular differences between grades of chondrosarcoma cells, taken from Kim *et al.*, 2011 [26].

Many grades may be seen within one tumour [12, 16], compounding the challenge of making accurate diagnoses and estimation of prognoses. Additionally, local recurrences often appear, requiring further surgical interventions [20]. Grade I chondrosarcomas shows a 10-year survival rate of 80-90%, Grade II, 65-80% and Grade III, 30-40% [16].

Due to the non-responsive nature of chemotherapy and radiation therapy, chondrosarcoma has a high metastatic potential, especially at higher grades [24, 35], and secondary tumours most commonly metastasizes to the lungs (as seen in **Figure 1.5)**, causing a 50% 5-year survival likelihood [18].

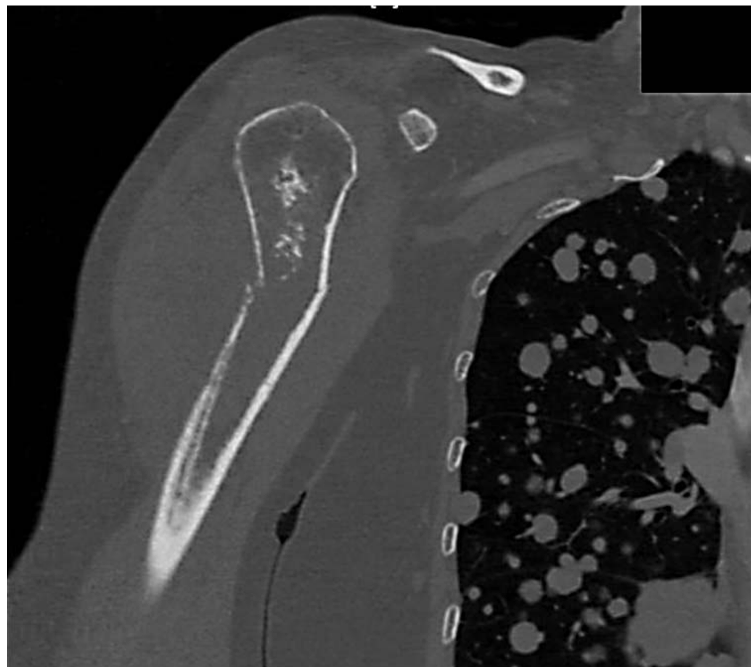


Figure 1.5: CT scan showing chondrosarcoma of the humerus with several secondary lung metastatic tumours.

Metastases are usually unresectable [36], causing lethal disease [30]. It is noteworthy, that of recurrent tumours, 10-13% display higher grades than the original tumour [18].

Current Treatments

Chondrosarcoma is a unique type of bone cancer, as it is unresponsive to chemotherapy (CT) or radiation therapy (RT) [15, 20, 23, 30, 35]. Chondrosarcoma is classically poorly vascularized, which causes difficulty in delivering drugs to the core of the tumour [12, 30, 37]. Many believe that the presence of the transmembrane ATP-mediated p-glycoprotein pump (encoded by the Multiple Drug Resistance gene; *MDR-1*) within the chondrosarcoma cell causes delivered drugs to be evacuated from the cell [19, 20, 30, 35, 38]. Ninety percent of malignant chondrosarcoma cells have shown to express this protein on their cell membrane [27, 35] and studies have determined that drugs including vinblastine and taxol are all shuttled out of the chondrosarcoma cell [39]. Interestingly, Kim *et al.* showed that the p-glycoprotein is also expressed by healthy chondrocytes, speculating that they too are chemotherapy (CT) resistant [35]. Through the inhibition of anti-apoptotic proteins Bcl-2, XIAP and p-glycoprotein, chemosensitivity increased over 4-fold across numerous chondrosarcoma cell lines [35], suggesting a possible mechanism to overcome this issue.

Chondrosarcoma has been used to study evasion mechanisms of radiation therapy (RT), and many target genes and proteins have been discovered to play a role in this process [19, 22]. RT works on the premise that the gamma radiation causes cellular damage by the production of reactive oxygen species (ROS), causing cytotoxicity [19]. ROS cause damage to cells through single and double stranded DNA breaks, inducing repair, apoptosis, senescence or necrosis of the cells [19, 38]. However, this situation only exists if the repair mechanisms are in proper functioning order, which is not always the case in many cancer types [19].

Doxorubicin is currently being investigated for the treatment of chondrosarcoma, as it a cytotoxic agent [40] which interchelates within the DNA, obstructing replication [19]. After dosing JJ012 chondrosarcoma and swarm rat chondrosarcoma cell lines with varying concentrations of doxorubicin, distinguishable amounts of the drug were found within the cells [35]. This lead the authors to hypothesize those anti-apoptotic genes are differentially expressed and protecting against CT in these cells [35].

Research on the pro- and anti-apoptotic factors in chondrosarcoma is on-going. Studies of endogenous Bcl-2 and XIAP protein levels were detected in healthy chondrocytes as well as chondrosarcoma in approximately even amounts [22]. Interestingly, articular cartilage cells are also RT resistant, which prompted

the authors to suggest that anti-apoptotic proteins may be linked to RT resistance in chondrosarcoma [22]. When RT damages the cells, they compensate by overexpressing their anti-apoptotic proteins to prevent death [22]. After radiating the chondrosarcoma cells, Bcl-2, Bcl-xL and XIAP became overexpressed, and in result, apoptosis levels decreased, as detected by histology [22]. When Kim *et al.* silenced Bcl-2 and Bcl-XL with small interfereing RNA (siRNA), apoptosis was restored [22], proposing that with pharmacological intervention aimed at down-regulating anti-apoptotic proteins in chondrosarcoma, the cells would become more sensitive to RT [19] and the tumour would shrink. Pro-apoptotic genes and their statuses in chondrosarcoma have not yet been characterized; however, pro-apoptotic drugs are currently in use to attempt to destroy chondrosarcoma cells *in vitro* [19, 35].

As chondrosarcoma is poorly vascularized, hypoxia is an important issue to consider [30, 37]. When radiation produces ROS, the low levels of oxygen within the tumour core yielded insignificant results [19, 38]. Acridine orange (a dye), is often used in conjunction with RT in chondrosarcoma cases, as the chemical can produce ROS in low oxygen supply environments

Onishi *et al.* review many possibilities as to why chondrosarcoma may be resistant to CT and RT [19]. Mutations in the p16 tumour suppressor gene are one such possibility [19]. p16 functions in G1 progression as a cyclin-dependent

kinase inhibitor to control apoptosis when irreparable DNA damage occurs [38]. When it is mutated, the rate of apoptosis decreases [19], causing changes in cellular populations. Chondrosarcoma commonly shows high numbers of p16 mutations, [38] while benign cells do not [41]. *In vitro*, p16-deficient chondrosarcoma cells showed more sensitivity to RT than healthy chondrocytes [41]. Being able to target chondrosarcoma cells specifically and to introduce functional p16 may show promise as adjuvant therapy to radiation or a method to overcome anti-apoptotic protein expression.

Wide surgical resection of the affected bone and surrounding soft tissue areas remains the standard treatment for chondrosarcoma [15, 18, 23, 34]. Unfortunately, resection is not always possible depending on patient comorbidities and the anatomic extent of the tumour within the skeleton and surrounding soft tissue [19, 20, 42]. The lack of available treatment may resort to amputation [19]. Without resection, chondrosarcoma will likely lead to local and metastatic recurrences [12]. Recurrence rates range between 10-70%, depending on the grade of the primary tumour [16], often requiring future surgical intervention. High-grade chondrosarcomas show a 50% 5-year survival rate, which declines to 29% by 10 years [15, 19, 30]. There is no known universal treatment for chondrosarcoma [22, 30, 35].

1.3 Matrix Metalloproteinases

The Matrix Metalloproteinase Family

The matrix metalloproteinase (MMP) family of proteins is comprised of over 20 known members in humans as well as in other species. This family is characterized by their zinc binding site within the catalytic domain of the enzyme and are capable of degrading extracellular matrix target proteins (See **Supplemental Table B)** [43-45]. These proteases are implicated in ranges of biological functions including wound healing, angiogenesis [46, 47], vascular remodeling [48], activation of downstream cellular signaling [49], as well as in correlation to cancer [50-55]. MMPs are differentially located; in the cytoplasm, tethered to the cell membrane or secreted, depending on its function [48].

Matrix Metalloproteinase 2 Protein Structure

Matrix metalloproteinase 2 (MMP2) is encoded by the *MMP2* gene on chromosome 16 yielding a 72kDA pre-protein and a 62kDa active form (see **Supplemental Table C** for more MMP2 information). Matrix metalloproteinases are highly conserved across species; MMP2 has over 95% protein homology to the rhesus monkey, chimpanzee, grey wolf, mouse, rat and cow (**Figure 1.6**).

Pairwise Alignment Scores

Gene		Identity (%)	
Species	Symbol	Protein	DNA
H.sapiens	**MMP2**		
vs. P.troglodytes	MMP2	99.5	99.2
vs. M.mulatta	MMP2	99.7	97.4
vs. C.lupus	MMP2	97.1	91.6
vs. B.taurus	MMP2	95.0	90.7
vs. M.musculus	Mmp2	95.9	89.4
vs. R.norvegicus	Mmp2	95.5	88.4
vs. G.gallus	MMP2	85.0	76.0
vs. D.rerio	mmp2	75.2	72.2

Figure 1.6: Homology analysis of MMP2 protein and DNA sequences in various species (NCBI HomoloGene search, Gene #3329).

MMP2 is composed of seven main sections; 1) a pre-domain and 2) a pro-domain, which are cleaved off to make up the active enzyme, 3) a catalytic region, which is responsible for the breakdown of various ECM proteins, 4) three collagen-fibronectin binding sites (II), 5) the zinc motif (Zn), 6) a hinge region (H) supplying flexibility linking the zinc motif, and finally 7) the hemopexin section containing a disulphide bridge, which assists in substrate binding (See **Figure 1.7**). The pro-domain encompasses a cysteine residue which binds the zinc atom in the proteins' inactive conformation [14]. Upon cleavage of the pro-domains, the sulphate bridge is broken, causing a conformational change of the protein, activating the zymogen [14].

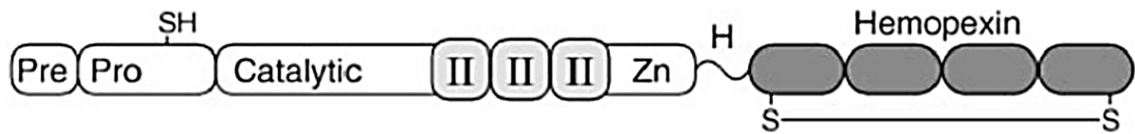


Figure 1.7: Schematic of MMP2 protein motifs, taken from Sternlicht and Werb, 2001 [48]. Pre: pre-domain, Pro: pro-domain, SH: sulfhydryl group, (II)(II)(II): fibronecting-binding domain, Zn: zinc atom, H: hinge region, S-S: disulphide bridge.

Tissue Inhibitors of Matrix Metalloproteinases

The tissue inhibitors of metalloproteinase (TIMP) group of four extracellular proteins can exert their forces either as activators or inhibitors of MMPs, depending on the surrounding conditions [56]. TIMPs are competitive inhibitors of MMPs, which bind the hemopexin domain of the MMP, preventing activation [56]. TIMPs contain regions that bind the catalytic pocket of the pro-MMP2 preventing their ability to become activated [45, 56]. TIMPs are also capable of binding membrane-type MMPs (MT-MMP), obstructing their binding to MMPs [56]. TIMP2 is the most common effector of MMP2, and functions in both as an activator and as an inhibitor [56].

Activation of MMP2

The *MMP2* promoter stretches from position -1704 to +1 [57], and contains binding sites for many transcription factors (TF), including AP-1 [56], sp1 and STAT [57]. The AP-1 TF is implicated in many signaling pathways, as well as in some cancers [58], and could contribute to the overexpressive nature of *MMP2*. The sp1 TF is capable of binding many different promoters. Like many TFs sp1, is involved with multiple signaling cascades, mostly relating to cell growth and maintenance (NCBI Gene ID: 6667). STAT6 (signal transducer and activator of transcription 6) cooperates with the *MMP2* promoter in the lung [59]. ETS TF binding sites are also present on the *MMP2* promoter [60] and will be discussed in later sections. As numerous TF binding sites are on the *MMP2* promoter, it is understandable that the resultant protein can have various roles within and external to the cells.

Once transcribed and shuttled to the cell membrane, the C-terminal end of the MMP2 zymogen is tethered to the exterior of the cell membrane via α5β3 integrin [45, 48], which allows activation to begin. Included in the MMP family, are the membrane-type MMPs (MT-MMP), containing a transmembrane portion and many are capable of activating MMP2 [48, 61]. MT1-MMP (also known as MMP14) protrudes through to the extracellular side of the cell which binds TIMP2 [11, 48]. Pro-MMP2 then binds TIMP2 through the hemopexin domain. The 37 amino acid pre-domain of pro-MMP2 is cleaved off [62-64] by a second MT1-

MMP [14, 45, 48]. This signals the auto-cleavage of the pro-domain between the Asn80-Tyr81 residues, causing a conformational change in the protein, revealing the catalytic region to produce a fully active MMP2 protein released into the ECM [14, 65, 66]. These processes are well illustrated by Sternlicht and Werb, 2001 [48] in **Figure 1.8.** A positive feedback loop exists; if the concentration of TIMP2 is too high, TIMP2 will return to acting as an inhibitor blocking MT1-MMP and MMP2, arresting the activation of MMP2 [67].

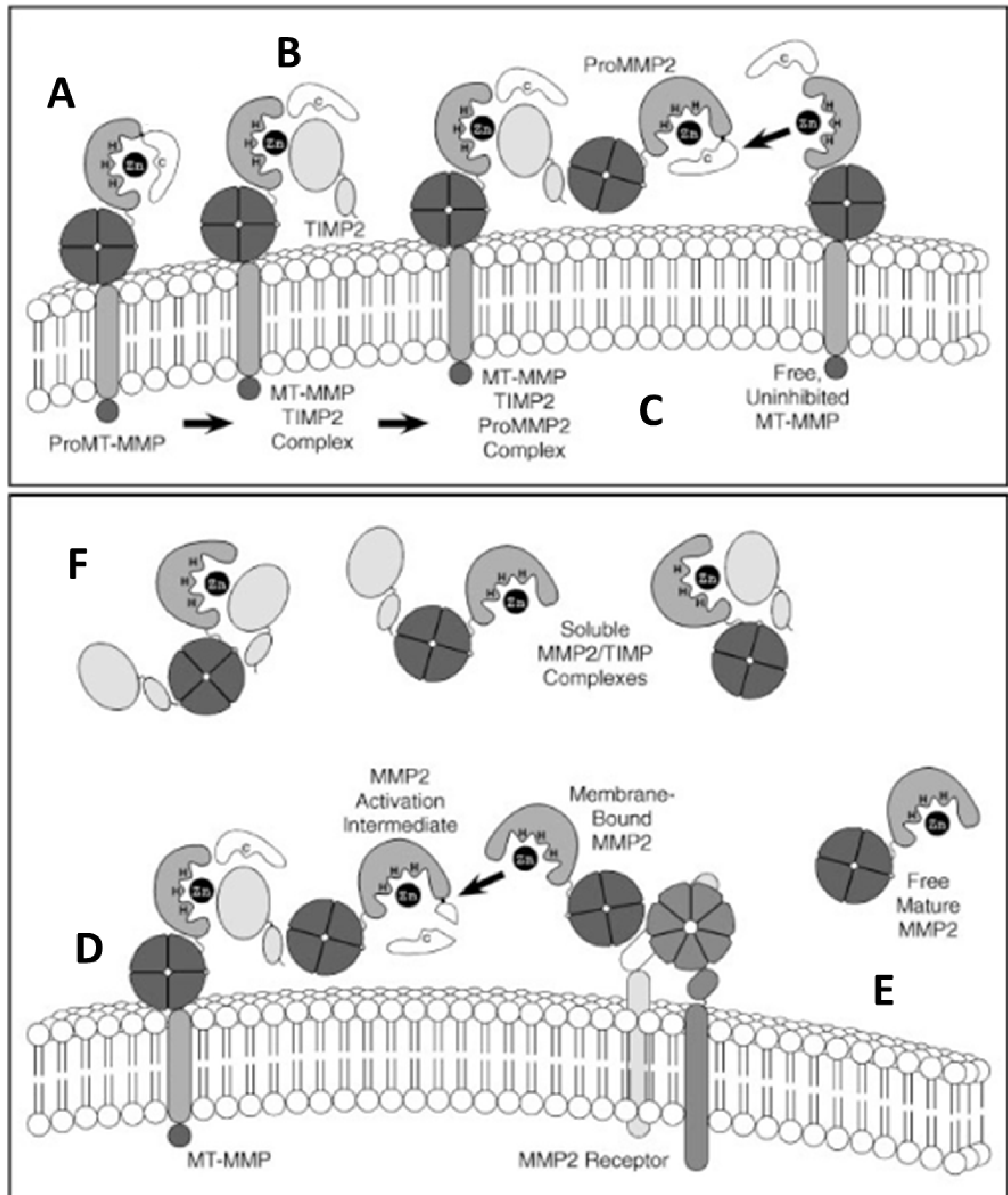


Figure 1.8: Activation of MMP2 taken from Sternlicht and Werb, 2001 [48]. (**A**) MT-MMP is tethered to the exterior of the cell membrane when it is (**B**) recruited to TIMP2 and inhibited via the hemopexin domain. (**C**) Pro-MMP2 bound by a

free MT-MMP becomes bound by TIMP2 at the c-terminal end creating a trimolecular intermediate. (**D**) The pre-domain of pro-MMP2 is cleaved off by a second MT-MMP then an active MMP2 removed the pro-domain (**E**) resulting in a free floating activated MMP2 protein. This process can also occur in the ECM with soluble TIMP2 (**F**).

Once activated, MMP2 is free in the extracellular space, where it can act on other targets, or dock at the membrane by an MMP2 receptor [48].

Wang *et al.* and others have shown that in TIMP2 deficient mice, pro-MMP2 activation occurs weakly, postulating that TIMP2 is necessary for efficient MMP2 activation. [66, 68, 69]. In MMP14 (MT1-MMP) null mice, *MMP2* expression was greatly reduced, also suggesting, that MMP14 is not required for the production of active MMP2 but plays a role in the stabilization of the MMP2 protein [14].

Growth factors (GF) also activate MMP2; vascular endothelial GF, [50, 56, 70] hepatocyte GF [71], fibroblast GF (FGF) family members [72] and many interleukins [56, 61, 67] have all shown to cause changes to *MMP2* expression in various cell types including chondrocytes [11, 72], melanocytes [71], and endothelial cells [50]. Pro-MMP2 can also be activated by pancreatic trypsin [73], and many members of the blood coagulation pathway [43, 67, 74].

Normal Physiological Functions of MMP2

The MMP family members are essential for cellular remodeling processes to developmental and protective roles within the cells and tissues.

Mammary glands branch out during various times of growth; at puberty, during pregnancy and lactation, in hormone-dependent manners [75]. This is known to be caused by changes in the basement membrane by particular MMPs. MMP2 functions alongside MMP3 to regulate mammary gland development in mammals [14]. Immunohistochemistry of mouse mammary tissues showed that MMP2 is mostly localized to the stromal section of the developing mammary ducts [75]. Upon observation of *MMP2*-/- mice, they lacked terminal end buds of the ducts, and showed shorter branching, causing phenotypic changes in the ductal network [14].

Angiogenesis is a crucial course for embryogenesis, however, deleterious in cancer. In healthy individuals, it has been suggested that MMP2 helps mediate neovascularization during embryogenesis [14, 57], as MMP2 mutant mice had reduced neoangiogenesis [46]. Interestingly, Kato *et al.* also found that in MT-MMP1 deficient mice, angiogenesis was perturbed, theorizing that the resultant change in phenotype was due to lower levels of active MMP2 [46]. This

was supported by evidence in other studies that also saw a decrease in blood vessel formation in *MMP2* double knock-out (KO) mice [49, 76, 77].

Apart from matrix degradation, MMPs can play dual roles in inflammation; anti and pro, depending on the needs of the cells and tissues [14], for instance in cell-to-cell communication by processing GFs and cytokines [78, 79]. MMP2 and MMP9 (also a gelatinase), regulate anti-inflammatory signals caused by allergens in the airways, as well as in asthma [14, 80]. *MMP2-/-* mice show losses in communication with eosinophils, causing the cells to remain in the interstitum of the airways, rather than migrate into the airways in the lungs for excretion through the mucus [80]. This can perpetuate difficulties breathing, as observed in asthmatic individuals [14]. MMP2 has been shown to offer a protective role against inflammation of the brain and spinal cord in cases of encephalomyelitis [49]. Furthermore, MMPs are also known to be overexpressed in the spinal cord as well as the brain in multiple sclerosis patients [81], suggesting that the overexpression of MMPs do not solely relate to cancer, but other physiological diseases in other body systems as well.

MMP2 has varied roles in bone development; it is secreted from both osteoblasts and osteoclasts [3, 78, 82, 83]. In mouse studies, *MMP2* KO mice show ranges of phenotypes due to defects in osteoblasts and osteoclast-mediated activities [84]. When *MMP2* is mutated in humans, they are at risk to

develop osteolytic “vanishing bone” disease caused by excessive bone degradation, as well as arthritis [14]. Other *MMP2* mutations can cause bone erosion, loss of bone mineralization, skeletal deformations, arthritis [84] and decelerated skeletal growth [48], causing multifaceted issues surrounding this one gene.

***Targets of* MMP2**

Of the entire MMP family, MMP2 has one of the largest ranges of ECM target molecules; it can digest over 30 different ECM proteins [48] (See **Supplemental Table D** for examples). As MMP2 is able to affect the integrity of many extracellular structural proteins within the matrix, it is understandable that downstream effects of its action are also quite diverse. More specifically, MMPs demonstrates the strongest affinity towards gelatin, and type I and IV collagens. MMP2 and MMP9 gelatinases are the only MMPs to have fibronectin-binding motifs within their catalytic domain [48], allowing them to bind to gelatin (or degraded type IV collagen), and cleaving them [45, 56].

MMP2 Inhibitors

There are two main classes of MMP2 inhibitors; biological and synthetic. The biological inhibitors are comprised of the TIMPs, as previously described, certain MMPs and other proteins naturally synthesized by the cell. RECK, a membrane glycoprotein, is a biological inhibitor of MMPs, MT-MMPs as well as

some TIMPs [20, 56, 61]. It functions by targeting MT1-MMP which is involved activation pathway of MMP2 [56]. Interestingly, RECK exhibits down-regulation in certain cancers [20, 56, 85].

Synthetic MMP inhibitors are typically chemicals, which Chaudhary *et al.* break down into four main classes: peptidomemetic, non-peptidic, natural matrix metalloproteinase inhibitors (MMIs) and tetracycline derivatives [61]. Peptidomemetic MMIs, are synthesized peptide-like molecules which act as collagen fibrils and combine with the zinc ion in the MMP catalytic site, impeding MMP activation [61]. Non-peptidic MMIs have superior specificity to the zinc binding site than the non-petidomemetic inhibitors which show more precise structure [61]. Natural MMIs, like neovastat work by inhibiting multiple stages in the angiogenesis pathway, including MMPs, and VEGF, causing apoptosis of the endothelial cells [86]. Prinomastat, a non-peptidic collagen-like MMP2 inhibitor was developed in the 1990s and reached phase II/III clinical trials [44]. Unfortunately, it did not progress from phase III clinical trials, as the outcome was not as favorable as expected, and the compound also caused unwanted musculoskeletal issues [44]. Finally, tetracycline derivatives are modified to no longer have antibacterial properties; instead they have been shown to inhibit bone resorption by inducing apoptosis of osteoclasts by binding to calcium, as well as acting as an MMP inhibitor [87].

As there is a great deal of crosstalk within the MMP family, certain MMPs can influence each other. It has been documented that MMP2 is able to activate MMP9 gelatinase and MMP13 collagenase [43], therefore, when attempting to target solely MMP2, there is the inherent risk of targeting many of the relatives, reducing the specificity of the inhibitor.

MMP2 and Cancer

Pro-MMP2 is secreted by several cell types including neurons, cardiomyocytes [57], endothelial [11, 57, 70], fibroblasts [11, 57, 62], epithelial [56, 62] and immune cells [11, 48, 88], then activated as previously described. *MMP2* has been observed in a multitude of studies to be overexpressed in invasive [20, 54, 70], metastatic [85, 89] and/or migratory [67] cancers, including osteosarcoma [90], prostate [91], breast [92] and ovarian cancers [53]. Furthermore, tumours with high levels of MMP2 show a more malignant phenotype and metastatic profile [62], contributing to a worse prognosis [89]. This concept has now been taken a step further, and relative levels of *MMP2* expression are being studied to correlate with higher prognosis for metastasis of colorectal cancer [85].

MMP2 and Chondrosarcoma

MMP2 is expressed in healthy cartilage [93], as well as in chondrosarcoma [18, 52, 94]. The link between *MMP2* and chondrosarcoma has not been

extensively studied. Soderstrom *et al.* analyzed patient chondrosarcoma cell samples to ascertain any trends of *MMP2* expression versus the grading of the tumour [11]. They recognized that *MMP2* is more highly expressed in chondrosarcoma than in enchondroma, adult cartilage, as well as fetal cartilage [11], which may assist in defining the invasiveness of the tumour as well as the grade. This information is quite helpful for determining treatment options and prognosis.

A set of studies performed by Lai *et al*., used alendronate, a bisphosphonate drug able to decelerate bone resorption, to determine the effects of *MMP2* expression and activity in JJ012 chondrosarcoma cell line [94]. The groups deduced that the effect of MMP2 inhibition by the drug decreased the invasion ability of the cells [94], and supported the concept that MMP2 may be an important mediator of metastasis in chondrosarcoma.

1.4 ETS Translocation Variant 5

ETS Family of Transcription Factors

The first member of the ETS family of transcription factors (TF) ETS-1 was named after a viral protein (v-ets) [95] three decades ago [96]. The v-ets was found in the E26 Avian Erythroblastosis virus which produced v-ets with

homology to the human ETS family of proteins [95]. ETS was given the same name as E Twenty Six (ETS) protein [95]. The ETS family of TFs is composed of over 20 members in humans having a conserved 85 amino acid DNA binding domain (DBD) [97] named the ETS domain [98, 99]. As well as being homologous to one another, ETS TFs also exist among the mouse, *Drosophila* and *C. elegans* [100]. Homology within and between the ETS domains vary, and the human ETS family has been divided into smaller groups based on their ETS sequences [101] (**Figure 1.9**).

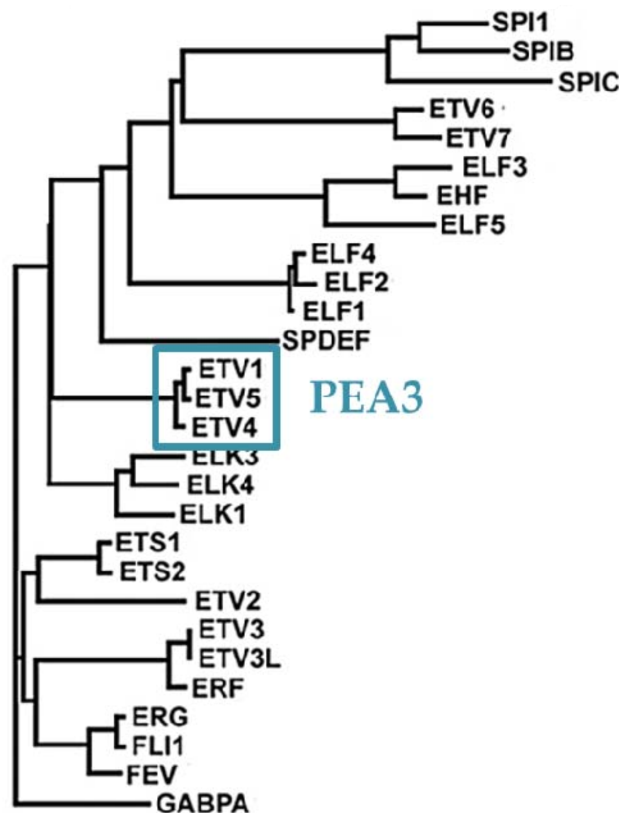


Figure 1.9: Phylogenetic tree illustrating the separation of the ETS family members, based on the homology of the ETS domain. The PEA3 subgoup is highlighted in the blue box. Taken from Oikawa and Yamada, 2003 [101].

The ETS domain recognizes a core 5'-GGA(A/T)-3' region on the target gene promoter [97-99, 102]. The ETS TF binds the promoter and depending on many pretenses, can activate or repress the expression of that gene [99]. Key determinants in dictating the TF functionality are the sequences flanking the core binding motif [99] as well as the presence of repression zones [97, 100]. The flanking sequences on the target promoter also influence binding capability; if one amino acid is mutated, the binding affinity can change [101]. Most ETS factors are activators; there are only a small number of repressors [97].

As TFs, the ETS family controls the expression of many genes of wide cellular function. The diverse functionality of the ETS group of TFs include cell proliferation, cellular differentiation, cell-cell and cell-matrix interactions, apoptosis [99, 101], as well as angiogenesis, invasion and metastasis, tumour suppression [103]. Among these functions, the main target genes for the ETS group of TFs are proteases of the extracellular matrix [104].

Polyomavirus Enhancer-Binding Activator (PEA3) Transcription Factors

The ETS family is divided into smaller groups based on the ETS domain homology to each other, functional motif configurations, and location of the DNA binding domain (DBD), which are illustrated in **Figure 1.10** [97, 103, 105].

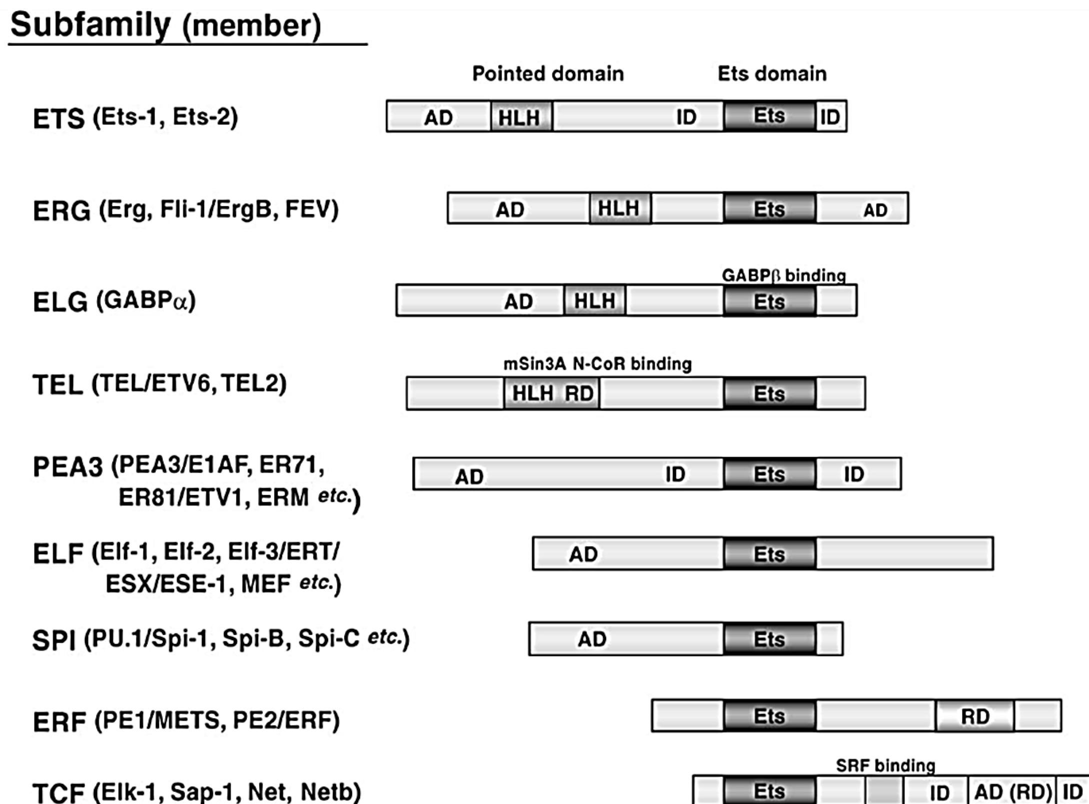


Figure 1.10: Schematics of ETS transcription family members, sorted by subgrouping. Illustrates various location of each group; **AD:** activation domain, **HLH:** helix-loop-helix, **ID:** (auto)-inhibitory domain, **Ets:** DNA binding domain, **RD:** repression domain. Taken from Oikawa and Yamada, 2002 [101].

Amid these subgroups is PEA3; a trio which contains three members, PEA3 (also known as ETV4), ER81 (ETS related protein 81, also known as ETV1) and ERM (ETS related molecule PEA-like, also known as ETV5) [36, 97, 98, 106]. These three TFs are over 95% identical in the DBD, over 85% in the transactivating motif [36, 106], and approximately 50% in the transcriptional segment [36]. The Pea3 subgroup members are transcriptional activators [95, 97] and have similar organization.

ETV5 Structure and Function

Under the PEA3 grouping resides the ETS Transcript Variant 5 (ETV5) TF. It is encoded by a gene on the third chromosome, at the q27-29 locus [97] which yields a 1533 amino acid protein of 38kDa (NCBI Gene #2219) (see **Supplemental Table C** for more ETV5 information). The ETV5 contains an ETS DBD, two transactivating domains (one at each terminal end), an inhibitory domain, and one transcriptional domain (see **Figure 1.11**) [97, 107, 108].

Figure 1.11: Schematic of the ETV5 protein. **TADn**: transactivating domain, N-terminal end, **NRD:** negative regulatory domain, **ETS**: DNA binding domain, **TADc:** transactivating domain, C-terminal end. Numbers correspond to the number of amino acid residues. Adapted from Lens *et al.,* 2010 [107].

The human ETV5 protein shows over 95% protein homology to chimpanzees, rhesus macaque, the grey wolf, the cow and mouse ETV5 protein sequences (**Table 1.12**).

Pairwise Alignment Scores

Gene		Identity (%)	
Species	Symbol	Protein	DNA
H.sapiens	**ETV5**		
vs. P.troglodytes	ETV5	99.4	99.4
vs. M.mulatta	ETV5	99.6	98.8
vs. C.lupus	ETV5	96.7	93.3
vs. B.taurus	ETV5	97.6	93.7
vs. M.musculus	Etv5	95.5	90.1
vs. R.norvegicus	Etv5	94.7	89.7
vs. G.gallus	ETV5	84.2	78.7
vs. D.rerio	etv5a	68.4	67.4

Figure 1.12: Human ETV5 Protein and DNA Homology percentages versus other species (NCBI HomoloGene Search, Gene #3276).

In humans, *ETV5* expression is found in several types of tissues including the brain, [101, 109] lung, testis [109], breast, colon [97], pancreas and heart [100]. *ETV5* and other ETS TFs are regulated by the fibroblast family of GFs [99, 110-112] as well as via many of the MAPK, RAS, ERK and PKA signalling pathways via activation through phosphorylation [95, 97, 98, 105, 106]. When

ETV5 becomes activated through various kinase pathways, it is able to bind DNA directly via the DBD. Analysis via nuclear magnetic resonance determined that the DBD has an α1-β1-β2-α2-α3-β3-β4 structure, creating a winged helix–turn–helix formation [101]. It is the α3 portion that binds the major groove on the DNA [101].

ETS factors often associate with other TFs or proteins for promoter binding-activity [95]. The specificity of ETS factor attraction to genes depends on a myriad of factors including TF post translational modifications such as phosphorylation, acetylation [97], sumoylation [101, 107, 113], methylation [114] that the protein can undergo [97]. Other parameters able to dictate target affinity include external stimuli [95], interactions with co-factors [101], TF concentration within the nucleus [100], and the presence of protein-protein interactions [95]. Different members of the ETS family have different targets [101], and the pairing made by the ETS factor and assistant proteins augment target specificity [102]. Little information is known as to which proteins bind to which location(s) on ETV5 to assist in ETV5 function and its regulation [101, 104].

ETV5 Inhibition

ETV5 (as well as ER81 and ETS-1) are able to be auto-inhibited, which involves different mechanisms for each member [107, 108]. The ETV5 protein contains inhibitory domains [107, 108], however, sources are contradicting,

indicating that the inhibitory domain(s) are in different locations (**Figure 1.11**). Lens *et al.* claim that one region is adjacent to the N-terminal end, whereas Zhang *et al.* and Oikawa *et al.* claim that there are two areas flanking the ETS domain [107, 108]. This suggests that there exists the potential for numerous inhibitory domains along the protein which may function differently depending on the upstream signaling. It is believed that these regions prevent ETV5 binding to the DNA or to accessory proteins [115]. Most commonly, phosphorylation of specific lysine and serine residues change the conformation of the protein, concealing the DBD thus, rendering it inactive [101]. Interestingly, many kinases are dysregulated in chondrosarcoma, causing downstream changes to various enzymes; this may account for sporadic ETV5 activation observed in cancer [30]. This warrants further study to better understand the inhibitory mechanisms of ETV5 and which consequences affect its inhibitory actions.

As a TF, the ETV5 protein is localized to the nucleus [97]. Nonetheless, studies have shown that in some instances, the nuclear export or nuclear localization signals found in certain ETS TFs can be mutated, causing them to be unable to translocate into the nucleus and thus, remain in cytoplasm [101]. This situation has not yet been assessed in regards to ETV5 specifically, though the possibility is plausible.

ETV5 can be ubiquitinated at multiple sites, as well as other members of the PEA3 group, therefore once it has completed its' function, it undergoes degradation via the ubiquitin pathway [97, 116], further regulating its activity.

ETV5 and Development

The two main roles for ETV5 are in spermatogenesis and embryogenesis. During embryogenesis, ETV5 is implicated in the development of many tissues, including the mammary glands [97, 117], salivary glands, lung [117], kidney [117, 118], as well as the central and peripheral nervous systems [98, 101]. *ETV5* has also been shown to be expressed in the developing small intestine in the mouse [99]. ETV5 has high involvement in ureteric [118] and limb bud [97, 111] formation during embryogenesis as well as in vertebrate development [111].

ETV5 and Spermatogenesis

Spermatogenesis is a self-renewing process in which the spermatogonial stem cells (SSC) mature into spermatozoa [105]. The SSC reside in the seminiferous epithelium in contact with the basement membrane within the seminiferous tubules of the testis [105]. Also present in the seminiferous tubules of the testis are Sertoli cells, which help regulate spermatogenesis. Spermatogenesis occurs in four steps; SC self-renewal to replenish the stock of germ cells, spermatogonial proliferation and differentiation, meiosis of the cells into spermatocytes and differentiation and morphogenesis from spermatids into

spermatozoa [105]. The first three parts occur in the seminiferous tubules, at the basement membrane [105] then the last step ensues in Sertoli cells [105].

Sertoli cells secrete glial cell line-derived neurotrophic factor (GDNF) which is required for the self-renewal capabilities of SSC [105, 110]. *ETV5* is expressed by the germ and Sertoli cells in the testis [119]. Interestingly, *ETV5* is regulated by GDNF in the Sertoli cells [119] while EGF and FGF2 regulate its expression in the germ cells [119]. ETV5 is thought to assist in regulating the environment in which spermatogenesis takes place [120] and migratory processes of the developing sperm cells [121]. ETV5 is also a player in homing processes of the SSCs within the testis by producing agents to keep a balance in the SSC niche [105, 119, 121]. The specific mechanism to which ETV5 regulates the spermatogonial niche is not fully understood [105, 110, 122, 123].

ETV5-/- KO mice survive to birth, advocating that ETV5 is not essential for survival [105, 109]. In *ETV5*-/- mice, the first wave of spermatogenesis is achieved early on in life, however, this leads to the cessation of SSC production [122] and the loss of spermatogonia [110, 120, 122] causing infertility. Testicular size is also reduced in *ETV5* -/- mice, however no other anatomical differences are seen between KO and wild-type (WT) mice [109, 123]. *ETV5*+/- mice are fertile [109]. Conversely, GDNF-/- mice expire days after birth due to absent kidneys and neuronal defects [105]. Interestingly, in *ETV5*-/- mice, GDNF levels

are the same as detected in WT mice, indicating that ETV5 does not regulate GDNF [124]. The production of GDNF is dictated by levels of follicle stimulating hormone [105, 110, 122, 123] while the production of *ETV5* is regulated in part by epidermal growth factor, but interestingly, not testosterone or follicle stimulating hormone [121].

ETS Transcription Factors and Cancer

ETS factors activate several oncogenesis-related pathways including tumour suppressor genes, apoptosis-related genes, differentiation-related genes, angiogenesis-related genes, as well as invasion and metastasis-related genes [103]. Therefore it is understandable that mutations in the ETS TF genes could contribute to tumourigenesis when dysregulated. The specific actions of PEA3 members in cancer is unknown [105], however it is recognized that their expression is dysregulated in many cancers [117].

ETV5 is sporadically overexpressed in colon carcinoma [99], and in the endometrioid endometrial carcinoma Hec-1A cell line, *ETV5* is upregulated and exhibits a metastatic phenotype [60]. *ETV5* overexpression is seen at the invasive fronts in endometrial carcinoma [97], and moreover, *ETV5* is overexpressed in 80% of breast carcinomas attributing to a poor prognosis [117]. Finally, cell invasion is perpetuated in prostate epithelial cell lines in which *ETV5* is overexpressed [125, 126].

Additionally, *ETV5* can fuse or crossover with other genes, altering certain regulator and cell proliferation signalling pathways [96]. The most common gene fusion with *ETV5* is TMPRSS2, a transmembrane serine protease found in prostate cancers and has an unknown function [125]. Although ETS fusion proteins are present in 50% of prostate cancer cases, the TMPRSS2:*ETV5* fusion encompasses less than 8% of all cases of prostate cancer [125].

Interestingly, mRNA copy numbers of ETV5 vary dependant on the cell line. For example, Hollenhorst *et al.* determined that mRNA copy numbers of ETV5 vary across cell lines, from normal to malignant types [100]. Healthy prostate cells showed an average of three copies of ETV5 per cell, while the PC3 prostate cancer cell line showed 34 copies, suggesting a common amplification [100]. Healthy colon cells produced one copy of ETV5 while its malignant equivalent the HCT116 colorectal carcinoma cell line displayed 34 copies of ETV5 mRNA [100]. *ETV5* copy numbers in healthy and diseased bone have not yet been quantified.

ETV5 and Chondrosarcoma

No studies with respect to *ETV5* implicated in chondrosarcoma in any capacity have been reported.

CHAPTER TWO: STUDY RATIONALE

2.1 ETV5 and MMP2 in Cancer

Previous findings have illustrated that PEA3, a cousin to ETV5 has corresponded to *MMP2* expression in breast cancer tumorigenesis [106, 127]. Many members of the MMP family can be regulated by the ETS family of transcription factors [60]. Several studies indicated that ETV5 has a regulatory relationship with *MMP2* in breast [55] and ovarian cancers [128]. High expression levels of *ETV5* have been linked to higher degrees of invasiveness and metastatic ability in additional cancers [60, 97, 106, 125, 126]. Qin *et al.* have shown that the AIB1 transcriptional activator assists Pea3 in expressing of *MMP2* as well as *MMP9* in human breast cancers, further perpetuating the disease [55].

As mentioned earlier, the *MMP2* promoter region carries putative ETV5 binding sites (5'-GGA(A/T)-3') [60, 129], suggesting a possibility for a direct interaction between the ETV5 protein and *MMP2* (see **Supplemental Figure A** for partial MMP2 promoter sequence containing potential ETV5 binding sites). There also exists the likelihood that ETV5 binds the *MMP2* promoter at other regions, as ETV5 can also act as a co-factor binding to AP-1 sites on the

promoter [101, 102, 119]. With three ETS-1 sites and three AP-1 sites, ETV5 could have the opportunity to bind the *MMP2* promoter at many different loci to initiate transcription [56, 101].

Monge *et al.* showed that when *ETV5* was overexpressed in endometrial endometrioid carcinoma, MMP2 protein levels increased [60]. Interestingly, it has been illustrated that both ETV5 and MMP2 are present at strong levels at the invasive edges of endometrial endometrioid carcinoas [128], and that cell lines overexpressing the PEA3 TF family members showed high invasive capability, which correlated to the expression of MMP2 [97]. For these reasons, further studies to correlate ETV5 and MMP2 in cancer cells would be valuable, especially for chondrosarcoma, where little is known about the cause of its metastatic nature.

2.2 Hypothesis

ETV5 activates *MMP2*, upregulating the expression of *MMP2* in human chondrosarcoma. This further perpetuates the gelatinase activity and bone resorption capacities of chondrosarcoma, contributing to metastatic ability of these cells. This is illustrated in **Figure 2.1**.

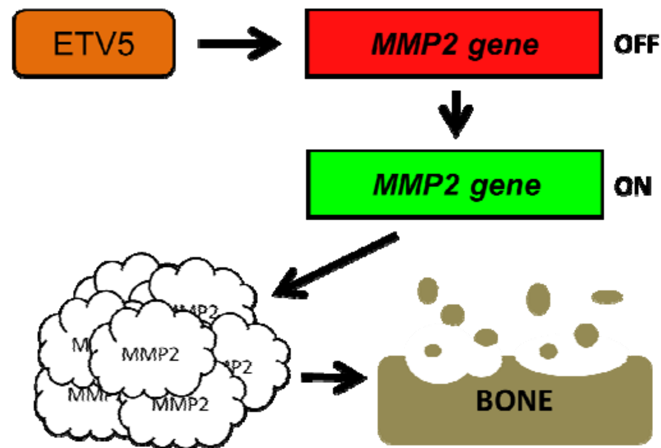


Figure 2.1: Diagrammatic representation of hypothesis. ETV5 activates *MMP2*, increasing the expression of *MMP2* in human chondrosarcoma. This further perpetuates the gelatinase activity and bone resorption capacities of chondrosarcoma, contributing to metastatic ability of these cells.

2.3 Objectives

1. Assess endogenous expression of *ETV5* and *MMP2* in human fetal osteoblastic (hFOB) and KC chondrosarcoma cells.
2. Knockdown and overexpress *ETV5* in KC cells and determine the effect on *MMP2* expression.
3. Measure bone resorption differences by chondrosarcoma cells with and without an MMP2 inhibitor within the growth media.
4. Explore the *ETV5* and *MMP2* expression patterns in primary chondrosarcoma cell lines.

CHAPTER THREE: MATERIALS AND METHODS

3.1 Cell Culture

We received human 105KC (KC) cells (grade II conventional chondrosarcoma) as a generous gift from Dr. Joel Block (Rush, Chicago, IL). The human fetal osteoblastic (hFOB, ATCC #CRL-11372) and RAW264.7 (RAW, ATCC #TIB-71) cell lines were acquired from the American Type Culture Collection (ATCC). hFOB cells were often used as controls for experiments as they are non-malignant cells from the skeletal system of mesenchymal origin. RAW cells, when exposed to receptor activator of NF-κB ligand (RANKL), develop bone resorption capabilities, acting as the positive control for the bone resorption assay. The KC and RAW cells are grown at 37°C and the hFOB cells are grown at 34°C in optimal media in humidified air with 5% CO_2. KC chondrosarcoma media is composed of 40% D-MEM, 40% minimum essential media alpha (MEM-α), 10% F-12, 10% fetal bovine serum (FBS), 1% ascorbic acid (2.5mg/mL), penicillin (100U/mL), streptomycin (100mg/mL), 0.1% of recombinant human insulin (4mg/ml), and hydrocortisone (50µM). RAW media is composed of D-MEM, penicillin (100U/mL), streptomycin (100mg/mL), and 10% FBS. hFOB media is composed of D-MEM, penicillin (100U/mL), streptomycin (100mg/mL), 10% FBS, 1% ascorbic acid (2.5mg/mL) and 10mM β-

glycerophosphate. Patient cell lines generously provided by Dr. Rebecca Gladdy at Mount Sinai Hospital (Toronto, ON) are grown in D-MEM:F12 with penicillin (100U/mL), streptomycin (100mg/mL) and 10% FBS at 37°C in humidified air with 5% CO_2. (See **Supplemental Table E** for Patient Case Information and **Supplemental Figure B** for cell morphologies when grown in T75 flasks in optimal conditions.).

3.2 RNA Isolation and cDNA Synthesis

Total RNA extraction was performed according to the manufacturer's protocol (E.Z.N.A. Total RNA Kit I, Omega). Concentration and purity of the samples were evaluated by the ratio of optical density 260:280 using a spectrophotometer (Beckman DU® 530). One microgram of total RNA was used for complimentary DNA (cDNA) synthesis following manufacturer's instructions (qScript cDNA Supermix, Quanta BioSciences). The cDNA samples were used directly for quantitative real-time-PCR (qRT-PCR) (MJ Mini Gradient Thermocycler, Bio-Rad) or stored at -20°C. qRT-PCR conditions for cDNA synthesis: 25°C for 5 minutes, 42°C for 30 minutes, followed by 5 minutes at 85°C.

3.3 Quantitative Real-Time Polymerase Chain Reaction

Quantitative real-time polymerase chain reaction (qRT-PCR) was performed by real-time monitoring of increases in fluorescence of the SYBR Green dye (Molecular Probes) using the MiniOpticon Real-Time polymerase chain reaction (PCR) system (Bio-Rad). Following initial denaturation at 95°C for 5 min, PCR was performed at 95°C for 30s, at specific annealing temperature (56–62°C) for 30s, and 72°C for 30s. Primer sequences are listed in **Supplemental Table F**. A melt curve was then performed from 65°C to 95°C in increments of 0.5°C. Cycle values were then normalized to the values yielded by β-actin. All qRT-PCR experiments are performed in triplicate and $2^{-\Delta\Delta Ct}$ data was analyzed using Microsoft Excel 2010 software.

3.4 Immunofluorescence Microscopy

Cells were grown on cover slip, fixed with 4% paraformaldehyde for 20 min at room temperature, and permeabilized with 0.1% Triton X-100 in phosphate buffered saline (PBS) for 5 min. Subsequently, these slides were blocked with 10% bovine serum albumin (BSA) in PBS, and incubated for 1h at room temperature with antibodies (1:200 anti-MMP2, Calbiochem). Slides were further incubated in secondary antibody (1:700 Alexa 488 goat anti-mouse antibodies,

Invitrogen) for 1h at room temperature. Slides were washed and incubated with DAPI for 3 min at room temperature and mounted with 50% glycerol and visualized under a light microscope (Leica DM IRB).

3.5 siRNA Knockdown (KD) of ETV5

KC cells were trypsinized and transfected with ETV5 small interfering RNAs (siRNAs) via electroporation (Bio-Rad Gene Pulser II). Briefly, the cells were washed in PBS, trypsinized, and resuspended in optimal media. The cell suspension was then mixed with either 600uM ETV5 siRNA (Invitrogen), 20μg of a positive Silencer™ siRNA control against GAPDH (Invitrogen) or 20μg of a Negative Universal Control (Invitrogen). Cells with the siRNA mixture were incubated on ice then electroporated under a single-pulse with optimized combination of voltage and capacitance. Cells were then plated in T25 culture flasks with optimal media. Media was changed 24h post-transfection and at 48h post-transfection, RNA was isolated from cells as described above, and cDNA was produced for use in qRT-PCR following the manufacturer's instructions as described above (see **Supplemental Table F** for primer sequences). β-actin was selected for normalization for qRT-PCR analysis, and values are compared to the negative control values. This was completed in triplicate and the statistical

analysis (Student's t-Tests) was rendered through Microsoft Excel 2010. **Supplemental Figure D** illustrates the siRNA binding sites on the ETV5 mRNA.

3.6 Plasmid Construction for ETV5 Overexpression

An *ETV5* plasmid was purchased from OriGene. The *ETV5* open reading frame was amplified by PCR from using oligonucleotides (see **Supplemental Table F**) and cloned into TOPO TA® cloning vector (Invitrogen). After *BamHI* and *EcoRI* digestion, the open reading frame was ligated into a pcDNA3.1 mammalian expression vector and confirmed by sequencing.

3.7 Overexpression of ETV5

$1x10^5$ KC cells per well were plated in 6-well plates in optimal media the day before transfection. On the day of the transfection, 200ng of pcDNA3.1-ETV5 was transfected into the KC cells as directed by the manufacturer's instructions (Effectene Transfection Reagent Kit, Qiagen). The cells were incubated at 37°C for 72 hours then RNA was harvested (see above) to produce cDNA (see above) for qRT-PCR. This was completed in triplicate and the

statistical analysis (Student's t-Tests) was rendered through Microsoft Excel 2010.

3.8 Western Blotting

Cells were grown to 80% confluence. Cells were lysed and 20ug of protein was run on a 7.5% SDS-PAGE gel, transferred to PVDF membrane and blocked with 10mg/mL BSA in 1X TBS-T. The membrane was incubated in primary mouse anti-MMP2 antibody (1:1000, Calbiochem) overnight at 4°C. Following membrane washes in 1X TBS-T, the membrane was incubated in the horseradish peroxidase-conjugated secondary anti-mouse antibody (1:1000, Promega) for 1 hour at room temperature followed by protein visualization on audioradiography film. Densitometry performed on the resultant blots were normalized to actin controls using ImageJ software.

3.9 MMP2 Gelatinase Activity Assay

This assay works on the principle that the fluorescently-tagged gelatin when broken down fluoresces, which can be detected and quantified by plate reader. The amount of measured fluorescence is proportional to the gelatinase

activity in the sample. KC cells were grown to 80% confluence, and then the media was harvested and concentrated (Microsep™ Advance Centrifugal Device, Pall Corporation). The concentrated media was quantified, and 1000μg of protein was used for the activity assay following optimization (EnzChek® Gelatinase/Collagenase Assay Kit, Molecular Probes). Briefly, concentrated media was incubated with a 1:1 volume of 5uM 4-aminophenylmercuric acid (APMA) in PBS for 3.5hrs at 37°C. Samples were mixed with reaction buffer, 100μg DQ gelatin in 96-well plates and incubated at 37°C. The relative absorbance was read with a fluorescent plate reader (CytoFluor® Series 4000, PerSeptive Biosystems) at time zero and at 60 minutes. Statistical analysis was performed on Microsoft Excel 2010.

3.10 Bone Resorption Assay

Bone resorption assays were performed as described in Mak *et al.* (2010) [130]. Briefly, bovine femoral cortical bone pieces were cut into 200μm slices. Subsequent slices were cut into ¼-inch circles and sterilized using 70% ethanol and 50% bleach in separate washes. Bone chips were rinsed in PBS to remove any ethanol or bleach residue and placed into individual wells of a 48-well plate. $5x10^3$ KC cells per well were plated on top of the bone chips in media either containing or lacking 12.5nM MMP2 inhibitor III (Calbiochem) (See **Supt. Figure**

G for more information). The positive control cells for bone resorption used were a monocytic cell line; RAW264.7 cells (ATCC# TIB-71™**)** induced with RANKL at 50µg/mL (Gibco) to differentiate into osteoclastic cells. hFOB cells were used as a negative control as they are known not to resorb bone. Media was changed every 2-3 days as needed and the conditioned media was collected and frozen at -20°C. The bone chips were harvested after six weeks of incubation. Internal negative controls comprised of wells containing: media only, media and bone chips without cells, media and cells without bone chips. See **Supplemental Figure C** for bone chip assay diagram.

3.11 Bone Chip Processing

On the last day of the bone resorption assay, the media was removed and the bone chips were rinsed in 50% bleach followed by PBS. The chips were then decalcified for two weeks (TBD-2 Decalcifier, Thermo Scientific) and processed (Shandon Citadel 2000 Tissue Processor). The bone chips were embedded in paraffin wax, cross sectioned and mounted to microscope slides for hematoxylin staining. Chip cross sections were visualized under a light microscope and assessed for resorption pits.

3.12 Enzyme Linked Immunosorbent Assay (ELISA)

The conditioned media from the bone resorption assay was collected every 2 to 3 days as needed into separate Falcon™ tubes, frozen and the wells were refilled with fresh media (with the exception of the RAW cells with RANKL-containing media; only half is removed, and the same volume replaced). At the end of the six weeks, the collected media was thawed and used for the ELISA assaying for human C-telopeptides of type I collagen fragments following manufacturer's directions (Human C-telopeptide of Type I Collagen, CTX-1 ELISA kit, NovoTeinBio). This was completed in triplicate and the statistical analysis (Student's t-Tests) was rendered through Microsoft Excel 2010.

3.13 Histology

Hematoxylin & eosin staining on patient chondrosarcoma tissue samples was used to detect resorption pits. Histology on patient chondrosarcoma tissue samples embedded in paraffin blocks after fixation with formalin was performed. Tissue slides were deparaffinized in xylene and rehydrated in ethanol before rinsing in water, stained with hematoxylin and eosin, and examined under a light microscope equipped with a CCD camera.

CHAPTER FOUR: RESULTS

4.1 Endogenous Expression of ETV5 and MMP2

Endogenously expressed *ETV5* and *MMP2* in chondrosarcoma (KC) and human fetal osteoblastic (hFOB) cells lines were evaluated using quantitative real-time PCR (qRT-PCR). The hFOB cell line was chosen as a control to represent a non-neoplastic mesenchymal cell line originating from the human skeleton. It was found that endogenous *ETV5* expression in KC cells is 2.6-fold greater than that of hFOB cells (**Figure 4.1A**). *MMP2* also shows considerably greater endogenous expression in KC cells than in the hFOB cells with a 184.1-fold higher expression level (**Figure 4.1B**).

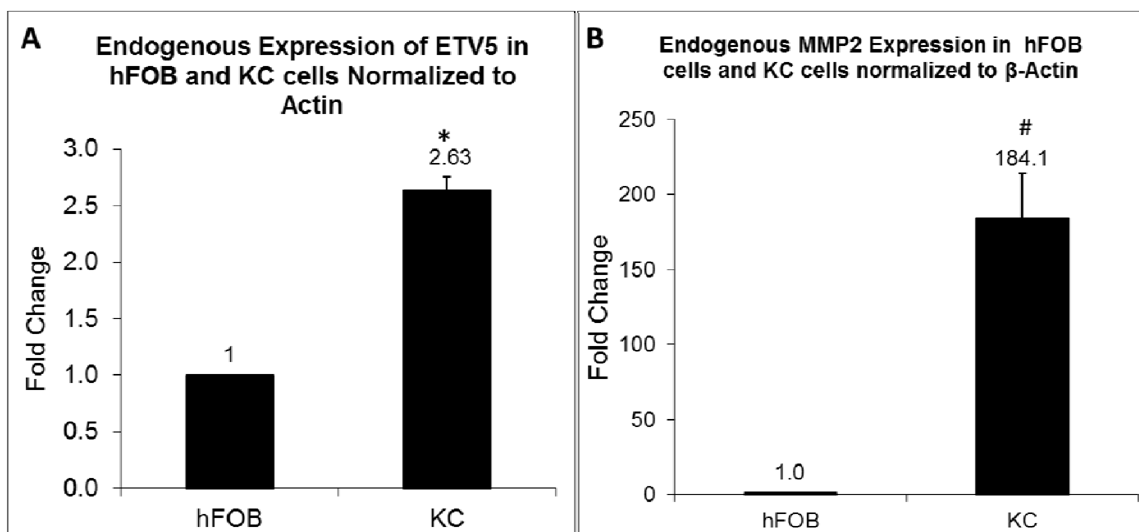


Figure 4.1: Endogenous expression of **(A)** *ETV5* and **(B)** *MMP2* in KC cells compared to hFOB cells as determined by quantitative real-time PCR after normalization to β-actin (P=0.002 (*), P=0.007 (#)), (n=4).

As MMP2 is membrane-bound before secretion, immunofluorescence studies displayed that MMP2 is concentrated within the KC cells in the cytoplasm (**Figure 4.2**). When compared to the immunofluorescence of MMP2 in hFOB cells, the intensity of expression is clearly much weaker, validating the qRT-PCR data.

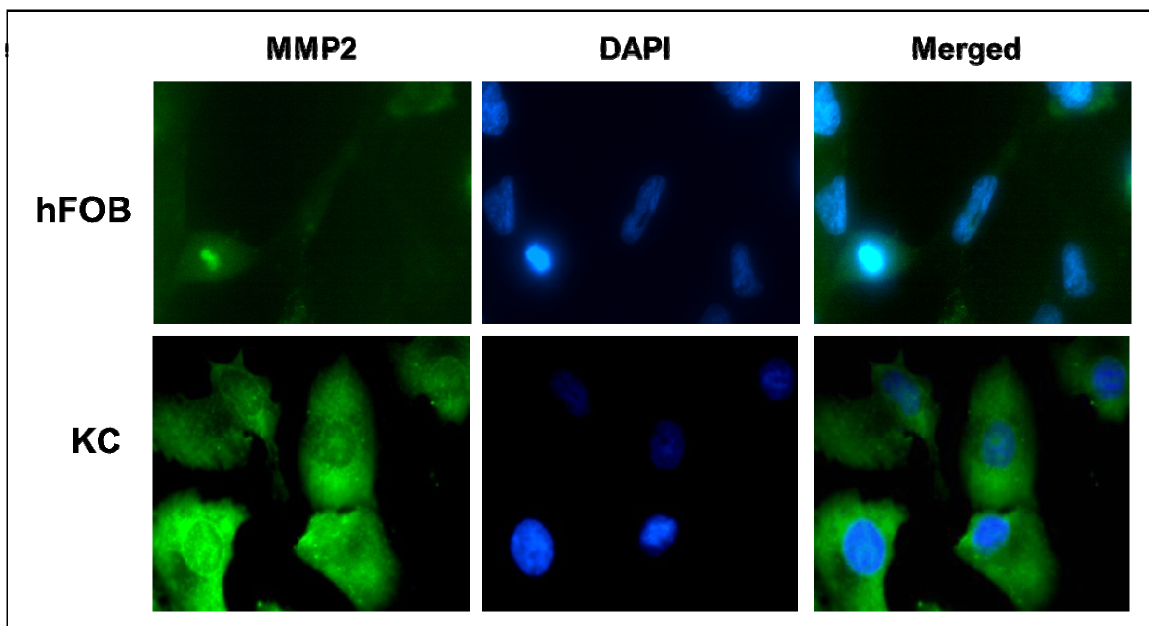


Figure 4.2: MMP2 cellular localization in hFOB and KC cells (Green: MMP2, Blue (DAPI): nuclei), 100x magnification.

4.2 The ETV5-MMP2 Regulatory Relationship

To assess the regulatory relationship between ETV5 and MMP2 in KC cells, *ETV5* was knocked-down using ETV5-specific siRNA, and the resultant change in ETV5 and MMP2 mRNA expression were measured with qRT-PCR. With an average of 18% *ETV5* expression in knocked-down KC cells, *MMP2* expression decreased significantly to 43% compared to untransfected control cells (**Figure 4.3A**). Conversely, when the pcDNA3.1-ETV5 expression vector was transfected into KC cells, gene expression of *ETV5* increased by 93%, while that of *MMP2* increased by 116%, compared to untransfected control cells (**Figure 4.3B**). Although not statistically significant, the trend is still persisting. It is possible that *MMP2* is already functioning at a maximum expression rate, thus, cannot be further overexpressed.

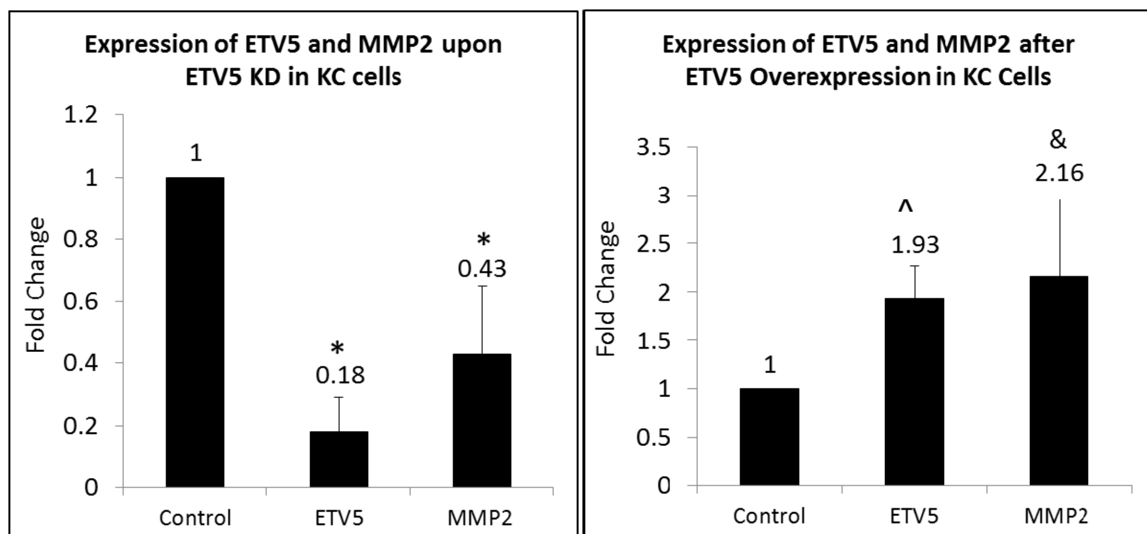


Figure 4.3: *ETV5* and *MMP2* expression levels **(A)** after *ETV5* knockdown by siRNA **(B)** and *ETV5* overexpression in KC cells via transfection of 100ng of pcDNA3.1-ETV5 construct, as determined by qRT-PCR after normalization to β-actin. Expression levels are relative to untreated control cells. (Asterisk denotes P<0.05, **"^"** denotes P=0.059, "&" denotes P = 0.19), (n=3).

To determine if the relationship seen at the RNA level translated to a change in protein expression, protein lysates were collected from control, *ETV5* knocked-down and *ETV5* overexpressed cells for immunoblot analysis. The ETV5 blot verified that both methods to manipulate *ETV5* expression functioned as expected; *ETV5* knockdown (KD) shows less protein expression than the control, while the overexpression vector does indeed increase protein production of ETV5 in the KC cells (**Figure 4.4A, B**). The trend that ETV5 regulates *MMP2* proportionally in an upstream manner was also observed at the protein level, as shown by immunoblot analysis (**Figure 4.4C, D**). When *ETV5* was KD in the KC cells, MMP2 protein levels decreased and inversely, MMP2 protein expression increased upon *ETV5* overexpression (OE).

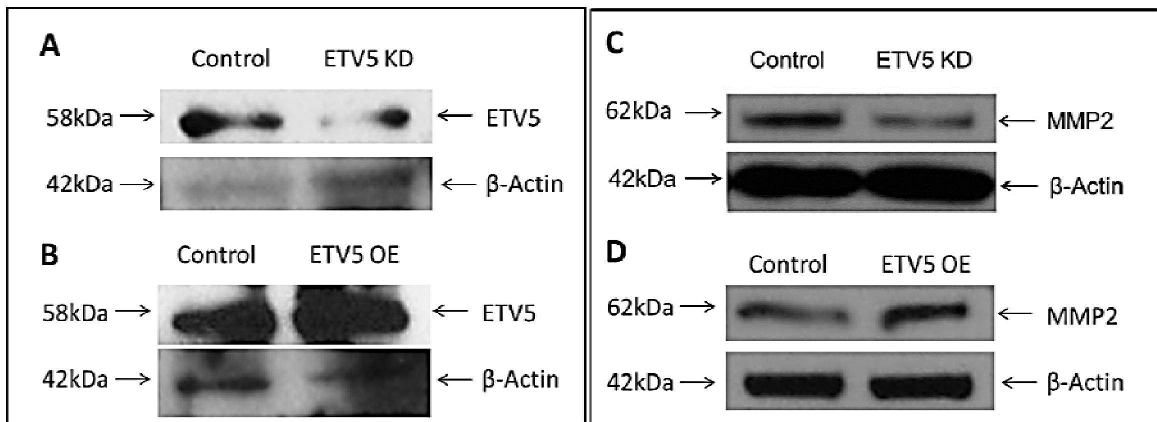


Figure 4.4: Western blots of **(A)** ETV5 protein levels following *ETV5* knockdown via siRNA, **(B)** ETV5 protein levels following *ETV5* overexpression via pcDNA3.1-ETV5 construct transfection, **(C)** MMP2 protein levels following *ETV5* knockdown via siRNA, and **(D)** MMP2 protein levels following *ETV5* overexpression via pcDNA3.1-ETV5 construct transfection.

Densitometry on the MMP2 blots showed that on average, MMP2 protein levels decrease by 41% upon *ETV5* knockdown in KC cells and that overexpressing *ETV5* resulted in a 43% increase in MMP2 protein production (data not shown).

A commercial enzyme activity assay kit was used to determine the change in gelatinase activity (a representation of MMP2 activity) upon *ETV5* KD. The assay kit contains gelatin conjugated to a fluorescent tag, which fluoresces upon molecular breakdown. The relative florescence of the samples equates to the

activity of active gelatinase within the sample. With *ETV5* knockdown, these assays determined that gelatinase activity decreased significantly by 20% compared to controls (**Figure 4.5**), showing that the alteration in protein leads to a change in gelatinase activity.

The use of 4-aminophenylmercuric acid (APMA) to activate any pro- forms of MMP2 was used to quantify the total amount of MMP2 protein produced. As the control and KD samples were treated equally with the activator, the results are still relative to the difference in expression levels of *MMP2* between the two samples. This assay was not performed when *ETV5* was overexpressed as it was felt that the expression of *MMP2* was endogenously at its threshold, and overexpression of *ETV5* would not result in any substantial change in MMP2 activity.

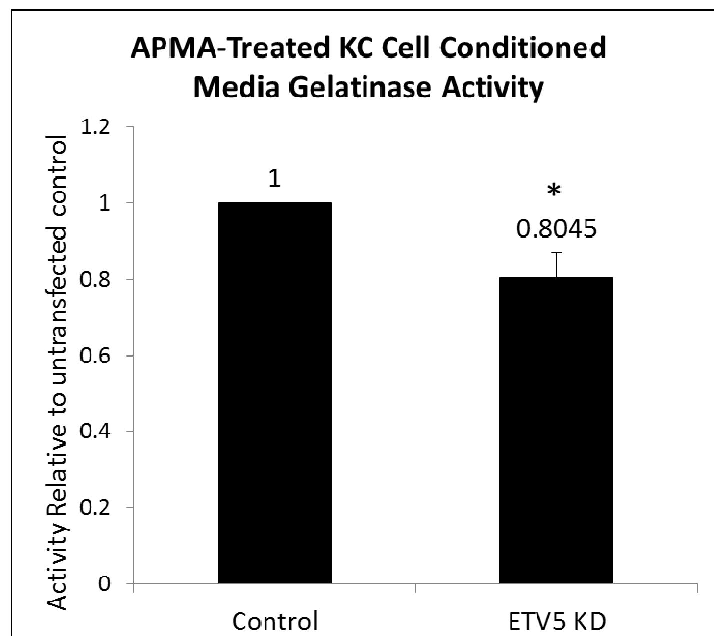


Figure 4.5: Change in gelatinase activity following *ETV5* knockdown in KC cells, normalized to control cells, after APMA activation. (P=0.034 (*)), (n=3).

Therefore, ETV5 affects all tested aspect of *MMP2* expression levels in an upstream manner; at mRNA, protein and activity levels in KC cells.

4.3 Bone Resorption Assays with MMP2 Inhibition

To visualize the capability of bone resorption by KC cells, bovine cortical bone chips incubated with KC cells in media were rinsed, decalcified, processed, cross sectioned and visualized for the identification of resorption pits. In the absence of osteoclasts, KC chondrosarcoma cells are independently capable of resorbing bone *in vitro*, as confirmed by a Bone Pathologist (**Figure 4.6**).

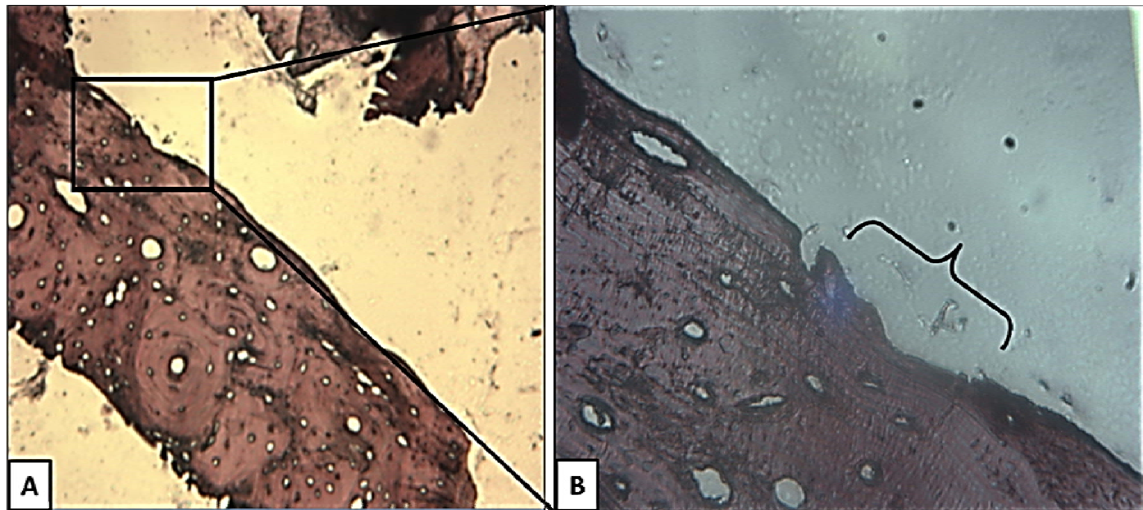


Figure 4.6: Hematoxylin-stained cross sections of bone chips after incubation with KC cells. (**A:** 100x, **B:** 400x magnification). Black bracket indicates a bone resorption pit created by the KC cells in normal growth media.

In order to assess the functional significance of MMP2 in chondrosarcoma causing bone resorption, a synthetic MMP2 inhibitor was present in the KC cell media for the entire incubation period of the bone chip assay at a concentration that selectively inhibits solely MMP2. hFOB cells were used as negative controls as they do not resorb bone. RAW cells treated with RANKL were used as positive cells for bone resorption, as this causes the monocytes to differentiate into osteoclastic cells. Bone chips were rinsed, decalcified, processed, cross sectioned and hematoxylin-stained for identification of bone resorption pits (**Figure 4.7**).

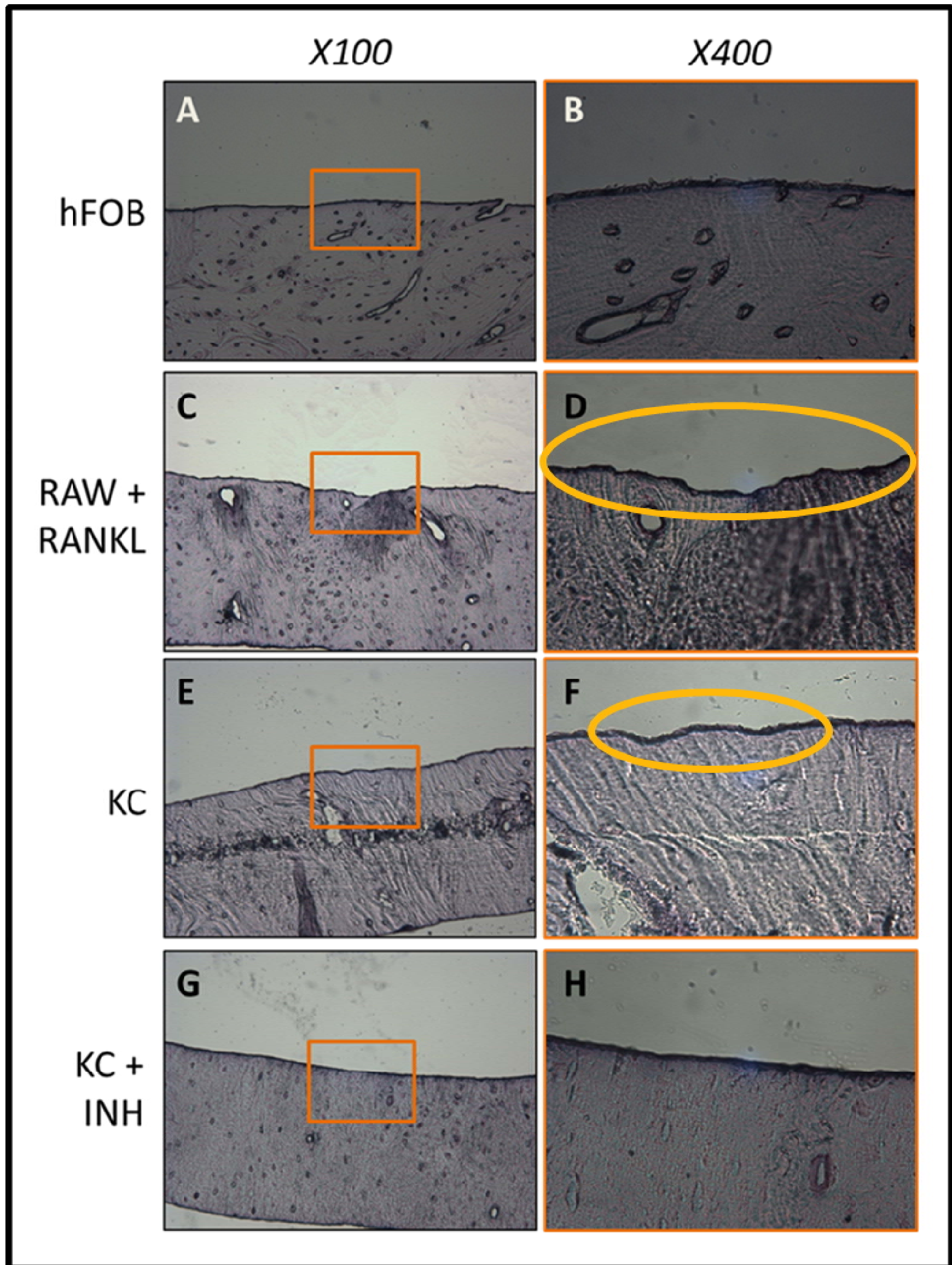

X100
X400
A
B
hFOB
C
D
RAW +
RANKL
E
F
KC
G
H
KC +
INH

Figure 4.7: Hematoxylin-stained bone chip cross sections after incubation with **(A, B)** hFOB cells, **(C, D)** RAW cells with RANKL (50μg/mL), **(E, F)** KC cells and **(G, H)** KC cells with MMP2 inhibitor (12.5nM), at 100x **(A, C, E, G)** and 400x magnification **(B, D, F, H)**. Orange box at 100x magnification denotes the field of view at magnified to 400x. Yellow ellipses highlight areas of bone resorption.

MMP2 is capable of degrading unravelled type I collagen fibrils [10, 131]. For this reason, a type I collagen fragment ELISA was performed on the collected conditioned media, as a manner to quantify bone resorption occurring by the KC cells. It was found that cells incubated with media containing the MMP2 inhibitor released significantly less type I collagen from the bone chips than the cells without exposure to the inhibitor (**Figure 4.8**).

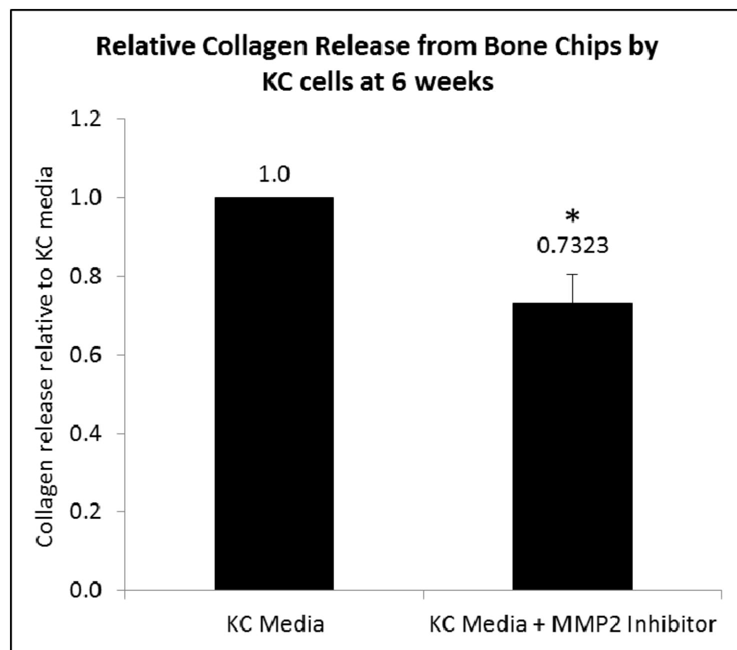


Figure 4.8: Relative amounts of released collagen by KC cells treated or untreated with MMP2 inhibitor (12.5nM) during the bone resorption assay as determined by type I collagen ELISA (P=0.023 (*)), (n=3).

To confirm that the MMP inhibitor was not causing death of the cells, contributing to a lesser amount of bone resorption, pictures were captured seven days into the bone resorption assay to assure that the cells attached to the bone and assess that the cell numbers appeared even in both types of media (**Supplemental Figure D**). Indeed, the KC cells attached to the bone chips and the bone was left untouched in no-cell control wells.

MMP2 has important collagenase capabilities causing bone resorption *in vitro*, and that inhibiting MMP2 inflicts less destruction of the bone at the cell-bone interface. Although it is feasible to assume that many proteases are acting on the bone chips, the specificity of the inhibitor was controlled to solely target MMP2 by using the inhibitor at a specific concentration, without any effects on cellular toxicity (as determined quantitatively). This resulted in a controlled assessment of changes to bone resorption due to changes in MMP2 and no other MMP family members that may also be playing a role in bone resorption.

4.4 Primary Cell Lines Show Similar Trends to KC Cells

Four patient chondrosarcoma cell lines (PT1, PT2, PT3 and PT4) were obtained and endogenous expression of *ETV5* and *MMP2* were assessed to determine if the expression profile seen with the KC cells is relative to primary patient cells. Compared to hFOB, the patient samples expressed similar levels of endogenous *ETV5* and *MMP2* (**Figure 4.9**). They emit a range of expression relative to hFOB; between 0.4 and 1.89-fold in *ETV5* expression **(Figure 4.1A)** and a range of 98.2 to 400.6-fold increase in *MMP2* expression over hFOB cells **(Figure 4.1B)**. When compared endogenous expression MMP2 levels from KC cells, two patients exhibit lower and two patients had higher levels of MMP2, as compared to KC cells. Interestingly, for *ETV5* (**Figure 4.1A**), KC shows the highest expression comparison of all five chondrosarcoma cell lines tested, as compared to hFOB cells (see **Supplemental Figure F** for values relative to KC expression levels).

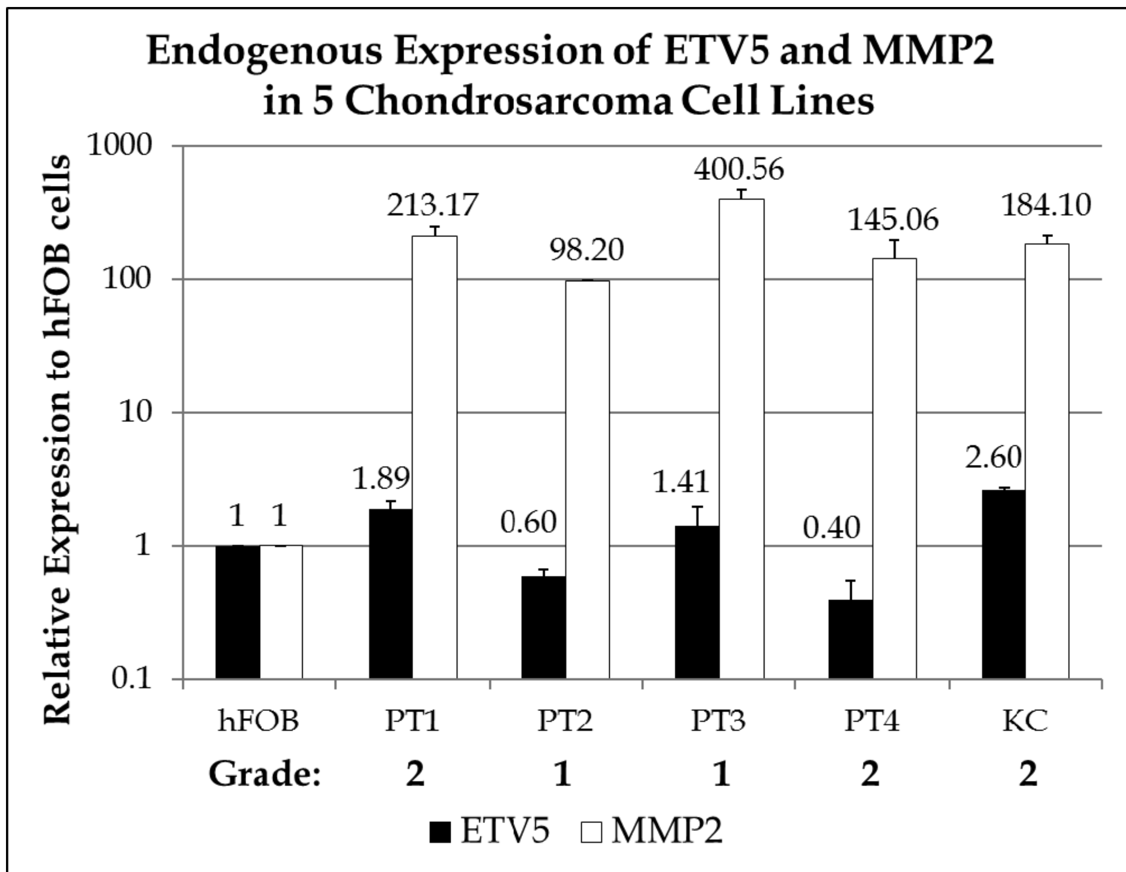


Figure 4.9: Endogenous expression of **(A)** *ETV5* and **(B)** *MMP2* in patient cell lines, relative to hFOB cells, after normalization to β-actin expression (n=3). Black bars on graphs highlight KC cells in the ranking of expression levels for *ETV5* and *MMP2* across the chondrosarcoma cell lines assessed.

Observing that *ETV5* and *MMP2* expression trends are similar to KC cells, the KD experiment was conducted on these cells to see if our observed ETV5-MMP2 relationship also occurs in the patient cells. When *ETV5* was KD in patient samples 1-4, the same association is seen; when *ETV5* was KD in all four cell lines, *MMP2* expression decreases (**Figure 4.10**). It is interesting to note that

although the four lines reproduce the correlation between ETV5 and MMP2 measured in the KC cells, the degree of KD of *ETV5* and its difference in *MMP2* expression level changes varies quite a great deal. This could be due to the fact that the endogenous levels are not of the same magnitude as seen in KC, as well as differential grading represented in the patient cell lines, therefore KD experiments could yield various amounts of expressional changes.

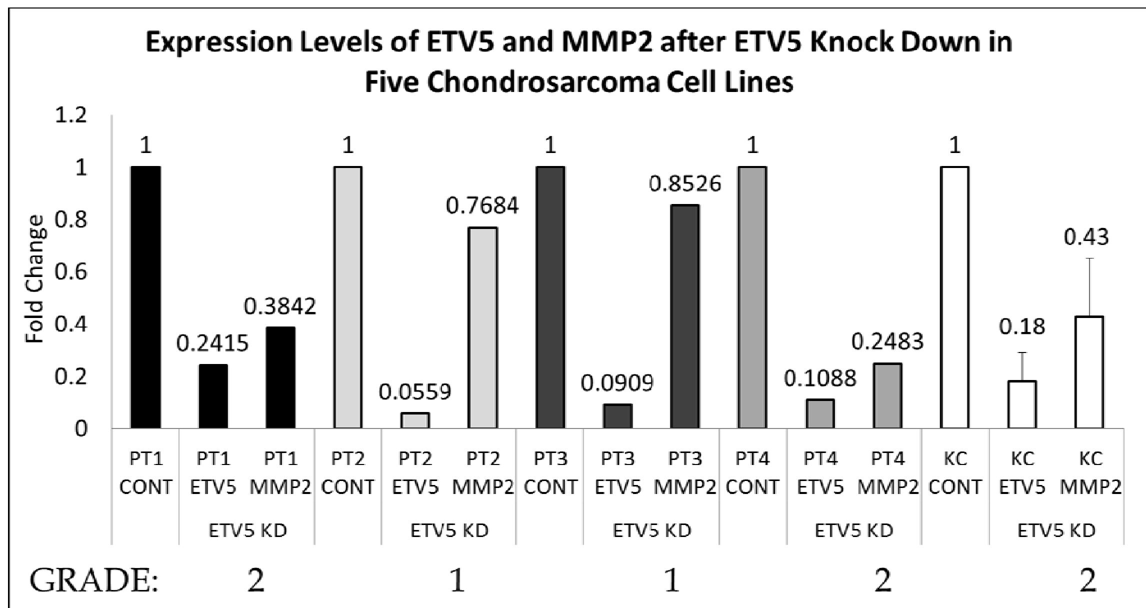


Figure 4.10: Expression levels of *ETV5* and *MMP2* after *ETV5* KD via siRNA in patient cell lines, relative to untransfected controls (n=1 for patient (PT) sample cells, n=4 for KC cells).

This research strongly supports the hypothesis; ETV5 does regulate *MMP2* across all five chondrosarcoma cell lines tested, at the RNA level and this is further exemplified at the protein and activity levels in KC cells. The duties of *ETV5* in chondrosarcoma, nor the relationship between *ETV5* and *MMP2* in chondrosarcoma have never been previously studied. This data provides novel information which can be used to build upon the knowledge on metastatic and invasion cascades in chondrosarcomas as well as in other types of malignant tumours.

CHAPTER FIVE: DISCUSSION

It was found that *ETV5* has a role upstream in the regulation of *MMP2* expression *in vitro* in chondrosarcoma cells, as well as on MMP2 activity and bone resorption. The fact that both *ETV5* and *MMP2* were highly expressed in chondrosarcoma cells suggests that both of these entities are important for the cancerous bone resorption capabilities of chondrosarcoma.

5.1 Bone Resorption: Healthy vs. Diseased Processes

Osteoclasts and cancer cells resorb bone through different mechanisms. How osteoclasts and osteoblasts regulate bone degradation and formation is a balanced process which is well studied. Cancer however, causes dysregulated, accelerated bone destruction though processes that are not fully understood [83] **Figure 5.1** shows bone resorption in chondrosarcoma.

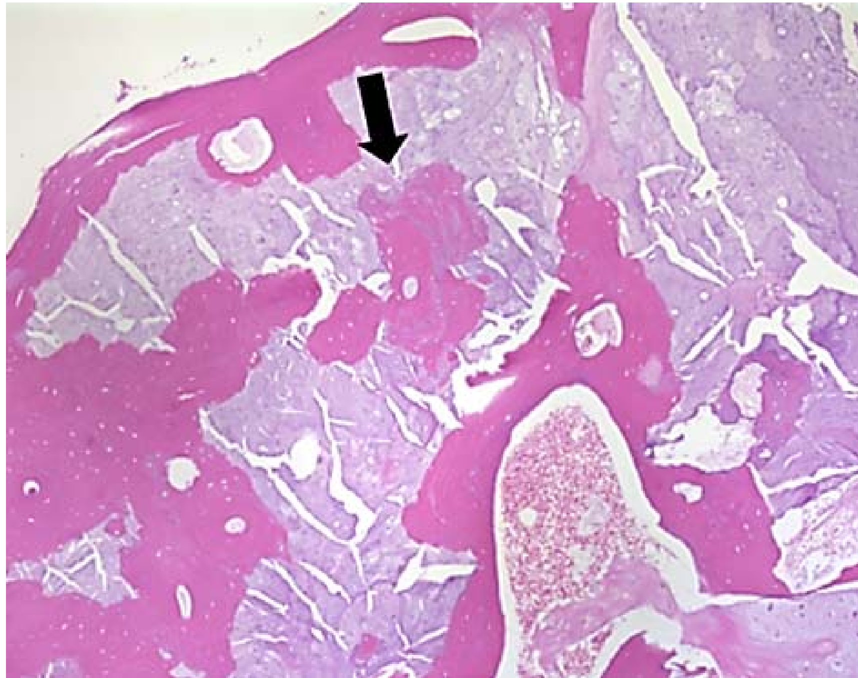


Figure 5.1: Histological visualization of chondrosarcoma patient tissue specimen. Black arrow depicts an area of bone resorption by chondrosarcoma as seen by bone scalloping. (Hematoxylin and eosin stained; pink: cortical bone, blue: malignant cartilage.) 100x magnification.

Normal bone turnover by osteoclasts and osteoblasts is a tightly regulated and balanced process. Osteoclasts are large, multinucleated cells of monocyte/macrophage linage of mesenchymal origin produced in the bone marrow [87, 132]. They excrete GFs and cytokines eroded from the bone which cue the latching of osteoblasts to the bone to lay down fresh matrix. Osteoblasts

secrete matrix to form fresh bone which eventually becomes mineralized, as signalled by the osteoblast cells [13].

As osteoblasts express RANKL and osteoclasts express the RANK receptor, osteoblasts recruit osteoclasts to the site of resorption via RANKL-RANK receptor binding, activating the NF-κB signalling pathway in the osteoclast [8]. When osteoclasts get recruited to the bone site, it binds the bone via α5β3 integrins, creating a seal [2, 82]. This causes the osteoclastic membrane to change shape, forming a tight seal on the bone preventing leakage of corrosive materials into the ECM [8]. Actin reorganization allows the polarized portion of the cell in contact with the bone to become ruffled and increase surface area, then secrete various enzymes and chemicals to cause erosion of the bone, liberating calcium, GFs and hydroxyapatite into the ECM [82]. Hydroxyapatite $[Ca_{10}(PO_4)_6(OH)_2]$ stores 99% of the body's calcium and is what gives bone its strength and hardness [133].

The two principal destructive enzymes include cathepsin K and carbonic anhydrase, along with protons which are secreted from the cell to create a low pH environment [9, 78, 82]. Cathepsin K is an acidophilic collagenase that can cleave triple fibril type I collagen [2, 130]. The majority of the organic bone matrix is comprised of type I collagen [3], which allows cathepsin K to have a strong

affinity to the bone, dissolving hydroxyapatite off the bone [2, 51]. Cathepsin K KO mice show a drastic decline in bone resorption, often showing osteopetrotic phenotype of dense, thick, heavily mineralized bone in the long bones [2]. This increases the risk of fracture as well as a higher chance for infections, as the bone invades the marrow space constricting the space in the marrow for hematopoiesis to take place [134]. To inhibit bone resorption, osteoblasts secrete osteoprotegerin, a decoy ligand for the RANKL receptor, blocking activation of the osteoclastic activities [82].

In healthy conditions, osteoblasts can express MMPs to recruit and anchor cells and materials to the bone for the osteoblasts to use, as well as regulate the coupling mechanism of the osteoblast-osteoclast routine [2, 83, 135]. Specific collagenases, namely MMP1, MMP8 and MMP13 are secreted by these cells to breakdown collagen in the bone [10]. For normal physiological purposes, MMP2 and MT-MMP1 are also capable of degrading bone converted into gelatin, though, to a lesser extent [10]. Osteoblasts do secrete MMP2 however its principal role seems to be more of a recruiter of materials and cells to the site of the bone resorption than for its degradative activities [78].

In conjunction with osteoclast and osteoblast signalling, normal bone resorption is also mediated by mechanical force upon the bones, hormone levels,

exercise, weight and circulating cytokines [4, 136]. The bone remodelling process is on-going; adults can have over one million foci of resorption occurring concurrently at any given time [136]. Normal bone turnover is the attraction of osteoblasts to cortical and trabecular bone, where they attach, following the osteoclasts, and lay down fresh matrix [48, 82]. The new matrix becomes mineralized over time; this process varies in duration; there is an approximate two week delay between the laying down of fresh matrix and its mineralization [13]. Mineralization of new matrix is performed by osteonectin, a small, bone-specific protein which in serum, is often used to measure the body's osteoblastic activity levels [137]. Osteonectin is synthesized and secreted by osteoblasts, which also assists osteoclasts binding to bone [138].

Cancer cells, in comparison, can resorb bone in neutral pH environments, indicating that this method is not reliant on the release acidic chemicals into the resorption pocket [9, 10]. This was demonstrated in the bone resorption assay, as osteoclasts were not present yet, degradation still occurred (**Figure 4.6** and **Figure 4.7 E, F**). Cancer cells show upregulation of degradative enzymes which breakdown the ECM and surroundings tissues, including bone, contributing to angiogenesis and metastasis. MMP2 is one of these enzymes that has been previously shown to resorb bone [82]. This is illustrated in **Figure 4.7 G, H**; bone resorption decreases as the amount of active MMP2 is reduced in the bone extracellular environment. Cytokines such as IL-6, IL-8 and IL-11 secreted from

the breast cancer cells can also signal the initiation of bone resorption [3, 82]. Furthermore, physical pressure by the tumour on the underlying bone can also cause damage depending on the location of the tumour and other patient circumstances [82]. In cancer, bone is generally destroyed and not rebuilt due to the upregulation of osteoclastogenic signals, and it causes of advanced disease, bone formation halts, causing only bone resorption to continue [82, 139], which may illustrate the overexpression profile of MMP2 in the chondrosarcoma cell lines tested. Some of these pro-resorption signals are RANKL [139], transforming GFβ (TGF) [140], insulin-like GF, and vascular endothelial GF [3, 20, 82]. Many of these proteins present in the ECM in their inactive form become activated upon MMP2 cleavage, and also contribute to the bone resorption process [82].

There are no off-switches for resorption to cease and osteoblasts to takeover in cancer, causing significant bone damage at the tumour site. The "Vicious Cycle" is a term used when the growth of a tumour causes a positive feedback loop at the tumour-bone interface, increasing the growth capabilities of the tumour [78, 135]. The tumour hijacks the use of the growth factors (specifically TGFβ) relinquished from the bone matrix, causing the growth of the tumour to perpetuate and for bone resorption to increase as a result [78, 135]. This is observed in chondrosarcoma; although it is believed by some that osteoclasts are necessary for the bone resorption process, the bone resorption

activity of cancer cells contributes to their ability to invade surrounding tissue, enter the bloodstream and become metastatic. Our bone resorption assays confirmed that chondrosarcoma cells are able to produce resorption pits in non-decalcified bone chips in an environment deficient in osteoclasts, as confirmed by a bone pathologist (**Figure 4.6**).

5.2 MMP2 as a Target for Chondrosarcoma Therapy

It has long been known that bisphosphonates such as alendronate and zolendronic acid have been successful at inhibiting the growth of various malignant cell types including chondrosarcoma [12, 82]. They are synthetic MMP inhibitors which work by binding hydroxyapatite on the bone surface, become engulfed by osteoclasts causing apoptosis and ceasing bone resorption [61, 82, 141]. However, osteoclastic cellular function resumes once the treatment is discontinued, and these therapies can have negative effects on the skeletal system after long time use by affecting normal osteoblastic development and function [82]. Though these drugs have been used for decades, the understanding of its mechanism of action is still incomplete [61, 82].

As MMP2 is known to be involved in the bone resorption process [82, 135], it has been a popular target for anti-cancer treatments. As realized here, in all five chondrosarcoma cell lines studied displayed at least 98-fold higher expression level of *MMP2* than the osteoblastic cells (**Figure 4.9B**), showing that *MMP2* expression is in overdrive. Higher levels of circulating MMP2 levels in the serum have been correlated to malignant thyroid cancer [142], and some have begun to use the circulating MMP2 concentration as a measurement for grading chondrosarcoma [11]. Current clinical trials are attempting to target bone resorption, invasion and metastasis by several MMP-2 inhibitors with mixed success [30, 94]. Most clinical trials using MMIs have failed due to the causation of excessive tissue damage [44], accelerated metastatic rate of the tumours, or even by creating new tumours [44, 143]. Certain compounds which caused negligible surrounding damage to other tissues also did not show improved effectiveness over what is currently being used to treat the tumours or the dose was not physiologically plausible; they did not continue on to further phases of clinical trials [30, 44].

The goal of chondrosarcoma therapy is to inhibit MMPs locally at the tumour site, leaving healthy cells uninjured, but the lack of MMP2 in the body as a whole can contribute to diseases and disorders, which systemic inhibition of MMPs could mimic. Intriguingly, the majority of these are bone-related problems including osteolytic and arthritic-like issues [59], bone deformations [144] and

joint erosion [84]. *MMP2*-/- bones have shown to have lower densities than healthy ones [83] and they show trouble with bone growth [144]. Furthermore, *MMP2*-/- mice have shown to be smaller at birth that WT mice [14, 84]. This suggests that MMP2 has a role in bone maintenance and structure, in addition to its traditional proteolytic actions [84]. Mouse *MMP2*/*MMP14* double KO studies have been proven to be lethal mutations, which caused the authors to propose that this duo is required for the growth of the skeleton [14]. *MMP2*/*MMP9* double KOs are viable mutations in the mouse, however, the deficit in these proteins caused significant changes to the bone architecture, by way of bone decreased mineralization and density [83]. This supports the idea that MMP2 is used not only for its degradative capabilities, but also to support and maintain the bone architecture.

As previously described, other proteins play roles in *MMP2* regulation, expression and activity, for example, TIMP2 [11, 48]. As the TIMP family are homologous to one another [43], any potential TIMP inhibitor used would need to be highly specific, as with the MMP family, they have many overlapping functions and regulate various signalling pathways within different cells [45, 48, 56, 67]. Previous studies in our lab have shown that TIMP 1, 2, and 3 are expressed in KC cells (unpublished data). How their expression levels compare to hFOB cells is yet to be quantified.

Therefore, anti-MMP or anti-TIMP treatments to treat or control cancer do not seem feasible, given their varying roles in the bone, as well as in other tissues. Most of the MMP inhibitors have failed, as they are not specific enough to limit the amount of damage caused to healthy cells [89]. As many of the tested drugs are administered orally, issues arise with targeting malignancies through such ubiquitous signalling pathways as the drugs may affect other systems, thus causing side effects or secondary tissue damage.

5.3 ETV5 as a Target for Chondrosarcoma Therapy

Until now, the role of *ETV5* in bone resorption and remodelling had not been previously assessed. It was determined here for the first time that ETV5 affects MMP2 expression and activity in chondrosarcoma, which in turn plays a significant role in bone resorption.

The possibility of targeting ETV5 to inhibit chondrosarcoma migration, proliferation and metastasis may be a feasible route [103], though, as it also has a myriad of targets, not only MMP family members, perhaps not the best direct route to target chondrosarcoma cells. It is understood that male ETV5-/- mice show markedly reduced fertility due to signalling and developmental problems in gametogenesis [105, 109]. It is noteworthy, that targeting ETV5 past

reproductive age has not yet been assessed. Furthermore, when *ETV5* was knocked-down *in vitro*, the production of spermatogonial stem cells declined by 40%, suggesting that decreased quantities of ETV5 is still able to produce viable gametes [145].

Although the downregulation of ETV5 in the chondrosarcoma cells resulted in significant decreases in MMP2 expression and activity, Oikawa *et al.* suggest that as ETS TFs play such essential and mixed roles in cancer cells, that specific targeting of these TFs could attack the cancer at multiple pathways including apoptosis, GF receptor genes, metastasis-promoting genes as well as cell cycle proteins [103]. Nevertheless, the authors do caution that supressing these actions may not inhibit them completely and the tumour could compensate for the changes in signal transduction and gene expression [103]. Targeting *ETV5* may have more systemic side effects on overlapping signalling cascades as the ETS family members are quite homologous in their DNA-binding domain and catalytic motifs [36, 98, 99].

Few reports have shown success with inhibiting TFs via chemotherapy, as they are too broad a target [103]. There has also been a debate whether the PEA3 members are pro- or anti-tumourigenic. These TFs seem to work differently and show overlapping functions given different environmental stresses [98, 100]. This, coupled with the fact that many different ETS factors can be

expressed in the same cell [99], attempting to repress their action may cause damage in tissues that require higher amounts of the TF, like Sertoli and germ cells. Further studies are needed to assess changes to other genes affected by the downregulation in chondrosarcoma before targeting ETV5 can become a plausible treatment for this type of cancer.

Current research is attempting to treat chondrosarcoma via assorted venues of therapy, including monoclonal antibodies [12] proton therapy, carbon ion therapy, new MMP inhibitors, cryosurgery, proton beam radiotherapy [146], as well as various receptor-targeting drugs [19, 30]. Inhibitors of the Indian Hedgehog pathway are currently in use to attempt to control the growth of chondrosarcoma in humans [12, 147, 148].

The five chondrosarcoma cell lines studied here displayed a large range of *ETV5* expression profiles, ranging from 0.4 times that of hFOB cells to 2.6-fold the levels of hFOB (**Figure 4.9A**). Despite the fact that the five cell lines are all equally categorized as chondrosarcoma, their molecular background are remarkably different, and one therapy for one patient may work in inhibiting the cancer, and not work or increase metastasis than for another. Perhaps the use of personalized therapy for chondrosarcoma could have fewer side effects and function better at targeting the specific needs of the patients' genomes.

5.4 Chondrosarcoma and Other Matters

Patient comorbidities also factor into the progression and treatment of chondrosarcoma. For example, Ollier's disease is characterized by the presence of multiple enchondromas, usually presenting on one side of the body [149]. PT3 was diagnosed with this, in addition to chondrosarcoma (See **Supplemental Table E** for patient information). This patient displayed the highest level of *MMP2* expression across all five cell lines evaluated, suggesting that MMP2 also played a role in the pathogenesis of Ollier's disease.

Additionally, KC cells are a grade II chondrosarcoma, as well as two of the patient cell lines, while two of the four patient cells are grade I, which can exhibit different cellular processes and expression levels (refer to **Figure 1.4** for cellular changes between grading). Multiple benign osteochondromas were first seen in PT2, which then transformed into malignant chondrosarcoma. This cell line showed *ETV5* expression levels lower than that of the hFOB cells, perhaps demonstrating that there are other TF that regulate *MMP2* in those cases. PT2 was diagnosed as having grade I chondrosarcoma, which could account for the low expression profile of *ETV5*, as grade I chondrosarcomas are not usually metastatic, thus, not as commonly invasive. With the lowest ETV5 and second lowest MMP2 expression levels compared to hFOB, PT4 was 80 years old at

time of biopsy; perhaps this cancer was slower than average to develop, thus not being as aggressive as the heist two patients who showed higher expression levels of *ETV5* than hFOB cells. Perhaps the endogenous levels of MMP2 in patient cells could serve as a prognostic tool to assist pathologists to provide better estimations of stages of disease, leading to more effective treatments.

These patient profiles exemplify that additional medical issues compound the attempt to alleviate symptoms and rid the body of the tumours, as their genetic and medical histories add additional obstacles for treatment options.

CHAPTER SIX: FUTURE DIRECTIONS

6.1 Improvements on Completed Experiments

A variety of techniques have been employed here, however other procedures could have proved efficient as well. Recombinant protein transfection into the chondrosarcoma cells could potentially allow for a better overexpression profile of the cells versus the overexpression vector, as extra time is needed for transcription of the vector and for the post translational modifications of the protein to occur which are not applicable when using the recombinant protein. This may be a more efficient method to use, as cloning would not have been necessary and could allow for easier reproducibility of the experiment.

Correspondingly, it would be interesting to create a stable *ETV5* KD or KO chondrosarcoma cell line using shETV5 (small hairpin RNA), in the KC cells and repeat the bone resorption assay to realize any direct effects that ETV5 may have on the bone resorption process.

In addition to the fluorescent gelatinase activity assay performed here, a zymogen gelatinase assay (zymogram) could also distinguish the ratios of

inactive-to-active gelatinase in the cell lysis and media samples, which the fluorescent activity assay performed here could not.

6.2 Future Experiments

There are many future directions for this project. The next step in forming a more comprehensive picture of the ETV5-MMP2 relationship in human chondrosarcoma would be to determine whether or not ETV5 binds directly to the *MMP2* promoter, or if there are other co-factors, TFs or assistant proteins involved in this partnership. Electric mobility gel shift assays could be performed to determine if/where ETV5 binds the *MMP2* promoter. If there is/are such sites, luciferase assays could be completed to analyze gene regulatory sequences within the *MMP2* promoter, such as TF binding sites; which would be helpful in locating any ETV5-specific sites on *MMP2*, if one or more are present. This method encompasses a great deal of cloning of the various portions of the *MMP2* promoter, and control sequences to succeed in defining where the binding motifs reside. Afterwards, the determination of protein-protein interactions with ETV5 could be elucidated through co-immunoprecipitation experiments, followed by mass spectrometry analysis for the identification of the co-immunoprecipitated proteins.

Due to time constraints, TIMPs were not studied here as they were not the focus of this project. It is possible to hypothesize that the dysregulation of *MMP2* is caused also by aberrations in *TIMP2*. Assessing this would be of interest; perhaps overexpressed TIMPs are also the culprit in the high levels of active MMP2 seen in chondrosarcoma.

Finally, characterizing chondrosarcoma through flow cytometry or tissue microarray to identify any chondrosarcoma-specific markers may be employed to determine a chondrosarcoma cell-specific treatment option.

CHAPTER SEVEN: CONCLUSIONS

The results of this study allow for a molecular mechanistic view of the ETV5-MMP2 pathway in human chondrosarcoma, as well as the role of ETV5 in chondrosarcoma, which had never been studied.

In conclusion, *MMP2* is highly expressed in KC and patient chondrosarcoma cells, while ETV5 shows a range of expression levels when compared to hFOB controls across all five chondrosarcoma cell lines. By RNA, protein and activity assays, it is seen that ETV5 regulates MMP2 upstream in a proportional manner in KC cells and that the ETV5-MMP2 duo work similarly in the patient cell lines as the KC cells. That is, when *ETV5* is knocked-down, so becomes MMP2.

It was also shown here that KC chondrosarcoma cells can resorb bone without the presence of osteoclast cells, and when a matrix metalloproteinase inhibitor is added to the growth media with bone chips, that bone resorption decreases significantly, as determined both quantitatively and qualitatively. This indicates that MMP2 plays an important role in local tissue invasion and metastasis in chondrosarcoma. Finally, patient chondrosarcoma cell lines displayed the same ETV5-MMP2 dynamics as in the KC cells, as found by *ETV5* knockdown experiments.

As more information becomes known about the molecular pathways of metastasis of chondrosarcoma, patient-specific treatments could be developed to assist in inhibiting further growth of the primary tumour as well as its metastasis, while preventing neighbouring signalling cascades from becoming affected.

Furthering the current understanding of the ETV5-MMP2 pathway is needed, and the genetic factors affecting bone resorption could eventually lead to targeted treatment, which is desperately needed for patients affected by chondrosarcoma.

REFERENCE LIST

1. Apatech. *Cross sections of a bone*. 2012 [cited 2012 June 11th, 2012]; Available from: http://www.apatech.com/investors/bonaandspineanatomy.asp?selectorId=18.

2. Delaisse, J.M., et al., *Matrix metalloproteinases (MMP) and cathepsin K contribute differently to osteoclastic activities.* Microsc Res Tech, 2003. **61**(6): p. 504-13.

3. Orr, F.W., et al., *Pathophysiologic interactions in skeletal metastasis.* Cancer, 2000. **88**(12 Suppl): p. 2912-8.

4. Yingst, S., et al., *Characterization of collagenous matrix assembly in a chondrocyte model system.* J Biomed Mater Res A, 2009. **90**(1): p. 247-55.

5. Sigma-Aldrich, *Collagen*, 2011.

6. Alberts, J., Lewis, Raff, Roberts and Walter, *Molecular Biology of the Cell*. 4 ed2002, New York, NY, USA: Garland Science. 1463.

7. Shum, L. and G. Nuckolls, *The life cycle of chondrocytes in the developing skeleton.* Arthritis Res, 2002. **4**(2): p. 94-106.

8. Lodish, B., Matsudauram Kaiser, Krieger, Scott, Zipursky and Darnell, *Molecular Biology of the Cell*. 5 ed2004, New York, NY, USA: W. H. Freeman and Company. 973.

9. Milner, J.M., et al., *Fibroblast activation protein alpha is expressed by chondrocytes following a pro-inflammatory stimulus and is elevated in osteoarthritis.* Arthritis Res Ther, 2006. **8**(1): p. R23.

10. Young, D.A., et al., *Histone deacetylase inhibitors modulate metalloproteinase gene expression in chondrocytes and block cartilage resorption.* Arthritis Res Ther, 2005. **7**(3): p. R503-12.

11. Soderstrom, M., et al., *Expression of matrix metalloproteinases and tissue inhibitors of metalloproteinases in human chondrosarcomas.* APMIS, 2001. **109**(4): p. 305-15.

12. Jamil, N., S. Howie, and D.M. Salter, *Therapeutic molecular targets in human chondrosarcoma.* Int J Exp Pathol, 2010. **91**(5): p. 387-93.

13. Cotran, K.a.C., *Pathologic Basis of Disease*. 6 ed1999, Philadelphia, PA, USA: Robbins. 1425.

14. Page-McCaw, A., A.J. Ewald, and Z. Werb, *Matrix metalloproteinases and the regulation of tissue remodelling.* Nat Rev Mol Cell Biol, 2007. **8**(3): p. 221-33.

15. Lechler, P., et al., *The antiapoptotic gene survivin is highly expressed in human chondrosarcoma and promotes drug resistance in chondrosarcoma cells in vitro.* BMC Cancer, 2011. **11**: p. 120.

16. Rozeman, L.B., A.M. Cleton-Jansen, and P.C. Hogendoorn, *Pathology of primary malignant bone and cartilage tumours.* Int Orthop, 2006. **30**(6): p. 437-44.

17. Springfield, D.S., M.C. Gebhardt, and M.H. McGuire, *Chondrosarcoma: a review.* Instr Course Lect, 1996. **45**: p. 417-24.

18. Zyada, M.M. and A.A. Shamaa, *Is collagenase-3 (MMP-13) expression in chondrosarcoma of the jaws a true marker for tumor aggressiveness?* Diagn Pathol, 2008. **3**: p. 26.

19. Onishi, A.C., A.M. Hincker, and F.Y. Lee, *Surmounting chemotherapy and radioresistance in chondrosarcoma: molecular mechanisms and therapeutic targets.* Sarcoma, 2011. **2011**: p. 381564.

20. Clark, J.C., et al., *New clinically relevant, orthotopic mouse models of human chondrosarcoma with spontaneous metastasis.* Cancer Cell Int, 2010. **10**: p. 20.

21. Fitzgerald, M.P., et al., *Human Chondrosarcoma Cells Acquire an Epithelial-Like Gene Expression Pattern via an Epigenetic Switch: Evidence for Mesenchymal-Epithelial Transition during Sarcomagenesis.* Sarcoma, 2011. **2011**: p. 598218.

22. Kim, D.W., et al., *Targeting of cell survival genes using small interfering RNAs (siRNAs) enhances radiosensitivity of Grade II chondrosarcoma cells.* J Orthop Res, 2007. **25**(6): p. 820-8.

23. Rizzo, M., et al., *Chondrosarcoma of bone: analysis of 108 cases and evaluation for predictors of outcome.* Clin Orthop Relat Res, 2001(391): p. 224-33.

24. Nishida, K., et al., *Chondrosarcoma and peroxisome proliferator-activated receptor.* PPAR Res, 2008. **2008**: p. 250568.

25. Capps, E., et al., *Chest wall chondrosarcoma.* Proc (Bayl Univ Med Cent), 2009. **22**(4): p. 362-5.

26. Kim, M.J., et al., *Chondrosarcoma: with updates on molecular genetics.* Sarcoma, 2011. **2011**: p. 405437.

27. Terek, R.M., et al., *Chemotherapy and P-glycoprotein expression in chondrosarcoma.* J Orthop Res, 1998. **16**(5): p. 585-90.

28. Richardson, R.B., *Stem cell niches and other factors that influence the sensitivity of bone marrow to radiation-induced bone cancer and leukaemia in children and adults.* Int J Radiat Biol, 2011. **87**(4): p. 343-59.

29. Moradi, B., et al., *The impact of pathological fractures on therapy outcome in patients with primary malignant bone tumours.* Int Orthop, 2010. **34**(7): p. 1017-23.

30. Bovee, J.V., et al., *Cartilage tumours and bone development: molecular pathology and possible therapeutic targets.* Nat Rev Cancer, 2010. **10**(7): p. 481-8.

31. WHO, *Pathology and Genetics of Tumours of Soft Tissue and Bone*. World Health Organization Classification of Tumors, ed. K.a. Sobin2002, Lyon, France: IARCPress. 427.

32. Olsson, L., et al., *Clonal evolution through loss of chromosomes and subsequent polyploidization in chondrosarcoma.* PLoS One, 2011. **6**(9): p. e24977.

33. Vincourt, J.B., et al., *C-propeptides of procollagens I alpha 1 and II that differentially accumulate in enchondromas versus chondrosarcomas regulate tumor cell survival and migration.* Cancer Res, 2010. **70**(11): p. 4739-48.

34. Fong, Y.C., et al., *Absence of a correlation between the presence of a single nucleotide polymorphism in the matrix metalloproteinase 1 promoter and outcome in patients of chondrosarcoma.* Clin Cancer Res, 2004. **10**(21): p. 7329-34.

35. Kim, D.W., et al., *siRNA-based targeting of antiapoptotic genes can reverse chemoresistance in P-glycoprotein expressing chondrosarcoma cells.* Mol Cancer, 2009. **8**: p. 28.

36. Keld, R., et al., *The ERK MAP kinase-PEA3/ETV4-MMP-1 axis is operative in oesophageal adenocarcinoma.* Mol Cancer, 2010. **9**: p. 313.

37. Moses, M.A. and Y. Shing, *Production of matrix metalloproteinases and a metalloproteinase inhibitor by swarm rat chondrosarcoma.* Biochem Biophys Res Commun, 1994. **199**(1): p. 418-24.

38. Moussavi-Harami, F., et al., *Intrinsic radiation resistance in human chondrosarcoma cells.* Biochem Biophys Res Commun, 2006. **346**(2): p. 379-85.

39. Sorrentino, B.P., *Gene therapy to protect haematopoietic cells from cytotoxic cancer drugs.* Nat Rev Cancer, 2002. **2**(6): p. 431-41.

40. Ashkenazi, A. and R.S. Herbst, *To kill a tumor cell: the potential of proapoptotic receptor agonists.* J Clin Invest, 2008. **118**(6): p. 1979-90.

41. Asp, J., et al., *Changes of the p16 gene but not the p53 gene in human chondrosarcoma tissues.* Int J Cancer, 2000. **85**(6): p. 782-6.

42. Riedel, R.F., et al., *The clinical management of chondrosarcoma.* Curr Treat Options Oncol, 2009. **10**(1-2): p. 94-106.

43. Mandal, M., et al., *Clinical implications of matrix metalloproteinases.* Mol Cell Biochem, 2003. **252**(1-2): p. 305-29.

44. Purcell, W.T., M.A. Rudek, and M. Hidalgo, *Development of matrix metalloproteinase inhibitors in cancer therapy.* Hematol Oncol Clin North Am, 2002. **16**(5): p. 1189-227.

45. Stamenkovic, I., *Matrix metalloproteinases in tumor invasion and metastasis.* Semin Cancer Biol, 2000. **10**(6): p. 415-33.

46. Kato, T., et al., *Diminished corneal angiogenesis in gelatinase A-deficient mice.* FEBS Lett, 2001. **508**(2): p. 187-90.

47. Nam, J., et al., *Sequential alterations in catabolic and anabolic gene expression parallel pathological changes during progression of monoiodoacetate-induced arthritis.* PLoS One, 2011. **6**(9): p. e24320.

48. Sternlicht, M.D. and Z. Werb, *How matrix metalloproteinases regulate cell behavior.* Annu Rev Cell Dev Biol, 2001. **17**: p. 463-516.

49. Esparza, J., et al., *MMP-2 null mice exhibit an early onset and severe experimental autoimmune encephalomyelitis due to an increase in MMP-9 expression and activity.* FASEB J, 2004. **18**(14): p. 1682-91.

50. Chetty, C., et al., *MMP-2 alters VEGF expression via alphaVbeta3 integrin-mediated PI3K/AKT signaling in A549 lung cancer cells.* Int J Cancer, 2010. **127**(5): p. 1081-95.

51. Dong, Z., et al., *Matrix metalloproteinase activity and osteoclasts in experimental prostate cancer bone metastasis tissue.* Am J Pathol, 2005. **166**(4): p. 1173-86.

52. Hou, C.H., et al., *WISP-1 increases MMP-2 expression and cell motility in human chondrosarcoma cells.* Biochem Pharmacol, 2011. **81**(11): p. 1286-95.

53. Kenny, H.A., et al., *The initial steps of ovarian cancer cell metastasis are mediated by MMP-2 cleavage of vitronectin and fibronectin.* J Clin Invest, 2008. **118**(4): p. 1367-79.

54. Maatta, M., et al., *Differential expression of matrix metalloproteinase (MMP)-2, MMP-9, and membrane type 1-MMP in hepatocellular and pancreatic adenocarcinoma: implications for tumor progression and clinical prognosis.* Clin Cancer Res, 2000. **6**(7): p. 2726-34.

55. Qin, L., et al., *The AIB1 oncogene promotes breast cancer metastasis by activation of PEA3-mediated matrix metalloproteinase 2 (MMP2) and MMP9 expression.* Mol Cell Biol, 2008. **28**(19): p. 5937-50.

56. Ghajar, C.M., S.C. George, and A.J. Putnam, *Matrix metalloproteinase control of capillary morphogenesis.* Crit Rev Eukaryot Gene Expr, 2008. **18**(3): p. 251-78.

57. Singh, N.K., et al., *AP-1 (Fra-1/c-Jun)-mediated induction of expression of matrix metalloproteinase-2 is required for 15S-hydroxyeicosatetraenoic acid-induced angiogenesis.* J Biol Chem, 2010. **285**(22): p. 16830-43.

58. Isern, E., et al., *The activator protein 1 binding motifs within the human cytomegalovirus major immediate-early enhancer are functionally redundant and act in a cooperative manner with the NF-{kappa}B sites during acute infection.* J Virol, 2011. **85**(4): p. 1732-46.

59. Greenlee, K.J., Z. Werb, and F. Kheradmand, *Matrix metalloproteinases in lung: multiple, multifarious, and multifaceted.* Physiol Rev, 2007. **87**(1): p. 69-98.

60. Monge, M., et al., *ERM/ETV5 up-regulation plays a role during myometrial infiltration through matrix metalloproteinase-2 activation in endometrial cancer.* Cancer Res, 2007. **67**(14): p. 6753-9.

61. Chaudhary, A.K., et al., *Genetic polymorphisms of matrix metalloproteinases and their inhibitors in potentially malignant and malignant lesions of the head and neck.* J Biomed Sci, 2010. **17**: p. 10.

62. Poulsom, R., et al., *Stromal expression of 72 kda type IV collagenase (MMP-2) and TIMP-2 mRNAs in colorectal neoplasia.* Am J Pathol, 1992. **141**(2): p. 389-96.

63. Azar, D.T., et al., *Effect of MT1-MMP deficiency and overexpression in corneal keratocytes on vascular endothelial cell migration and proliferation.* Curr Eye Res, 2008. **33**(11): p. 954-62.

64. Cao, J., et al., *Membrane type matrix metalloproteinase 1 activates pro-gelatinase A without furin cleavage of the N-terminal domain.* J Biol Chem, 1996. **271**(47): p. 30174-80.

65. Deryugina, E.I., et al., *MT1-MMP initiates activation of pro-MMP-2 and integrin alphavbeta3 promotes maturation of MMP-2 in breast carcinoma cells.* Exp Cell Res, 2001. **263**(2): p. 209-23.

66. Wang, Z., R. Juttermann, and P.D. Soloway, *TIMP-2 is required for efficient activation of proMMP-2 in vivo.* J Biol Chem, 2000. **275**(34): p. 26411-5.

67. Klein, T. and R. Bischoff, *Physiology and pathophysiology of matrix metalloproteases.* Amino Acids, 2011. **41**(2): p. 271-90.

68. Lafleur, M.A., A.M. Tester, and E.W. Thompson, *Selective involvement of TIMP-2 in the second activational cleavage of pro-MMP-2: refinement of the pro-MMP-2 activation mechanism.* FEBS Lett, 2003. **553**(3): p. 457-63.

69. Lluri, G., et al., *Tissue inhibitor of metalloproteinase-2 (TIMP-2) regulates myogenesis and beta1 integrin expression in vitro.* Exp Cell Res, 2008. **314**(1): p. 11-24.

70. Pratheeshkumar, P. and G. Kuttan, *Nomilin inhibits tumor-specific angiogenesis by downregulating VEGF, NO and proinflammatory cytokine profile and also by inhibiting the activation of MMP-2 and MMP-9.* Eur J Pharmacol, 2011. **668**(3): p. 450-8.

71. Damm, S., et al., *HGF-promoted motility in primary human melanocytes depends on CD44v6 regulated via NF-kappa B, Egr-1, and C/EBP-beta.* J Invest Dermatol, 2010. **130**(7): p. 1893-903.

72. Krejci, P., et al., *Interaction of fibroblast growth factor and C-natriuretic peptide signaling in regulation of chondrocyte proliferation and extracellular matrix homeostasis.* J Cell Sci, 2005. **118**(Pt 21): p. 5089-100.

73. Lindstad, R.I., et al., *Pancreatic trypsin activates human promatrix metalloproteinase-2.* J Mol Biol, 2005. **350**(4): p. 682-98.

74. Du, J., et al., *O-fucosylation of thrombospondin type 1 repeats restricts epithelial to mesenchymal transition (EMT) and maintains epiblast pluripotency during mouse gastrulation.* Dev Biol, 2010. **346**(1): p. 25-38.

75. Wiseman, B.S., et al., *Site-specific inductive and inhibitory activities of MMP-2 and MMP-3 orchestrate mammary gland branching morphogenesis.* J Cell Biol, 2003. **162**(6): p. 1123-33.

76. Itoh, T., et al., *Reduced angiogenesis and tumor progression in gelatinase A-deficient mice.* Cancer Res, 1998. **58**(5): p. 1048-51.

77. Sano, H., et al., *The Foxc2 transcription factor regulates tumor angiogenesis.* Biochem Biophys Res Commun, 2010. **392**(2): p. 201-6.

78. Thiolloy, S., et al., *Osteoclast-derived matrix metalloproteinase-7, but not matrix metalloproteinase-9, contributes to tumor-induced osteolysis.* Cancer Res, 2009. **69**(16): p. 6747-55.

79. Mbeunkui, F. and D.J. Johann, Jr., *Cancer and the tumor microenvironment: a review of an essential relationship.* Cancer Chemother Pharmacol, 2009. **63**(4): p. 571-82.

80. Corry, D.B., et al., *Decreased allergic lung inflammatory cell egression and increased susceptibility to asphyxiation in MMP2-deficiency.* Nat Immunol, 2002. **3**(4): p. 347-53.

81. Yong, V.W., et al., *Elevation of matrix metalloproteinases (MMPs) in multiple sclerosis and impact of immunomodulators.* J Neurol Sci, 2007. **259**(1-2): p. 79-84.

82. Chen, Y.C., D.M. Sosnoski, and A.M. Mastro, *Breast cancer metastasis to the bone: mechanisms of bone loss.* Breast Cancer Res, 2010. **12**(6): p. 215.

83. Nyman, J.S., et al., *Differential effects between the loss of MMP-2 and MMP-9 on structural and tissue-level properties of bone.* J Bone Miner Res, 2010.

84. Mosig, R.A., et al., *Loss of MMP-2 disrupts skeletal and craniofacial development and results in decreased bone mineralization, joint erosion and defects in osteoblast and osteoclast growth.* Hum Mol Genet, 2007. **16**(9): p. 1113-23.

85. Oshima, T., et al., *Clinicopathological significance of the gene expression of matrix metalloproteinases and reversion-inducing cysteine-rich protein with Kazal motifs in patients with colorectal cancer: MMP-2 gene expression is a useful predictor of liver metastasis from colorectal cancer.* Oncol Rep, 2008. **19**(5): p. 1285-91.

86. Boivin, D., et al., *The antiangiogenic agent Neovastat (AE-941) induces endothelial cell apoptosis.* Mol Cancer Ther, 2002. **1**(10): p. 795-802.

87. Franco, G.C., et al., *Inhibition of matrix metalloproteinase-9 activity by doxycycline ameliorates RANK ligand-induced osteoclast differentiation in vitro and in vivo.* Exp Cell Res, 2011. **317**(10): p. 1454-64.

88. Shimokawa, K., et al., *Complexes of gelatinases and tissue inhibitor of metalloproteinases in human seminal plasma.* J Androl, 2003. **24**(1): p. 73-7.

89. Durrant, J.D., C.A. de Oliveira, and J.A. McCammon, *Including receptor flexibility and induced fit effects into the design of MMP-2 inhibitors.* J Mol Recognit, 2010. **23**(2): p. 173-82.

90. Fromigue, O., Z. Hamidouche, and P.J. Marie, *Blockade of the RhoA-JNK-c-Jun-MMP2 cascade by atorvastatin reduces osteosarcoma cell invasion.* J Biol Chem, 2008. **283**(45): p. 30549-56.

91. Dissanayake, K., et al., *ERK/p90(RSK)/14-3-3 signalling has an impact on expression of PEA3 Ets transcription factors via the transcriptional repressor capicua.* Biochem J, 2011. **433**(3): p. 515-25.

92. Stellas, D., A. El Hamidieh, and E. Patsavoudi, *Monoclonal antibody 4C5 prevents activation of MMP2 and MMP9 by disrupting their interaction with extracellular HSP90 and inhibits formation of metastatic breast cancer cell deposits.* BMC Cell Biol, 2010. **11**: p. 51.

93. Djouad, F., et al., *Microenvironmental changes during differentiation of mesenchymal stem cells towards chondrocytes.* Arthritis Res Ther, 2007. **9**(2): p. R33.

94. Lai, T.J., et al., *Alendronate inhibits cell invasion and MMP-2 secretion in human chondrosarcoma cell line.* Acta Pharmacol Sin, 2007. **28**(8): p. 1231-5.

95. Sharrocks, A.D., et al., *The ETS-domain transcription factor family.* Int J Biochem Cell Biol, 1997. **29**(12): p. 1371-87.

96. Wei, G.H., et al., *Genome-wide analysis of ETS-family DNA-binding in vitro and in vivo.* EMBO J, 2010. **29**(13): p. 2147-60.

97. de Launoit, Y., et al., *The Ets transcription factors of the PEA3 group: transcriptional regulators in metastasis.* Biochim Biophys Acta, 2006. **1766**(1): p. 79-87.

98. Baker, R., et al., *Pea3 transcription factors and wnt1-induced mouse mammary neoplasia.* PLoS One, 2010. **5**(1): p. e8854.

99. Jedlicka, P. and A. Gutierrez-Hartmann, *Ets transcription factors in intestinal morphogenesis, homeostasis and disease.* Histol Histopathol, 2008. **23**(11): p. 1417-24.

100. Hollenhorst, P.C., D.A. Jones, and B.J. Graves, *Expression profiles frame the promoter specificity dilemma of the ETS family of transcription factors.* Nucleic Acids Res, 2004. **32**(18): p. 5693-702.

101. Oikawa, T. and T. Yamada, *Molecular biology of the Ets family of transcription factors.* Gene, 2003. **303**: p. 11-34.

102. Ouyang, W., et al., *The Ets transcription factor ERM is Th1-specific and induced by IL-12 through a Stat4-dependent pathway.* Proc Natl Acad Sci U S A, 1999. **96**(7): p. 3888-93.

103. Oikawa, T., *ETS transcription factors: possible targets for cancer therapy.* Cancer Sci, 2004. **95**(8): p. 626-33.

104. Pastorcic, M. and H.K. Das, *Analysis of transcriptional modulation of the presenilin 1 gene promoter by ZNF237, a candidate binding partner of the Ets transcription factor ERM.* Brain Res, 2007. **1128**(1): p. 21-32.

105. Hofmann, M.C., *Gdnf signaling pathways within the mammalian spermatogonial stem cell niche.* Mol Cell Endocrinol, 2008. **288**(1-2): p. 95-103.

106. Bieche, I., et al., *Expression of PEA3/E1AF/ETV4, an Ets-related transcription factor, in breast tumors: positive links to MMP2, NRG1 and CGB expression.* Carcinogenesis, 2004. **25**(3): p. 405-11.

107. Lens, Z., et al., *Solution structure of the N-terminal transactivation domain of ERM modified by SUMO-1.* Biochem Biophys Res Commun, 2010. **399**(1): p. 104-10.

108. Zhang, Z., et al., *Preaxial polydactyly: interactions among ETV, TWIST1 and HAND2 control anterior-posterior patterning of the limb.* Development, 2010. **137**(20): p. 3417-26.

109. Chen, C., et al., *ERM is required for transcriptional control of the spermatogonial stem cell niche.* Nature, 2005. **436**(7053): p. 1030-4.

110. Ishii, K., et al., *FGF2 mediates mouse spermatogonial stem cell self-renewal via upregulation of Etv5 and Bcl6b through MAP2K1 activation.* Development, 2012. **139**(10): p. 1734-43.

111. Mao, J., et al., *Fgf-dependent Etv4/5 activity is required for posterior restriction of Sonic Hedgehog and promoting outgrowth of the vertebrate limb.* Dev Cell, 2009. **16**(4): p. 600-6.

112. Welsh, I.C., A. Hagge-Greenberg, and T.P. O'Brien, *A dosage-dependent role for Spry2 in growth and patterning during palate development.* Mech Dev, 2007. **124**(9-10): p. 746-61.

113. Guo, B., et al., *Dynamic modification of the ETS transcription factor PEA3 by sumoylation and p300-mediated acetylation.* Nucleic Acids Res, 2011. **39**(15): p. 6403-13.

114. Staun-Ram, E., S. Goldman, and E. Shalev, *Ets-2 and p53 mediate cAMP-induced MMP-2 expression, activity and trophoblast invasion.* Reprod Biol Endocrinol, 2009. **7**: p. 135.

115. Znosko, W.A., et al., *Overlapping functions of Pea3 ETS transcription factors in FGF signaling during zebrafish development.* Dev Biol, 2010. **342**(1): p. 11-25.

116. Hollenhorst, P.C., et al., *Oncogenic ETS proteins mimic activated RAS/MAPK signaling in prostate cells.* Genes Dev, 2011. **25**(20): p. 2147-57.

117. Firlej, V., et al., *Reduced tumorigenesis in mouse mammary cancer cells following inhibition of Pea3- or Erm-dependent transcription.* J Cell Sci, 2008. **121**(Pt 20): p. 3393-402.

118. Lu, B.C., et al., *Etv4 and Etv5 are required downstream of GDNF and Ret for kidney branching morphogenesis.* Nat Genet, 2009. **41**(12): p. 1295-302.

119. Kostereva, N. and M.C. Hofmann, *Regulation of the spermatogonial stem cell niche.* Reprod Domest Anim, 2008. **43 Suppl 2**: p. 386-92.

120. Morrow, C.M., et al., *Claudin 5 expression in mouse seminiferous epithelium is dependent upon the transcription factor ets variant 5 and contributes to blood-testis barrier function.* Biol Reprod, 2009. **81**(5): p. 871-9.

121. Simon, L., et al., *ETV5 regulates sertoli cell chemokines involved in mouse stem/progenitor spermatogonia maintenance.* Stem Cells, 2010. **28**(10): p. 1882-92.

122. Boyer, A., et al., *CTNNB1 signaling in sertoli cells downregulates spermatogonial stem cell activity via WNT4.* PLoS One, 2012. **7**(1): p. e29764.

123. Schlesser, H.N., et al., *Effects of ETV5 (ets variant gene 5) on testis and body growth, time course of spermatogonial stem cell loss, and fertility in mice.* Biol Reprod, 2008. **78**(3): p. 483-9.

124. Tyagi, G., et al., *Loss of Etv5 decreases proliferation and RET levels in neonatal mouse testicular germ cells and causes an abnormal first wave of spermatogenesis.* Biol Reprod, 2009. **81**(2): p. 258-66.

125. Tomlins, S.A., et al., *Role of the TMPRSS2-ERG gene fusion in prostate cancer.* Neoplasia, 2008. **10**(2): p. 177-88.

126. Vitari, A.C., et al., *COP1 is a tumour suppressor that causes degradation of ETS transcription factors.* Nature, 2011. **474**(7351): p. 403-6.

127. Xu, J., R.C. Wu, and B.W. O'Malley, *Normal and cancer-related functions of the p160 steroid receptor co-activator (SRC) family.* Nat Rev Cancer, 2009. **9**(9): p. 615-30.

128. Planaguma, J., et al., *Matrix metalloproteinase-2 and matrix metalloproteinase-9 codistribute with transcription factors RUNX1/AML1 and ETV5/ERM at the invasive front of endometrial and ovarian carcinoma.* Hum Pathol, 2011. **42**(1): p. 57-67.

129. Cao, S., Z. Xiang, and X. Ma, *Global gene expression profiling in interleukin-12-induced activation of CD8(+) cytotoxic T lymphocytes against mouse mammary Carcinoma.* Cell Mol Immunol, 2004. **1**(5): p. 357-66.

130. Mak, I.W., et al., *Evidence for the role of matrix metalloproteinase-13 in bone resorption by giant cell tumor of bone.* Hum Pathol, 2010. **41**(9): p. 1320-9.

131. Rabinovich, A., et al., *Matrix Metalloproteinase Activity in the Stromal Cell of Giant Cell Tumor of Bone.* Open Bone J, 2009. **1**: p. 46-52.

132. Kubo, T., et al., *Inhibitory effects of a new bisphosphonate, minodronate, on proliferation and invasion of a variety of malignant bone tumor cells.* J Orthop Res, 2006. **24**(6): p. 1138-44.

133. Mendelson, B.C., et al., *The fate of porous hydroxyapatite granules used in facial skeletal augmentation.* Aesthetic Plast Surg, 2010. **34**(4): p. 455-61.

134. Gowen, M., et al., *Cathepsin K knockout mice develop osteopetrosis due to a deficit in matrix degradation but not demineralization.* J Bone Miner Res, 1999. **14**(10): p. 1654-63.

135. Bruni-Cardoso, A., et al., *Osteoclast-derived matrix metalloproteinase-9 directly affects angiogenesis in the prostate tumor-bone microenvironment.* Mol Cancer Res, 2010. **8**(4): p. 459-70.

136. Boyce, B.F. and L. Xing, *Functions of RANKL/RANK/OPG in bone modeling and remodeling.* Arch Biochem Biophys, 2008. **473**(2): p. 139-46.

137. Lipton, A., et al., *The science and practice of bone health in oncology: managing bone loss and metastasis in patients with solid tumors.* J Natl Compr Canc Netw, 2009. **7 Suppl 7**: p. S1-29; quiz S30.

138. Atalay, S., et al., *Diagnostic utility of osteocalcin, undercarboxylated osteocalcin, and alkaline phosphatase for osteoporosis in premenopausal and postmenopausal women.* Ann Lab Med, 2012. **32**(1): p. 23-30.

139. David, E., et al., *The Bone Niche of Chondrosarcoma: A Sanctuary for Drug Resistance, Tumour Growth and also a Source of New Therapeutic Targets.* Sarcoma, 2011. **2011**: p. 932451.

140. Ferretti, G., et al., *Zoledronic-acid-induced circulating level modifications of angiogenic factors, metalloproteinases and proinflammatory cytokines in metastatic breast cancer patients.* Oncology, 2005. **69**(1): p. 35-43.

141. Garrett, I.R. and G.R. Mundy, *The role of statins as potential targets for bone formation.* Arthritis Res, 2002. **4**(4): p. 237-40.

142. Thrailkill, K.M., et al., *Physiological matrix metalloproteinase concentrations in serum during childhood and adolescence, using Luminex Multiplex technology.* Clin Chem Lab Med, 2005. **43**(12): p. 1392-9.

143. Kruger, A., et al., *Hydroxamate-type matrix metalloproteinase inhibitor batimastat promotes liver metastasis.* Cancer Res, 2001. **61**(4): p. 1272-5.

144. Egeblad, M., et al., *Type I collagen is a genetic modifier of matrix metalloproteinase 2 in murine skeletal development.* Dev Dyn, 2007. **236**(6): p. 1683-93.

145. Oatley, J.M., M.R. Avarbock, and R.L. Brinster, *Glial cell line-derived neurotrophic factor regulation of genes essential for self-renewal of mouse spermatogonial stem cells is dependent on Src family kinase signaling.* J Biol Chem, 2007. **282**(35): p. 25842-51.

146. National of Health, U.S. *ClinicalTrials.gov*. Available from: http://clinicaltrials.gov/.

147. Tiet, T.D., et al., *Constitutive hedgehog signaling in chondrosarcoma up-regulates tumor cell proliferation.* Am J Pathol, 2006. **168**(1): p. 321-30.

148. Schrage, Y.M., et al., *Aberrant heparan sulfate proteoglycan localization, despite normal exostosin, in central chondrosarcoma.* Am J Pathol, 2009. **174**(3): p. 979-88.

149. Kumar, C.a.R., *Basic Pathology*. 7 ed2003, Philadelphia, PA, USA: Saunders. 873.

SUPPLEMENTARY FIGURES AND TABLES

Tables

Supplemental Table A: Collagen classified by structural organization, adapted from Alberts *et al.*, 2002 [6] and Lodish *et al.*, 2004 [8].

COLLAGEN TYPES AND TISSUE LOCALIZATION

Structural Formation	Type	Tissue Types	Polymerized form
Fibrillar	I	Bone, skin, tendon, ligaments, internal organs	Fibril
	II	Cartilage	Fibril
	III	Skin, blood vessels, internal organs	Fibril
	V	Bone, skin, tendon, ligaments, internal organs	Fibril (with type I)
	XI	Cartilage	Fibril (with type II)
Fibril-associated	VI	Intertitial tissues	Associations with type I
	IX	Cartilage	Associations with type II
	XII	Tendons, ligaments	Associations with some type I
Network-forming	IV	Basal lamina	Sheet-like network
	VII	Beneath stratified squamous epithelia	Anchoring fibrils
Transmembrane	XIII XVII	Hemidesmosomes in the skin	Unknown
Other	XVIII	Basal lamina around the blood vessels	Unknown

Supplemental Table B: Matrix metalloproteinases and their targets. Taken from Stamenkovic, 2000 [45].

SUBSTRATES OF THE MEMBERS OF THE MATRIX METALLOPROTEINASE FAMILY

MMP#	Common name	Mr (kDa)	Substrates
1	interstitial collagenase, collagenase-1	52/43	collagen I, II, III, VII, X, IGFBP
2	gelatinase A	72/62	gelatin, collagen I, IV V, X, laminin, IGFBP latent TGF-β
3	stromelysin-1	52/43	collagen III, IV, V, IX X, gelatin, laminin proteoglycans, fibronectin, elastin, E-cadherin, IGFBP, HB-EGF, perlecan, proMMP-13
7	matrilysin	28/19	gelatin, fibronectin, laminin, collagen IV, proteoglycans FasL, HB-EGF, pro-MMP-1
8	neutrophil collagenase, collagenase-2	51/42	collagen I, II, III, VII, X
9	gelatinase B	92/83	gelatin, collagen I, IV,V, X, IGFBP, latent TGF-β
10	stromelysin-2	52/43	collagen III, IV, IX, X gelatin, proteoglycans laminin, pro-MMP-1 pro-MMP-13
11	stromelysin-3	51/44	α-1-antiprotease, IGFBP
12	metalloelastase	52/43	elastin, pro-MMP-13
13	collagenase-3	52/42	collagen I, II, III,IV,VII, X, XIV, tenascin, aggrecan, fibronectin, pro-MMP-9
14	MT1-MMP	64/54	gelatin, collagen I, fibrin, fibronectin, proteoglycans, laminin, pro-MMP-2. Pro-MMP-13
15	MT2-MMP	71/61	fibronectin, laminin, tenascin, pro-MMP-2 pro-MMP-13
16	MT3-MMP	66/56	collagen III, gelatin fibronectin, pro-MMP-2
17	MT4-MMP	54	unknown
18/19	RASI-1	55/47	unknown
20	Enamelysin	52/43	amelogenin

Supplemental Table C: Summary of ETV5 and MMP2 gene, mRNA and protein information. NCBI.

SUMMARY OF ETV5 AND MMP2 INFORMATION

Gene	ETV5	MMP2
Also Known As	ERM	Gelatinase A
Gene Name	ETS Variant 5	Matrix Metalloproteinase 2
Gene Location	3q28	16q13-q21
mRNA Size	1533	1833
# of amino acids	510	611
Protein Size	58kDa	72kDa
NCBI GENE ID	2119	4313
NCBI Gene accession number	NM_004454.2	NM_004530.4

Supplemental Table D: Substrates of matrix metalloproteinase 2 [45, 48, 61].

LIST OF MMP2 SUBSTRATES

α1-AC	Elastin	IGFBP	Osteonectin
α1-PI	Fibrillin	IL-1β	Plasminogen
C1p	Fibrin	Laminin	pro-TGFα
Collagen I, III, IV	Fibrinogen	Inactive TGFβ	Substance P
Collagen VII, X, XI	Fibronectin	Link protein	Tenascin
Decorin	Gelatin	Myelin	Vitronectin

Supplemental Table E: Primary chondrosarcoma (CS) patient records. "**+**" dictates the cells are positive for collagen 2A1, supporting the diagnosis of chondrosarcoma.

PRIMARY CHONDROSARCOMA CELL LINE PATIENT DEMOGRAPHICS

	Gender	Age of Biopsy	Location of Tumor	Grade	Type	Other Information	Collagen 2A1 Expression
PT1	M	55	Proximal left femur	2	Chondrosarcoma	Pain	+
PT2	F	50	Left iliac wing	1	Enchondroma / Chondrosarcoma	On and off pain, long standing history of osteochondroma of left iliac wing, but it underwent malignant transformation to low grade chondrosarcoma	+
PT3	M	41	Right hand, 5th finger	1	Chondrosarcoma	Patient has Ollier's deisease	+
PT4	M	80	Right groin	2	Chondrosarcoma	Pain	+

Supplemental Table F: Primer sequences for real-time polymerase chain reaction and cloning with amplicon size and melting temperature.

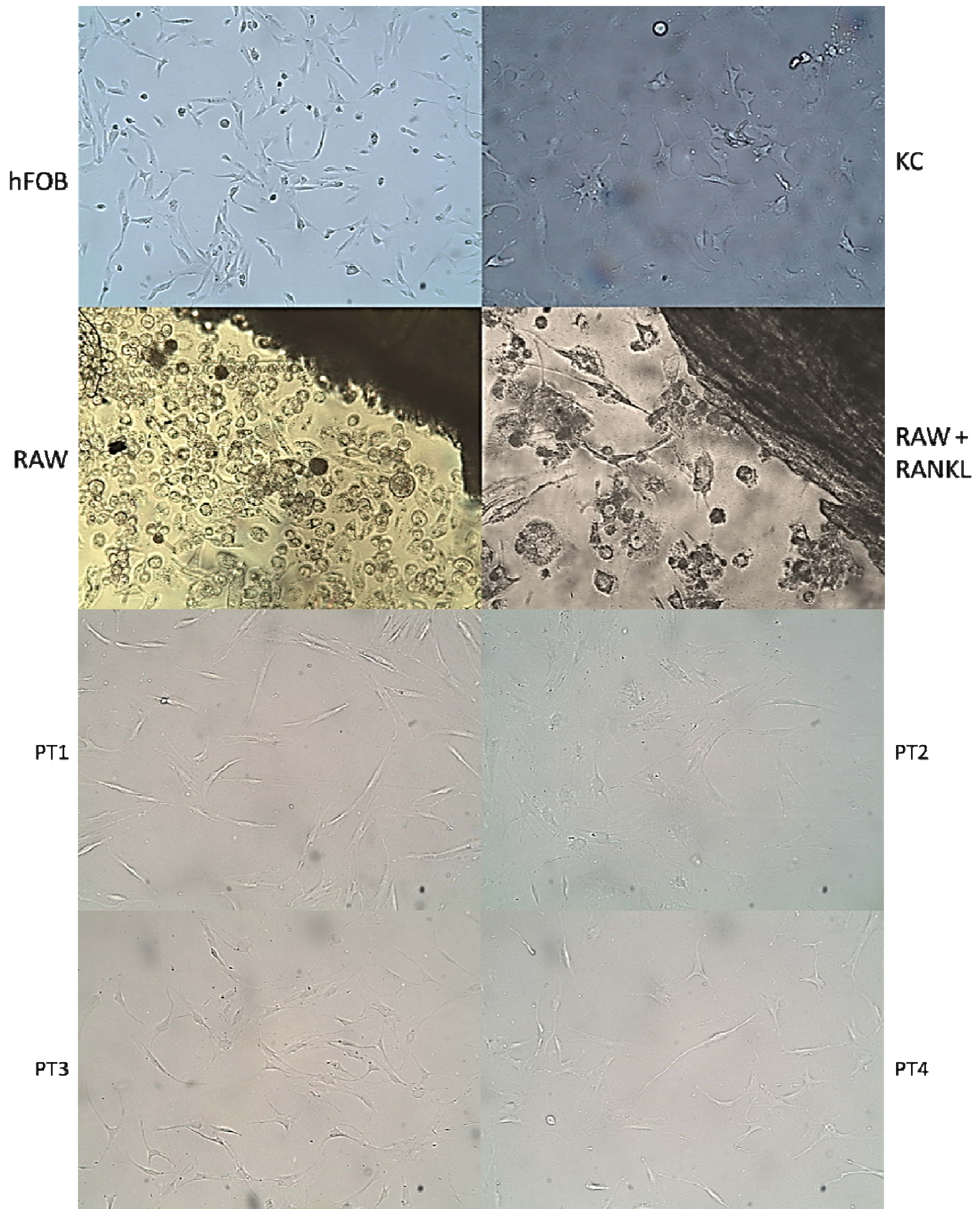
PRIMERS FOR REAL-TIME POLYMERASE CHAIN REACTION AND CLONING

Gene	Sequences	Amplicon Size (bp)	Tm (°C)
β-ACTIN	F GGTCATCACCATTGGCAATG R GGTAGTTTCGTGGATGCCACA	97	66.2 66.9
ETV5	F TCTGAGCTGTCGTCTTGTAGCC R GTTATTGGCTTGAACCCAGAGG	116	58.6 55.9
MMP2	F ACATCAAGGGCATTCAGGAG R CTGAGCGATGCCATCAAATA	131	64.0 63.7
GAPDH	F CATGAGAAGTATGACAACAGCCT R AGTCCTTCCACGATACCAAAGT	113	62.0 62.5
COL1A2	F CTGGAGAGGCTGGTACTGCT R CACCAGCAACACCTGGTAGA	145	63.5 63.8
COL2A1	F CCTGGACGAGAGGTGAGCAGT R TCTCTCCCCGACAACCTTCCC	181	60.9 60.8
Cloning Oligos	F ATGGACGGGTTTTATGATCAGCAAG R TTAGTAAGCAAAGCCTTAGGCATA		

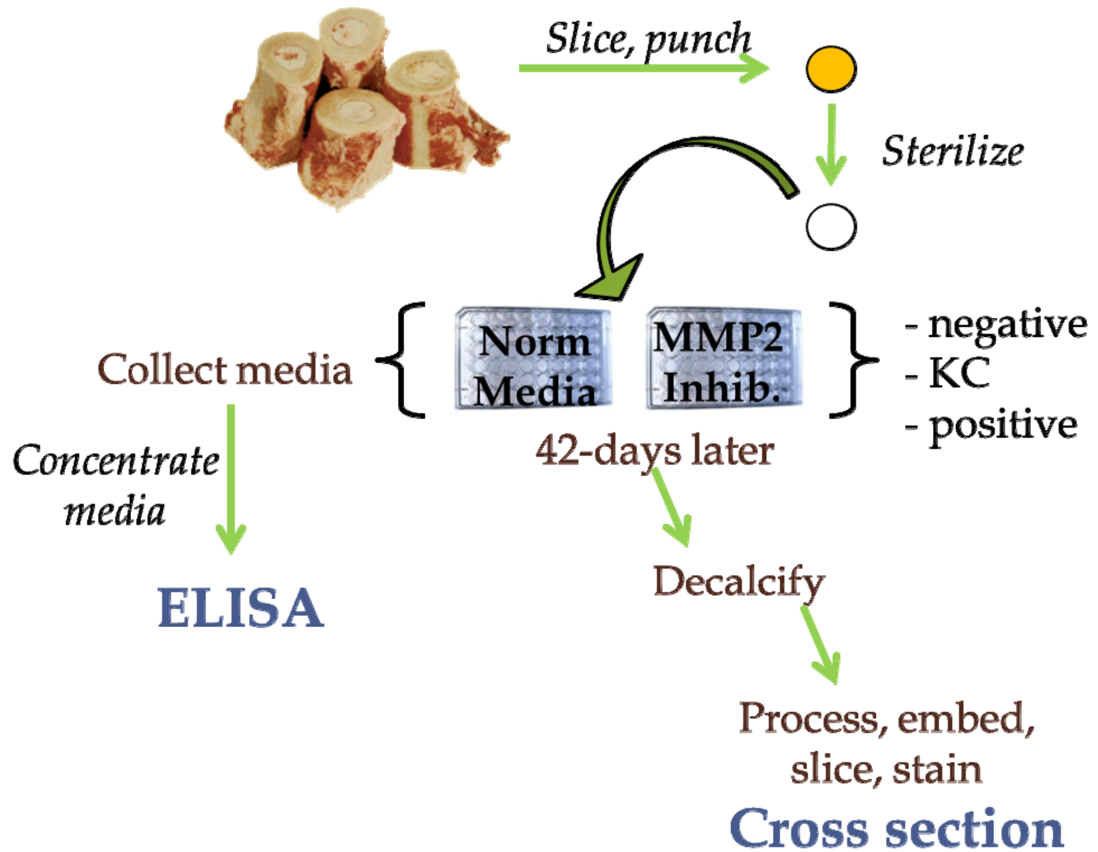
Figures

aaactttcttctccagtgcctcttgctgtttttcatctctgggccattgtcaatgttccctaaaa
cattccccatattcccc**acccagcactccacctcttt**agctcttcaggtctcagctcaga
MMP-2 P3F →
agtcacttcttccaggaagccttccttgattgtctttactagtttaggggctgaagtcaggc
Ets-1
gttcccaacagcctgctggagttccccatcacagcttatctctcaactgtctttcctgagag
agggagaagacattcctcagagacggttgtcacagggagaacttcaaaattgggatt
PEA3
cgacctgagaggccacatggattcttggcttggcgcaggaaaggattcaagagtgagt
ggggaattcgtggaactgagggctcctcccctttttagaccatatagggtaaacctcccc
acattgccatggcatttatacactgccatggc**actggtgggtgcttcctttta**acatgcta
ATGcat **← MMP-2 P3R**
→MMP-2 **PEA3**

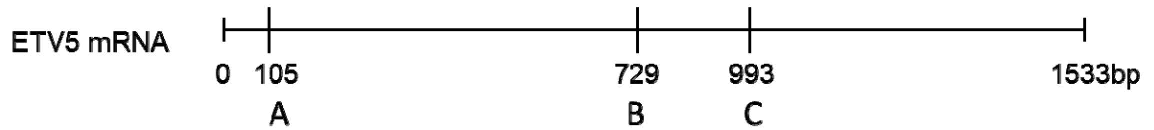
Supplemental Figure A: Portion of *MMP2* promoter region highlighting the PEA3 TF binding sites, taken from Monge *et al*., 2007 [60].



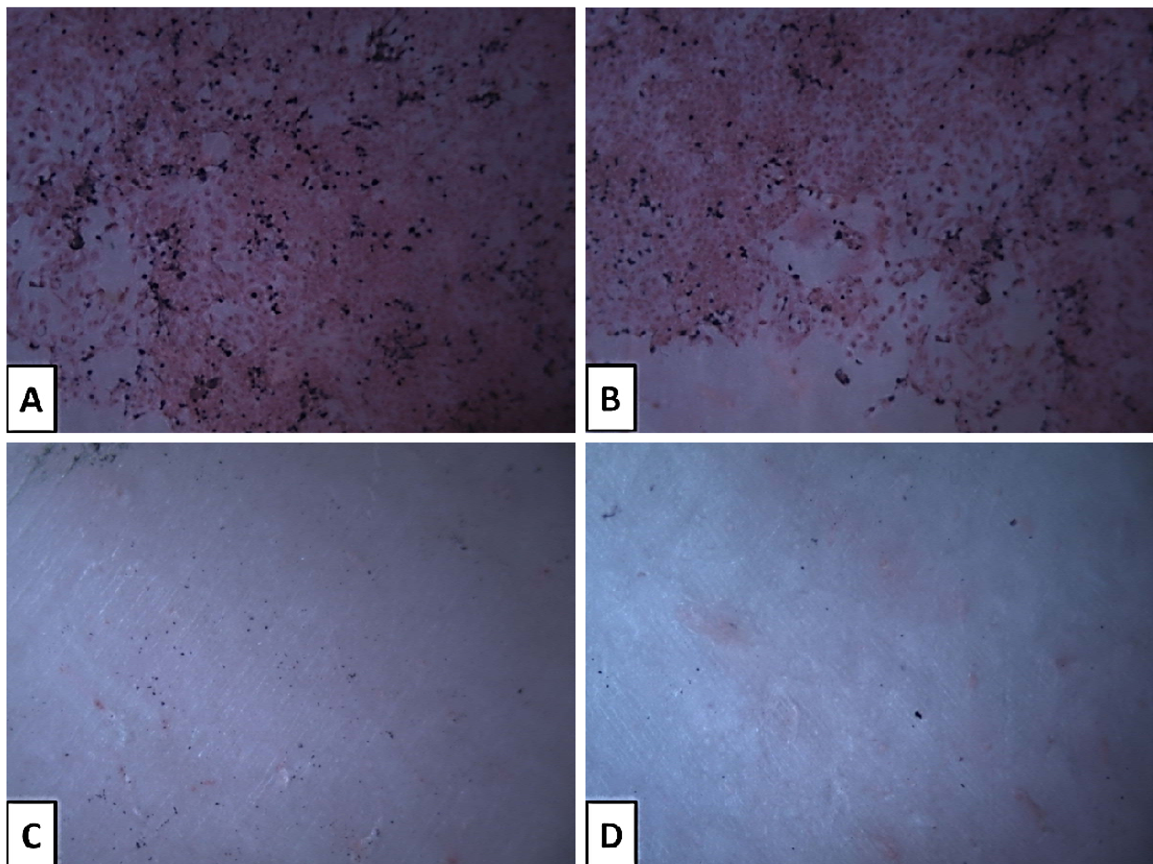
Supplemental Figure B: Images of hFOB, KC, RAW and RAW+RANKL (aside bone chips) and PT1-4 cell line images (100x).



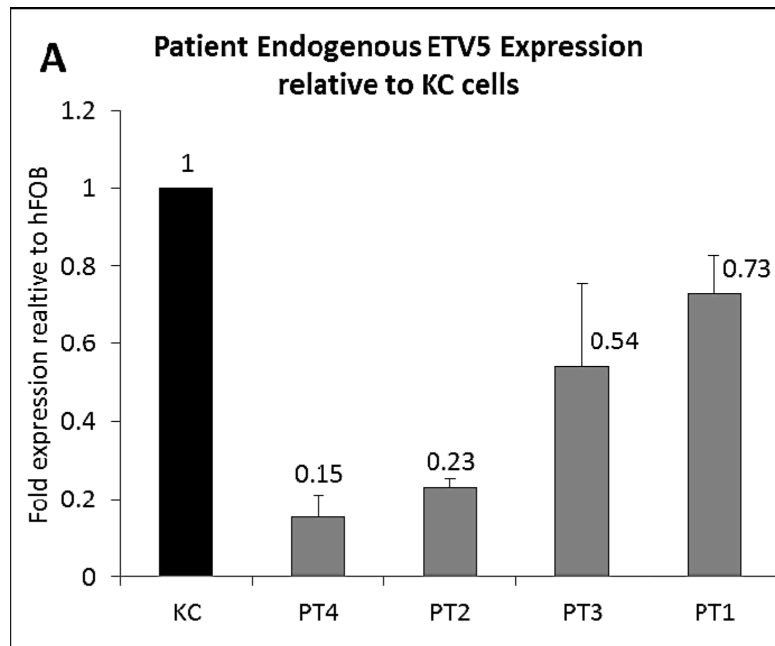
Supplemental Figure C: Bone chip methodology diagram. See section **3.10** for detailed protocol.

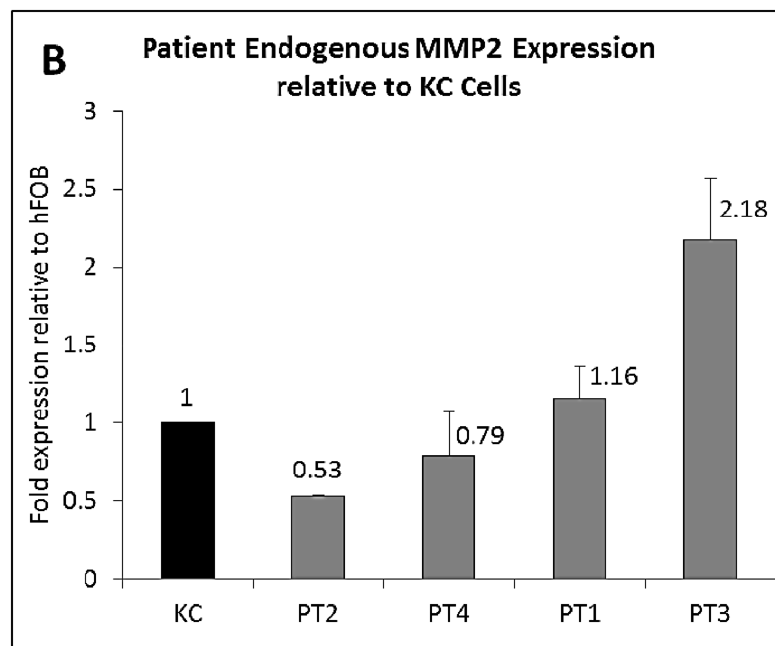


Supplemental Figure D: siRNA binding sites (A, B, C) on the *ETV5* mRNA.



Supplemental Figure E: Bone chip images after one week of incubation with **(A)** KC cells in normal media, **(B)** KC cells with MMP2 inhibitor in the media, **(C)** normal media without cells or **(D)** media with MMP2 inhibitor without cells.





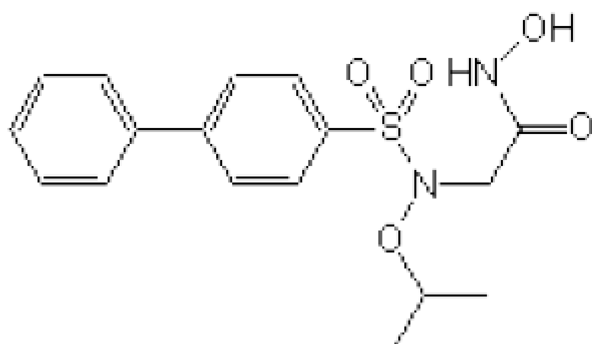
Supplemental Figure F: Patient *ETV5* and *MMP2* endogenous expression levels relative to KC cells (as highlighted in black), after normalization to β-actin (n=3).

Product Name: MMP-2 Inhibitor III, CalBiochem

Chemical Name:

2-((Isopropoxy)-(1,1′ -biphenyl-4-ylsulfonyl)-amino))-N-hydroxyacetamide

Chemical Structure:



Supplemental Figure G: Chemical information of the MMP2 inhibitor.