INVESTIGATION OF THE ROLE OF HACE1 IN OSTEOSARCOMA

by

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ABSTRACT

Osteosarcoma is a malignancy of childhood that is characterized by extensive genomic disruption within the tumour cells. It is proposed to have a close relationship with normal osteoblast development. *HACE1* is a gene located at 6q21 in humans that has been shown to be a potential tumour suppressor in a wide range of tumours. Disruption or loss of 6q21 is relatively common in osteosarcomas, and mice that are *Hace1*$^{-/-}$ and *p53*$^{+/-}$ develop osteosarcomas, amongst other tumour types, while those that are solely *p53*$^{+/-}$ do not.

Immunohistochemistry revealed that a number of osteosarcomas exhibit low expression of HACE1 protein, and where expression is low the protein is restricted to the cytoplasm, while in normal osteoblasts and high-expressing osteosarcomas the expression is nuclear and cytoplasmic. FISH results showed reduced 6q21 copy number in 45% of cases in one series, and in a second series one case out of 16 possessed a disruption in the 6q21 region.

To investigate *HACE1*'s role in osteosarcoma further we developed a novel model for human osteoblasts by harvesting and culturing cells from discarded bone taken as graft during adolescent scoliosis surgery. Comparing the expression of *HACE1* in these osteoblastic cells to osteosarcoma cells showed reduced levels of expression in osteosarcoma cells using qRT-PCR, but not by western blot analysis.

Re-expression of functionally normal *HACE1* in osteosarcoma cells using a lentiviral system significantly altered their behaviour in soft agar assays, Matrigel assays and

produced larger subcutaneous tumours in immunodeficient mice. We conclude that *HACE1* has a role in osteosarcoma as a growth regulator, and possibly as a tumour suppressor.

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LIST OF ABBREVIATIONS

Every effort has been made to minimize the use of abbreviations, and to use the most standard abbreviations where possible. The following abbreviations have been used in this manuscript.

ALP	Alkaline Phosphatase
ATCC	American Type Tissue Collection
BLAT	BLAST-like alignment tool
BMP	Bone Morphogenic Protein
BSP	Bone Specific Protein
CDKI	Cyclin Dependent Kinase Inhibitor
cDNA	Complementary DNA
CGH	Comparative Genomic Hybridisation
COL1A1	Collagen type 1, subtype A1
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethylsulfoxide
dNTP	Deoxyribonucleotide Triphosphate
DNA	Deoxyribonucleic Acid
FBS	Fetal Bovine Serum
FISH	Fluorescent In Situ Hybridisation
H&E	Hematoxylin and Eosin
HACE1	Hect-domain and Ankyrin-repeat Containing Enzyme1

kDa	KiloDalton
LOH	Loss of Heterozygosity
MEM	Modified Eagle's Medium
MSC	Mesenchymal Stem Cell
mRNA	Messenger RNA
NCI	National Cancer Institute
OCN	Osteocalcin
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline with Tween-20
qRT-PCR	Quantitative Reverse Transcription Polymerase Chain Reaction
RANK	Receptor Activator of Nuclear factor κ-β
RANKL	RANK Ligand
Rb	Retinoblastoma
RNA	Ribonucleic Acid
RT-PCR	Reverse Transcription Polymerase Chain Reaction
TP53	Tumour Protein 53
UCSC	University of California, Santa Cruz

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Chapter 1: INTRODUCTION

1.1 Osteosarcoma

Osteosarcoma is a malignant bone tumour that affects between 4 and 5 people per million of population per year, and although it is the most common bone or soft tissue malignancy of childhood[1,2] it is relatively rare overall, accounting for only 2% of all cancers[3].

It is predominantly a disease of adolescence and young adulthood, with 60% of patients aged under 25 years at diagnosis; however there is a second peak of incidence in later life, with 30% of patients being over 40 years of age[4]. Some authors report higher incidences in boys and African Americans[5,6]. The most common sites in young adults are the areas where most rapid bone growth occurs, such as the distal femur and proximal tibia[3,5,6].

There are several sub-types of osteosarcoma described, which have in common the production of osteoid by the malignant cells and the potential for systemic metastasis[6,7]. Conventional osteosarcoma, by far the most common sub-type of osteosarcoma, represents 70% of all osteosarcomas[7]. Conventional osteosarcoma is typically a high grade tumour showing anaplastic and pleomorphic characteristics. Besides conventional osteosarcoma, other subtypes include parosteal osteosarcoma, periosteal osteosarcoma and telangiectatic osteosarcoma, amongst

others[8], forming a clinically diverse group of diseases including some of low malignant potential and more of high malignant potential.

The extracellular matrix produced by osteosarcoma cells may include a mixture of osseous, chondroid and/or fibrous tissue. However, the diagnosis of an osteosarcoma is made on tissue biopsy and requires the identification of malignant osteoid, an amorphous extracellular material. Normal osteoid is secreted by osteoblasts to become the mineral component of normal bone, while malignant osteoid is secreted by the tumour cells, is poorly organized, and described as being 'lace-like'[7,9]. The extracellular matrix produced by the tumour cells may be a mixture of osseous, chondroid and/or fibrous tissue. Although tumours can be described according to the predominant form of matrix (for example, "chondrogenic osteosarcoma"), these have identical treatment algorithms and prognosis[8].

The typical clinical presentation is a combination of pain and swelling of the affected area, typically near the end of a long bone with the distal femur being the most commonly affected. Radiographs typically show a destructive lesion typified by the deposition of radio-opaque osteoid within the matrix of the lesion itself[10].

The central pillars of osteosarcoma management are chemotherapy and surgery. Surgery alone results in 20% survival, with adjuvant chemotherapy survival increases to over 60%[3,11,12]. The treatment plan is individualised for each patient, with the best results being seen when the patient is managed in a multidisciplinary care setting[13].

Chemotherapy regimes typically involve a combination of adriamycin, cisplatinum, ifosfamide or methotrexate[14,15].

Surgical resection should aim to achieve wide margins, and there has been an increase in the use of limb-sparing surgery over the past two decades due to the development of better reconstructive techniques[6,15]. A positive surgical margin is associated with higher rates of local recurrence and margins should not be compromised in the pursuit of function[16]. Radiation therapy has a role in the palliative setting[17].

1.2 Etiology and Molecular Pathology of Osteosarcoma

The etiology of osteosarcoma is poorly understood, and most cases are sporadic[18], but certain environmental and hereditary factors are associated with osteosarcoma, and this has helped in understanding the biology of the disease.

The most well-established environmental factor is ionising radiation, which is known to induce osteosarcoma both in therapeutic and non-therapeutic settings, [19]. Such patients have a similar prognosis to sporadic osteosarcoma[20,21]. Beryllium oxide has been shown to induce osteosarcoma in a rabbit model[22] but there is little other evidence to implicate chemical causes, and this compound is capable of inducing several different malignancies[23].

Certain hereditary syndromes are associated with osteosarcoma and these have contributed greatly to the understanding of osteosarcoma. These include Familial Retinoblastoma, Li Fraumeni syndrome, Rothmund Thomson syndrome, Blooms Syndrome and Werner syndrome[24]. In addition Paget's disease of bone may be inherited and is associated with an increased risk of osteosarcoma[25,26]. The rest of this section outlines the major molecular features associated with osteosarcoma in more detail.

1.2.1 Retinoblastoma

The retinoblastoma tumour suppressor (*RB1*) is the most well-characterized gene implicated in osteosarcoma. It is located at chromosome 13q14 and produces a 110kDa protein that negatively regulates progression of the cell cycle from G0/G1 to the S phase[27,28]. Loss of regulation of this pathway renders the cell unable to respond to anti-growth signals that would normally stop the cell cycle at this checkpoint. This insensitivity to anti-growth signals is one of the "hallmarks of cancer" outlined by Hanahan and Weinberg, the other hallmarks being self-sufficiency in growth signals, tissue invasion and metatasis, limitless replicative potential, sustained angiogenesis and evasion of apoptosis[29].

Patients with familial retinoblastoma have constituional loss of function of one copy of the retinoblastoma gene, and tumours arise in cells that sustain a second mutation, in accordance with the two-hit hypothesis proposed by Knudson for tumour suppressor genes[30]. Osteosarcoma is the second most common malignancy in patients with familial retinoblastoma, and these patients are 500 times more likely to develop osteosarcoma than the general population[31]. *RB1* has also been implicated in a wide range of other cancers[24].

As a result of the association of retinoblastoma and osteosarcoma, *RB1* has been investigated and shown to be important in sporadic osteosarcoma[31-34]. The mechanism is loss of heterozygosity (LOH) in 60% of cases, structural rearrangements in 30% and point mutations in 10% of cases[35]. LOH of the *RB1* locus

has been shown to be a poor prognostic factor and to occur in high grade osteosarcomas [34,36]. In osteosarcomas where there are no *RB1* alterations, other parts of the pathway such as *CDKN2A* are frequently disrupted[37].

1.2.2 Tumour protein 53, mouse double minute 2 and cyclin dependent kinase inhibitors

Li Fraumeni syndrome results from a germline mutation in the tumour protein 53 gene (*TP53)*. Patients with Li Fraumeni syndrome are predisposed to a number of malignancies, of which osteosarcoma is the second most common, occurring in up to 12% of patients[38]. Other tumours occurring in Li Fraumeni sydrome include leukemias, soft tissue sarcomas, and brain tumours[39]. Mutations in *TP53* are found in many human cancers[40].

Sporadic osteosarcomas also show alterations in *TP53*. Mechanisms of loss of *TP53* include allelic loss, point mutations and gene rearrangements[41,42]. Up to 60% of high grade osteosarcomas show *TP53* mutations, compared with 1% of low grade osteosarcomas[43,44]. However, the association between *TP53* status and prognosis is not clear[43].

TP53 is located at 17p13 and encodes a transcription factor, p53, that regulates genes involved in DNA repair, cell cycle control and apoptosis[45-47]. Following DNA damage p53 activation transcriptionally upregulates genes that halt the cell cycle, and if the damage is severe and p53 activation is prolonged, the cell will undergo apoptosis.

Other genes involved in the *TP53* pathway are also implicated in osteosarcoma, such as the mouse double minute 2 (*MDM2)* gene, located at 12q13. This gene negatively regulates the function of p53 by encoding a protein which is able to bind the p53 protein and block its transcriptional activities[46,48,49]. *MDM2* also has E3 ubiquitin ligase activity, and is able to direct the p53 protein for degradation[50].

MDM2 amplification reduces the function of *TP53* even when wild-type p53 protein is present[51,52], and amplification of *MDM2* been shown in as many as 17% of osteosarcomas[53,54]. Amplification of *MDM2* in osteosarcoma has been associated with metastasis and recurrent disease in several studies[44,54-56].

Cyclin dependent kinase inhibitors (CDKIs) are also implicated in this pathway. CDKIs inhibit progression through the cell cycle, and loss of these can lead to immortalisation[57]. *CDKN2A* at 9p21 encodes 2 major transcripts in different reading frames, $p14^{ARF}$ and $p16^{INK4A}$, which act by different mechanisms. The $p14^{ARF}$ transcript inhibits *MDM2* function by binding to the MDM2 protein and holding it within the nucleus so it cannot direct p53 for degradation. In this way deletion of *INK4A* has a similar effect to amplification of *MDM2*. Deletion at the *CDKN2A* locus has been shown in 10% of osteosarcomas[58]. The $p16^{INK4A}$ transcript causes arrest in the G1 phase of the cell cycle. Loss of expression of the $p16^{INK4A}$ transcript is associated with poorer outcomes in paediatric osteosarcoma[59].

1.2.3 RECQ helicases

Three syndromes with mutations affecting RECQ helicases are associated with osteosarcoma: Rothmund-Thomson syndrome, Werner syndrome and Bloom syndrome. All three show increased risk for osteosarcoma, as high as 32% in patients with Rothmund-Thomson syndrome[60]. Each of these syndromes has mutations affecting a different one of the five known RECQ helicases. RECQ helicases unwind DNA strands and are important in maintaining genetic stability by facilitating DNA repair[61]. However, the molecular biology of these syndromes is not yet fully understood[24].

1.2.4 Telomerase and telomeres

Telomeres are located at the ends of chromosomes, and are important structures for maintenance of genetic stability. In normal differentiated cells, telomeres shorten with each cell division, providing a limit to replication. In stem cells telomerase is expressed, which maintains telomere length despite cell division, an important requirement of immortality[62]. Tumour cells either express telomerase or utilize an alternate lengthening of telomere pathway (ALT) in order to become immortalized[63].

Telomerase expression and ALT have both been observed in osteosarcoma[64]. The ALT mechanism is associated with greater levels of genetic instability in osteosarcomas[65]. A subset of osteosarcomas show no evidence of telomerase expression or ALT, and these tumours seem to have a better prognosis[66].

1.2.5 Genetic instability in osteosarcoma

Bone and soft tissue sarcomas can be divided into two groups according to the nature of their chromosomal abnormalities: those with reciprocal translocations and balanced karyotypes such as synovial sarcoma or Ewing sarcoma, and those with complex rearrangements and disruption of p53 and Rb pathways such as osteosarcoma[5,7,19].

Analysis of osteosarcoma using standard cytogenetic techniques, comparative genetic hybridization (CGH) and array-based CGH has shown diverse findings, with multiple areas of loss, amplification and re-arrangement. This reflects genetic instability, which can result from increases in mutations due to poorly functioning DNA repair mechanisms or errors in chromosomal segregation during cell division leading to gross aneuploidy[24,67].

Some recurring patterns have been found amongst the complexity of the osteosarcoma genome. The most common abnormalities on standard cytogenetics are gain of chromsome 1, and loss of -9, -10, -13 and -17, with -6q partial or complete loss also reported[68]. Other studies have shown recurring loss of 5q, 6q, 10q, 11p, 15q, 16p and 22q[69].

CGH has shown 1p11-p13, 1q11-q12, 1q21-q22, 1q21-22, 11p14-p15, 14p11-p13, 15p11-p13, 17p and 19q13 to be the most common areas showing structural changes[55,68].

Array-based CGH has also shown multiple areas of loss or gain. Tarkkanen *et al* reported the most common copy number increases were at 1q21 (58%), 8q21.3-q22 (52%) and 8cen-q13 (45%), with copy number increases also at 14q24-qter (35%) and Xp11.2-p21 (35%) and the most common losses were at 6q16 (32%) and 6q21-q22 (32%)[70].

High density cDNA microarray CGH also shows 8q amplification, including the *MYC* oncogene and 17p amplification, including several genes involved in osteogenic morphogenesis, while three out of 9 showed loss of 6q21[71]. Spectral analysis showed 531 rearrangements and 300 breakpoints[72], demonstrating the complexity of the genetic changes in osteosarcoma.

1.2.6 Other genetic abnormalities in osteosarcoma

MYC is a proto-oncogene at 8q24 that regulates cell growth and DNA replication. Amplification of 8q is common[73,74], and using micro-array based CGH amplification of 8q24 was seen in 7 out of 9 osteosarcomas[71]. Expression levels of *MYC* are elevated in up to 42% of osteosarcomas[75]. Inactivation of *MYC* in a mouse model that develops osteosarcomas resulted in regression of the osteosarcomas[76].

Other oncogenes implicated in osteosarcoma include *FOS, MAPK7, PMP22/GAS3* and *TSPAN31/SAS*[24,53,77-80].

The Wnt family of proteins is involved in development and embryogenesis, are expressed on osteoprogenitors[81], and are implicated in many cancers. β-catenin (an integral part of the Wnt signaling pathway) is either overexpressed or localized to the nucleus in many osteosarcomas[82]. *TGF-β/BMP* signaling is also involved in osteogenesis and implicated in osteosarcoma.

1.3 Normal Osteoblastic Differentiation

Osteoblasts are defined by the ability to secrete osteoid, a substance which matures into the mineral component of bone. In conjunction with osteoclasts, which are cells capable of resorbing bone, osteoblasts are central to the production and homeostasis of the skeleton and calcium and phosphate metabolism. Their development and maturation is quite well understood, with excellent reviews by Aubin[83] and Thomas[84].

Mesenchymal stem cells (MSCs) are capable of differentiating into a number of different cell types including osteoblasts, chondrocytes, adipocytes, fibroblasts and myoblasts. Under the influence of Wnts, bone morphogenic proteins (BMPs) and parathyroid hormone, MSCs undergo a key transition to become osteoprogenitors[85]. These cells rapidly proliferate, but unlike MSCs they do not have unlimited self-renewal (Figure 1).

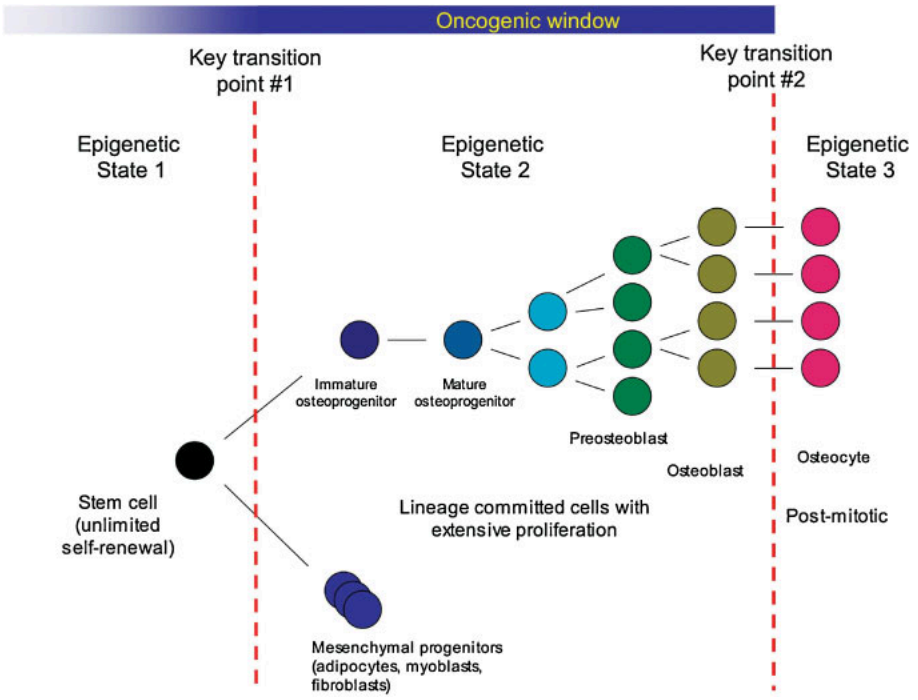


Figure 1. Model of osteoblast differentiation and tumorigenesis.

This diagram shows the three compartments of osteoblastic differentiation, and the two key transition zones between them. During the first transition the cells become committed to osteoblastic differentiation, and lose some of their stem-like capabilities, during the second transition they undergoes terminal differentiation. The oncogenic window shows the stages during which events can occur that allow for tumorigenesis. Reproduced with permission from Thomas et al[84].

Pre-osteoblasts are an intermediate phase between the population expansion of osteoprogenitors and mature osteoblasts. Pre-osteoblasts are not able to self-renew and are characterized by the expression of STRO-1, alkaline phosphatase (ALP), parathyroid hormone receptor and type 1 collagen[86]. The progression to mature osteoblasts is promoted by BMPs and dexamethasone, and inhibited by parathyroid hormone[83]. Mature osteoblasts are plump cells that line the new osteoid seams within bone, and are the cells responsible for bone matrix production. They have some replicative potential, and express ALP, osteocalcin, collagen type 1, bone sialoprotein (BSP) and osteopontin.

The second key transition is from mature osteoblasts to osteocytes, which are cells embedded within the bone of uncertain function. However, not all osteoblasts appear to terminally differentiate into osteocytes, some mature osteoblasts eventually undergo apoptosis without further differentiation[84].

An interesting consequence of this model is that, unlike in most tissues, it is not the terminally differentiated cells that are responsible for tissue production and homeostasis, but rather the late intermediate stage of the mature osteoblast. Changing the number of cells or activity of the cells located between the first transition of MSC to osteprogenitor and the second transition from osteoblast to osteocyte increases or decreases bone mass. Thus the control of these two key transitions is central to regulation of bone formation[87-89].

The key transcriptional regulator of osteoblastic differentiation is *RUNX2* (also known as CBFA1, Osf2, PEBP2a), which belongs to the runt family of transcription factors[83,84,90]. *Runx2* nullizygous mice form a chondrogenic phenotype, with no ossification and live only a short time after birth. *RUNX2* forms a transcriptional 'hub' for multiple incoming signals to direct osteoblastic differentiation, reviewed in detail by Lian et al[91].

RUNX2 appears to be responsible for inhibiting osteoblast proliferation and promoting terminal differentiation into osteocytes, and its expression levels probably vary with the cell cycle[92]. RUNX2 protein activity is frequently modified by other proteins[93]. One of the most interesting RUNX2 interactions is its co-activation by retinoblastoma protein pRb to facilitate terminal differentiation of osteoblasts, the second key transition discussed above[94]. pRb also plays other roles in osteogenic differentiation, and is implicated in osteosarcoma (discussed above).

1.4 Osteosarcoma as a Disorder of Osteoblastic Differentiation

Over the past few years the concept that tumorigenesis in osteosarcoma can be understood as a disorder of osteogenic differentiation has been expressed by several authors[3,84,95]. That osteosarcomas are related to osteoblasts is reflected in their similarities in cell markers and cellular pathways, as well as the deposition of osteoid that is required for a diagnosis of osteosarcoma; the only normal cell that also deposits osteoid is the osteoblast[96-98].

Tumorigenesis is likely related to the disruption of the normal processes that restrict the limitless replicative ability of stem cells as they become committed to a lineage, the first transition outlined above. Cancer cells and stem cells have many similar characteristics[99], and cells with stem-like characteristics have been demonstrated in osteosarcoma. These cells are able to grow in suspended culture and demonstrate markers of mesenchymal stem cells such as STRO-1, CD105 and CD44[100].

Disruption of the normal mechanisms responsible for terminal differentiation, the second transition, is probably also important. Terminal differentiation forces the cell out of the cell cycle, and the inability to do this leaves the cell in a variably differentiated state. If it has retained immortal stem-cell like abilities as well then it is exhibiting many of the characteristics of a malignant cell.

There is evidence that terminal differentiation is disrupted in osteosarcoma. As noted above, terminal differentiation is mediated by *RUNX2*, which causes the expression

of *p27*^{KIP1}, ultimately resulting in the dephosphorylation of pRb, and G1 cell cycle arrest. Loss of *p27*^{KIP1} is associated with dedifferentiation in high grade osteosarcomas[101].

In this model, tumorigenic events can occur any time prior to terminal differentiation (Figure 1). It is proposed that the closer the cell is to terminal differentiation, the more differentiated the final tumour. It also proposed that epigenetic changes may be partially responsible for the defective transitions from stem cell to pre-osteoblast and osteoblast to osteocyte[84]. The polycomb group of proteins are important in assigning epigenetic states through histone modifications[102]. One polycomb protein, Bmi-1, may play a role in the immortalisation of mesenchymal stem cells[103], however, overall there remains significant work to be done to understand the mechanisms controlling the key transition points in osteoblast differentiation and their relationship to osteosarcoma tumorigenesis.

1.5 HACE1

1.5.1 Identification and characterization of HACE1

HACE1 (HECT domain and ankyrin repeat-containing E3 ubiquitin-protein ligase 1) was originally identified in a Wilm's tumour in our lab, and reported by Anglesio et al in 2004[104]. The index patient, a 5 month old male with a Wilm's tumour of the kidney, was noted to have a non-constitutional chromosomal re-arrangement t(6;15)(q21;q21). 6q21 rearrangements had previously been described in Wilm's tumour, and 6q21 deletions are described in other tumours. This led the group to perform a more detailed analysis of the 6q21 region.

The position of the breakpoint in the index case was identified using polymorphic markers followed by Southern blotting. Using public sequence databases the group was able to identify a 2727bp open reading frame (ORF) approximately 50kb downstream from the breakpoint. An expressed sequence tag was identified, and the cDNA clone was obtained and then sequenced. Using the UCSC genome browser BLAT tool (http://genome.ucsc.edu/cgi-bin/hgBlat) they were able to predict that this ORF was organized into 24 exons.

Protein domain analysis was performed, revealing that a 6q21 ORF encoded a 909 amino acid protein (approximately 103 kDa) with six N-terminal ankyrin repeats and a C-terminal HECT domain. Ankyrin repeats are known to be involved in protein-protein interactions[105]. HECT is a domain with catalytic ability, in particular it is involved in ubiquitin-protein ligase activity[106]. *HACE1* was the first HECT family E3 ligase to be

associated with ankyrin repeats, although a second, *HECTD1* has now been described and implicated in neural tube closure[107].

Using the BLAT tool at UCSC, *HACE1* orthologues were found only in vertebrates. Other HECT domains are found in a wider variety of organisms, including yeasts. The *HACE1* HECT domain appears highly conserved across species, and also shows significant sequence similarity with other well characterized E3 ubiquitin ligases. HECT family E3 ligases are characterized by a consistent C-terminal cysteine residue which is required for thioester bond formation with ubiquitin. In *HACE1* this is represented by Cys-876.

1.5.2 Ubiquitylation and ubiquitin ligases

Ubiquitylation is the modification of a target protein by the addition of ubiquitin, a 76 amino acid protein. The ubiquitylation signal can lead a protein to a number of different fates, including proteasomal degradation, endocytosis and changes in subcellular localization[106].

The ubiquitylation system is largely involved with the metabolism of damaged or misfolded proteins, by marking these proteins for degradation within the proteasome. However, as ubiquitylation can control the levels of protein in the cell, and the subcellular localization of those proteins, it can also influence signaling within the cell.

There are three classes of enzyme that can help to transfer the ubiquitin substrate to a target protein, and were recently reviewed by Rotin et al[106]. E1 are ubiquitin-

activating enzymes, E2 are ubiquitin-conjugating enzymes and E3 are ubiquitin-protein ligases. The E3s are primarily responsible for the specificity of the ubiquitylation system, and as such there over 600 E3 ubiquitin ligases encoded in the human genome, with only 2 potential E1s and approximately 30 E2s[106].

E3 ubiquitin ligases transfer ubiquitin to one or more lysine residues in the target protein, either alone or in association with other proteins. Ubiquitylation can result in chains of ubiquitin linked to each other by successive ubiquitylation events in which the ubiquitin protein itself is ubiquitylated (polyubiquitination). The pattern of successive ubiquitylation events can determine the fate of the target protein; for example if the successive ubiquitin chains are directed at the Lys48 residue in ubiquitin, the protein is usually targeted for preoteasomal degradation.

The majority of E3 ubiquitin ligases are divided into RING and HECT types. RING types are the most common and act as scaffolds to bring E2 enzymes near the substrate, the E2 then performs the ubiquitylation. HECT types are much less common, representing approximately 5% of all E3 ubiquitin ligases. It is worth noting that downstream functions of most of these enzymes remains poorly understood as the identification of the specific substrate for E3 ligases is notoriously difficult.

The HECT ubiquitin ligases are named for the HECT domain of approximately 350 amino acids that occupies the carboxy terminus of the enzyme. At the amino terminus of the protein is a variable domain that determines the protein-protein or protein-lipid interactions of the enzyme. HECT ligases can be classified according to

their N-terminal domain features into three main groups. The first two are the Nedd4 family and the HERC family, and the last is a miscellaneous group of other enzymes. This last group includes *HACE1*, which along with *HECTD1* contains ankyrin repeats at the amino terminus[106].

1.5.3 HACE1 shows characteristics of a tumour suppressor gene

Tumour suppressor genes are genes which, when present and functioning, limit tumour growth. When their function is absent or reduced this control over tumour growth is lifted and tumours are able to be initiated or proliferate. Oncogenes exhibit their effects by being present, tumour suppressor genes exhibit their effects by being absent or non-functional[108].

Loss of function of a gene can occur at any one of multiple points. Epigenetic effects may result from methylation of CpG islands, silencing the gene in question. Mutations, truncations or loss of the gene can result in absent transcription of an mRNA product. Even if a protein is still produced, modifications to the structure may render the protein non-functional. These protein modifications may occur due to changes in amino acid sequence or in the post-translational modifications of the protein. If the protein is involved in complex interactions, then changes to other proteins may render the target protein effectively non-functional[108].

The level of expression of *HACE1* is reduced in many tumours compared to normal tissue. *HACE1* appears to be expressed in a wide range of normal tissues, although as the function of *HACE1* still remains to be determined it is difficult to be sure what a

normal level of expression is in any given cell at any state of differentiation. Anglesio et al showed that *HACE1* expression levels in Wilms tumour samples were decreased approximately 5 fold compared to patient-matched normal renal tissue when measured with quantitative RT-PCR[104]. This was confirmed in a second series in which 20 out of 26 Wilms tumours showed siginificant reductions in *HACE1* levels compared to normal kidney in terms of both mRNA expression and protein level[109].

cDNA arrays comprising paired tissue for a wide variety of tumour types were probed with full length *HACE1* cDNA and showed reduction in expression in multiple different tumour types[109]. The NCI60 panel of primary human cancer cell lines consists of 60 different tumours developed primarily for drug screening[110,111]. This panel was analysed by qRT-PCR for expression of *HACE1* mRNA, and this showed significant reductions in most cell lines compared to HEK293 cells (Figure 2) and published in Zhang et al[109]. HEK293 cells are derived from human embryonic kidney and reliably express *HACE1*[104].

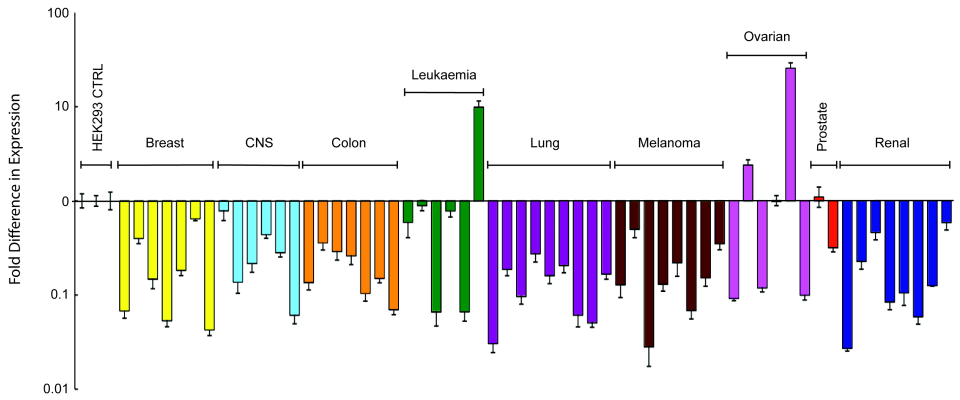


Figure 2. Relative expression of HACE1 mRNA in the NCI-60 panel of cell lines.

The NCI-60 panel of cell lines was obtained from the National Cancer Institute. RNA was isolated using Trizol® and qRT-PCR performed as outlined in the methods section. Expression differences are shown in fold difference relative to control HEK293 cells. Note the almost universal reduction in mRNA expression of *HACE1* across a broad spectrum of malignancies.

Loss of Heterozygosity in the region of 6q21 has been reported in multiple cancers, summarized in Zhang et al[109]. These tumours include ovarian carcinoma, non-Hodgkin's lymphoma, pancreatic carcinoma, prostate carcinoma and osteosarcoma[69].

1.5.4 HACE1 can be inactivated epigenetically

Genes are often inactivated due to mutation or deletion, but in the index case of the original paper describing *HACE1*, the chromosomal breakpoint did not occur through the *HACE1* gene itself. Anglesio went on to sequence the 6q21 region in a number of Wilm's tumours and was unable to demonstrate any mutations or deletions within the 24 *HACE1* exons[104].

Gene function can also be regulated epigenetically by methylation of CpG islands, which are regulatory structures that control access to the gene, regulate transcription, and are implicated in cancer[112]. Three CpG islands are associated with *HACE1*, lying proximal to gene itself. Using methylation-specific PCR, Anglesio et al were able to demonstrate that the CpG-177 island was more often hyper-methylated in tumour samples than normal tissue (73% v 35%), and that in more than half of those samples with CpG-177 hypermethylation, paired normal tissue showed complete absence of methylation[104].

Other groups have independently demonstrated lower levels of *HACE1* expression in human tumours, by looking for aberrant methylation. In 2008 Hibi et al used quantitative methylation specific PCR and found that 9 out of 32 primary colon

carcinomas exhibited aberrant methylation of the *HACE1* associated CpG islands[113]. Aberrant methylation was also associated with larger tumour size. In 2009 the same group found similar results with gastric carcinoma patients[114].

1.5.5 HACE1 in knockout mice

In order to further investigate the role of *HACE1* in normal physiology and oncogenesis knockout mice were created and reported on by Zhang et al[109]. *Hace1*$^{-/-}$ mice were born normal and fertile, but after a year they developed a range of tumours including lymphomas and angiosarcomas. Approximately 12% of *Hace1*$^{-/-}$ mice developed tumours compared to 1-2% of their *Hace1*$^{+/-}$ and *Hace1*$^{+/+}$ cohorts.

The relationship of *Hace1* loss to *p53* loss was investigated by creating double knockout mice. Addition of loss of one allele of *p53* to *Hace1*$^{-/-}$ mice greatly increased the number of tumours produced, and included osteosarcoma in 6 out of 69 total mice. When *p53*$^{-/-}$ mice were generated they predominantly developed thymic lymphomas but not osteosarcomas in this model. When these were crossed to produce mice which were *p53*$^{-/-}$ and *Hace1*$^{-/-}$ there was a much wider range of tumours produced, with several showing metastatic osteosarcoma amongst other sarcomas such as leiomyosarcoma and angiosarcoma and other various malignancies, which were seen with less frequency.

1.6 HACE1 in Osteosarcoma

The development of osteosarcoma in the transgenic mouse models suggested that *HACE1* may have an important but previously unreported role to play in osteosarcoma. We looked further into this by querying the Mitelman database (http://cgap.nci.nih.gov/Chromosomes/Mitelman) for recurrent genetic aberrations at 6q21 in osteosarcoma, which generated five cases. Searching for numerical aberrations in chromosome 6 in osteosarcoma showed 10 cases with increases in chromosome 6, and 24 cases with losses of chromosome 6.

Searching the literature revealed several studies implicating the 6q21 region as being deleted in osteosarcomas, as referred to above under the molecular biology of osteosarcoma section. Bridge reported cases with -6q partial or complete loss in some osteosarcomas on cytogenetic analysis, along with gain of +1, and loss of -9, -10, -13[68]. Other studies have implicated loss of 5q, 6q, 10q, 11p, 15q, 16p and 22q[69].

Tarkannen[70] examined 31 high-grade osteosarcomas and found losses affecting 6q21-q22 in 32%, which were the most frequent losses along with 6q16, which also showed loss in 32% of specimens. Fletcher et al[9] characterized the chromosomal abnormalities of 17 high-grade osteosarcomas, and while they found the karyotypes to be quite disrupted they reported “potential nonrandom deletions involved 6q21→qter, 9p21→pter, chromosome 10, chromosome 13, 17p12-pter, and chromosome 20”. Using high density cDNA microarray Squire et al showed loss of 6q21 in 3 out of 9 osteosarcoma cases[71].

In order to find microarray data on *HACE1* expression we queried the GEO database (http://www.ncbi.nlm.nih.gov/geo/) and identified two relevant datasets. The first set (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE14827) examined 27 osteosarcoma samples and showed low expression levels for *HACE1* compared to other genes using HG-U133 plus arrays. However, the only samples included were osteosarcomas, there was no comparison to normal controls.

A second dataset (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE12865) only had a single control sample and 6 osteosarcoma samples on Gene ST 1.0 arrays. Although this set showed high expression of *HACE1* in the tumour samples relative to the control, the presence of such small numbers prevents any firm conclusions.

The oncomine database was also queried (http://www.oncomine.org). This showed reduction in *HACE1* expression in tumour samples compared to normal samples in many datasets for tumours other than osteosarcoma. However, the sole osteosarcoma set had only three tumour samples, and compared these to other sarcomas, rather than normal samples. Nevertheless, they showed reduced expression of *HACE1*, ranking it in the 3% most underexpressed genes, and generated a p value of 0.004. However, it is not possible to draw firm conclusion from this alone with such a small sample of a tumour that is known for its cytogenetic heterogeneity.

Microarray analysis was also performed on a group of osteosarcoma patients by Dr Andrulis, University of Toronto, who compared gene expression profiles between patients presenting with metastases and those presenting without metastases, this has been presented at the Connective Tissue Oncology Society Meeting in 2007[115]. We asked Dr Andrulis' team to analyse their results specifically looking for *HACE1* expression levels, which they very kindly did. Overall *HACE1* did not show low levels of expression, of 63 patients only four had reduced levels of mRNA expression, 6 showed high expression and the rest were neutral. There was no difference in *HACE1* expression between the patients presenting with metastases and those presenting without metastases.

Although what we found was somewhat conflicting, we felt that overall, given the findings of increased frequency of osteosarcomas in the mouse models and the cytogenetic frequency of involvement of the 6q21 region that there was enough evidence to warrant further investigation of the potential role of *HACE1* in osteosarcoma.

1.7 Hypothesis and Aims

Our overall hypothesis is that *HACE1* plays a role in the biology of osteosarcoma. From this we generated the follwing specific hypotheses:

a) *HACE1* is differentially expressed in osteosarcoma cells compared with normal osteoblasts, and that these differences in expression may be driven by chromosomal changes, rearrangements or deletions, or at post-transcriptional or post-translational levels, and

b) Re-introduction of *HACE1* into osteosarcoma cell lines will abrogate their transformed phenotype, and

c) Human osteosarcoma samples will show absence or low levels of the HACE1 protein.

In order to test these hypotheses we set up the following aims:

1. Examine *HACE1* levels from DNA to protein in osteosarcoma cell lines and tissues.
2. Establish normal osteoblast cell culture model for comparision with osteosarcoma cell lines.
3. Examine the biologic effects of *HACE1* expression in osteosarcoma cell lines.

Chapter 2: MATERIALS AND METHODS

2.1 Immunohistochemistry for HACE1 in Osteosarcoma

2.1.1 Immunohistochemistry technique

Developing an effective protocol for immunohistochemistry was problematic. Firstly there was significant difficulty obtaining an effective antibody. HACE1 protein shares epitopes with numerous other proteins within the cell: neither the HECT domain nor the Ankyrin repeats are unique to HACE1. Multiple different antibody sources for immunohistochemistry and western blot were tried with varying degrees of success. As there are only a small number of researchers working with HACE1, only small amounts of antibody are produced at a time.

Secondly, osteosarcoma specimens by definition contain bone, or at least some quotient of abnormal osteoid. In order to facilitate cutting the specimen and slide preparation, samples are typically subjected to a decalcification process involving exposure to acid for varying amounts of time. This process alters epitopes on proteins within the specimen, potentially rendering them unrecognizable by an antibody that works perfectly well for other applications such as western blot.

This combination of difficulties with an unreliable antibody detecting an unreliable target antigen clearly makes for a difficult immunohistochemistry challenge, beyond the expertise of this author. The immunohistochemistry was undertaken by Joan Mathers within our lab and her persistence in getting this technique to work under

these difficult conditions is deeply appreciated. The final protocol for the detection of HACE1 Antibodies in FFPE using DAKO Envision+ Kit is summarized as follows.

1. De-paraffinization of slides. The slides were placed in oven at 60°C for 60 minutes and then de-waxed in two changes of 100% xylene for 5 minutes each. The slides were then re-hydrated by sequential exposure to 100%, 90% then 70% ethanol for 5 minutes each and rinsed twice in dH_2O.

2. Antigen Retrieval Process. The slides were placed in a coplin jar containing citrate buffer at a pH of 6.0 warmed to 95°C and placed in a steamer for 30 minutes. After allowing them to cool for 20 minutes they were washed 3 times in PBS.

3. Block Endogenous Peroxidase Activity. The slides were then incubated in a 3% hydrogen peroxide solution for 10 minutes at room temperature and then rinsed 3 times in PBS, for a total of 10 minutes.

4. Immunostaining. Using an ImmEdge pen, a hydrophobic barrier around the tissue was created and a bubble of DAKO serum-free protein block placed over the tissue. After incubation for 30 minutes at room temperature in a humidified container the excess protein block was drained and 20μl of primary antibody solution added as a bubble. A coverslip was applied, the edges sealed and the slides incubated overnight at 4°C.

The next morning, after allowing the slides to come to room temperature, they were rinsed in three changes of PBST, 200μl of secondary mouse or rabbit polymer applied and then placed in a humidification chamber at room temperature for 30 minutes. They were then washed again three times in PBST, 3 minutes each time.

5. Visualization. Using the Commercial DAB Kit (DAKO catalogue no. K 3468) 1 drop of DAB chromagen solution was added to each 1 ml of buffer solution. After mixing well, 200 μL of chromagen solution was added to each slide. Colour development was monitored using a microscope and the reaction (usually 3-5 min) stopped using tap water in a coplin jar.

The slides were counterstained in Gill's Hematoxylin for 30 sec, colour development stopped with tap water, and the slides washed in distilled water. The slides were dehydrated in a graded alcohol series and cleared in xylene before mounting.

The slides were then analysed by Dr. Amal el-Naggar and Dr. Torsten Nielsen.

2.1.2 Prospectively collected osteosarcoma specimens

We prospectively identified three patients undergoing urgent surgey for osteosarcoma. For various clinical reasons such as imminent pathological fracture these patients had not received preoperative chemotherapy. Fresh tissue was obtained from surgical specimens with patient consent and approval of the Research Ethics Board of the University of British Columbia.

These were patients of Dr Clarkson (author) and harvesting of the tissue was undertaken by Dr Clarkson at a minimum time after the specimen was excised (less than one hour), with care taken to sample the growing edge of the tumour and the interface with adjacent normal bone where possible. The tissue was placed into liquid nitrogen, transferred to the lab and then mounted in paraffin blocks. There was no decalcification performed on these specimens. Haemotoxylin and Eosin (H&E) stains were performed to assess adequate representation of tumour in the specimen prior to cutting sections for immunohistochemistry.

2.1.3 Retrospectively collected osteosarcoma specimens

Analysis of our database identified 37 patients who had osteosarcomas resected in the past 10 years. With further assistance from Dr Nielsen the paraffin blocks on these patients were reviewed to ensure that adequate quality and quantity of tumour and normal tissue with a minimal decalcification effect was present, reducing the number to 25 cases. Some of the samples were from patients who had received chemotherapy pre-operatively. Slides were cut from the paraffin blocks for H&E staining and immunohistochemistry.

2.2 Fluorescent In Situ Hybridization

2.2.1 FISH performed in Toronto

Fluorescent in situ hybridization (FISH) was performed on two sets of samples. The first group was performed by Dr Jeremy Squire, University of Toronto, on cases from the Children's Oncology Group. A centromeric probe for chromosome 6 (Vysis) and BAC clones RP11-809N15 and RP11-460L11, both of which map to the 6q21 region, were used.

2.2.2 FISH performed in Vancouver

Subsequently paediatric human osteosarcoma samples were obtained by kind donation from Dr Ken Brown, Orthopaedic Surgeon at BC Children's Hospital. There were 16 cases, all of which had fresh tissue available, albeit in small amounts. Touch preps were prepared and FISH analysis performed. The FISH itself was performed by Nataliya Melnyk in our lab, with my appreciation. A centromeric probe was used for chromosome 6 (Vysis) and BAC clones RP11-373022 and RP11-809N15, which map to the 6q21 region, were used to identify the *HACE1* locus.

2.3 Cell Culture and Sample Preparation

All cells were cultured in MEM with Earle's salts, supplemented with 1% non-essential amino acids, 20% fetal bovine serum (FBS), 1% L-glutamine and 1% penicillin-streptomycin-fungicide. Cells lines were stored in liquid nitrogen in 10% DMSO, 90% FBS when not in use.

Osteosarcoma cell lines HOS, MG63, SaOS-2 and U2OS were obtained from the American Type Culture Collection (ATCC, catalog numbers CRL-1543, CRL-1427, HTB-85 and HTB-96, respectively) all of which are established osteosarcoma cell lines. The osteosarcoma Takase (OST) cell line was kindly provided by Dr Tim Triche of the University of California, Los Angeles. In addition the cell lines 1544 and MNNG (ATCC, catalog numbers CRL-1544 and CRL-1547 respectively) were used, both of which were developed from the HOS cell line by inducing further transformation: 1544 was transfected by the Kirsten murine sarcoma virus and MNNG was exposed to the oncogenic agent N-Methyl-N'-Nitro-N-Nitrosoguanidine .

Human fibroblasts that had been immortalized by transfection with a telomerase-expressing construct were kindly provided by Dr Peter Lansdorp, British Columbia Research Centre and University of British Columbia. Other cell lines used were kindly provided from within the lab.

Protein samples were prepared by removing the culture media, rinsing the cells three times with sterile phosphate buffered saline (PBS), adding lysis buffer (10 mM Tris-HCl pH7.5, 1% NP40, 2 mM EDTA, 100 mM NaCl, 1 mM Na3VO4, and 50 mM NaF) with a Complete cocktail of proteases inhibitors (Roche) directly to the plate and then harvesting the lysate into an eppendorf tube. Lysates were cleared by centrifugation at 15,000 rpm for 10 min. The supernatants were collected and protein con centrations were determined using the Bio-Rad Bradford protein assay kit (Bio-Rad), and at each western blot experiment, an initial blot was run and stained with Ponceau Red to determine protein integrity and equality of protein loading in each lane. Western blots were run with 20μg of protein separated by SDS-PAGE and electrophoretically transfered to PVDF membranes (Invitrogen).

RNA samples were prepared by removing the culture media, rinsing the adherent cells three times with sterile PBS and then applying Trizol® reagent (Invitrogen, catalog number 15596-018) directly to the plate in a fume hood[116]. The sample was then harvested from the plate and vortexed, followed by 15 minutes of centrifugation in a cold centrifuge. The aqueous layer containing the RNA was transferred to a separate tube and the RNA precipitated with isopropanol. After further centrifugation the isopropanol was removed and the RNA pellet washed with 75% ethanol and then re-suspended in 20-30μl of RNAase-free water. RNA integrity and concentration was assessed by spectrophotometry and gel electrophoresis. RNA samples were then stored at -80°C until required.

Synthesis of cDNA was performed on 1μg RNA samples using SuperScript Reverse Transcriptase (Invitrogen), random primers and dNTPs. After cDNA synthesis was complete 1μl of RNAase H was added to each tube to remove any residual RNA, then inactivated by heating to 37°C for 20 minutes.

2.4 RT-PCR

Mic2 is a ubiquitously expressed gene, and was used to confirm successful cDNA synthesis on all cDNA samples. Primers were kindly provided by the staff at BC Children's Hospital's molecular laboratory. The conditions were (253 bp, forward: GATGACTTTGACTTAGGAGAT reverse: CGACAGCCCCCACAATCCCG at 100nM each), 1.0mM $MgCl_2$, 50mM dNTPs, 94°C 1 min, 65°C 1mi n, 72°C 2 minutes, for 35 cycles.

Primers for osteoblast markers were initially selected using the University of California Southern California genome browser (http://genome.ucsc.edu). Primers were designed to cross exon boundaries to avoid amplification from genomic material, obviating the need for DNAase treatment. RT-PCR conditions and final primer selection was then made using the online primer3 primer design software (http://frodo.wi.mit.edu/primer3/), and then subsequently optimized in the lab.

Conditions used were: Alkaline Phosphatase (196bp, forward: CCACGTCTTCACATTTGGTG reverse: AGACTGCGCCTGGTAGTTGT), Bone Specific Protein (171bp, forward: ACACTGGGCTATGGAGAGGA reverse: TGCTTCGCTTTCTTCGTTTT), Collagen 1A1 (231 bp, forward: CTGGTGCTAAAGGTGCCAAT reverse: CTCCTCGCTTTCCTTCCTCT) and Osteocalcin (175bp, forward: GTGCAGAGTCCAGCAAAGGT reverse: TCAGCCAACTCGTCACAGTC), all at 100nM with 1.0mM $MgCl_2$, 50mM dNTPs at 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds for 35 cycles.

Quantitative RT-PCR (qRT-PCR) was performed to assess *HACE1* mRNA levels using the TaqMan 5 prime exonuclease assay. The *HACE1* primer/probe set (forward: TCTTA CAGTT TGTTA CGGGC AGTT, probe: [6FAM]CAAAC CCACC ATGTG GGACC CTG[TAMRA], reverse: CAATC CACTT CCACC CATGAT) was multiplexed with the VIC-MGB labelled β-actin endogenous control primer/probe kit (Applied Biosystems). Both probe/primer sets were designed to cross exon boundaries, obviating the need for DNAase treatment of the source RNA. Reactions were performed in quadruplicate, and each experiment repeated twice. The reactions were run in an ABI 7000 sequence detection system (Applied Biosystems) and data subsequently analysed in Microsoft Excel.

2.5 Detection of Osteoblastic Phenotype

Alizarin red and was used to demonstrate deposition of calcium. Two grams of Alizarin Red S (Sigma-Aldrich, catalogue number 33010) was dissolved in distilled water and adjusted to pH of 4.2. The cells were prepared in six well plates to 80% confluency, the media removed an washed three times with sterile PBS. The Alizarin Red S solution was placed, and the reaction watched for 2 or 3 minutes until staining was complete. The excess dye was then removed and the plates washed with PBS and allowed to air dry.

Von Kossa staining was performed for phosphate deposition. After preparation of the cells in 6 well plates the media was removed and the plate rinsed 3 times with sterile PBS. 1% silver nitrate solution was added to the plates, and then incubated under ultraviolet light for 20 minutes. After thorough rinsing, the excess silver solution was removed with 5% sodium thiosulfate, rinsed with sterile water and then fixed with 1:1 acetone-xylene.

Semi-quantitative RT-PCR was performed for markers of osteoblastic activity, including alkaline phosphatase, collagen type 1A1, bone specific protein and osteocalcin. Western blot analysis for alkaline phosphatase was performed.

Akaline phosphatase staining of cells was performed using the Alkaline Phosphatase staining kit (Sigma-Aldrich, catalogue No. 86R-1KT). The cells were grown in 6 well plates until 60% confluent, and then placed in media containing BMP2/BMP7

heterodimer (R & D Systems, catalogue number 3229-BM) at a concentration of 25ng/ml for one week. The cells were then washed with 1x PBS and 1ml of fixative solution was added to each well for 45 seconds. The fixative was removed and cells washed with 1x PBS. 960µl of the staining solution (20µl Na Nitrate +20µl FRV alkaline + 900µl dH2O + 20µl Naphthol) was added to each well. The plate was left in the dark for 30 minutes at room temperature. The staining solution was removed and cells washed in 1x PBS. Positive immunoreactivity appears as a pink cytoplasmic colour.

2.6 HACE1 Re-expression in Osteosarcoma Cell Lines

Osteosarcoma cells were transfected with retrovirus to re-express *HACE1*. MSCV-based vectors had been produced by Dr Mike Anglesio[104], and were kindly provided for this research. Firstly, *HACE1* was tagged with haemagglutinin (HA) to make identification of expression with western blotting easier prior to the production of a reliable *HACE1* antibody. This was transferred into an MSCV vector carrying resistance to hygromycin. The proposed active site of *HACE1*, in the HECT domain, underwent site-directed mutagenesis converting the cysteine at residue number 876 to serine (C876S). This second construct acts as a non-functional control. Thus three vectors were used, firstly a control MSCV empty vector, secondly an MSCV vector containing functional *HA.HACE1*, and thirdly an MSCV vector containing the putatively non-functional *HA.C876S*.

These constructs were stably transferred into the host cell genome using a retroviral system. The plasmids were mixed with FuGENE 6 reagent (Roche) and incubated for 45 minutes before being added to the Phoenix A packaging cells along with 25μM of chloroquine. After 24 hours the media was changed, and then 48 hours later the virus-containing supernatant was collected and filtered to remove any stray packaging cells.

The target osteosarcoma cells were grown to 80% confluency and then grown in culture with 50% fresh media and 50% virus-containing supernatant for 24 hours, after which the media was discarded, the cells washed with PBS and then placed into normal cell culture medium. After 24 hours, selection with hygromycin at 250μg/ml

was initiated and maintained for 10 days. Western blot analysis for HA was then performed to confirm successful viral transfection.

2.7 Soft Agar Colony Assay

Soft agar assays were setup in 6-well plates with a final concentration of 0.4% agar in DMEM/FBS in the bottom “feeder” layer. Cells were then plated at a concentration of 8000 cells per well, suspended in 0.25% agar in DMEM/FBS on top of the feeder layer. The plates were checked to ensure that equal concentrations of cells had been obtained, and that the cells were suspended singly and not in contact with other cells.

The plates were then incubated at 37°C and supplemented with one or two drops of media every four days until colonies had started to form, and then the number of colonies per high power field was counted. Each cell line was plated across three wells and four high power fields counted for each well, giving 12 readings for each cell line. The whole experiment was then repeated in triplicate on separate days.

2.8 Matrigel 3-dimensional Colony Assay

Matrigel assays were set up in 6-well plates and performed as described by Debnath et al[117]. Growth factor reduced matrigel (BD Biosciences, catalog number 354230) was thawed overnight at 4°C in the fridge. After pre-wetting the plates 100μl of matrigel was placed into each well and then incubated at 37°C to solidify and form the bottom layer. Cells were harvested with trypsin at 90% confluency, washed and resuspended as a single cell suspension in assay media (DMEM/F10 supplemented with 10μg/ml Insulin, 20ng/ml Epidermal Growth Factor, 5% Horse Serum and 1% Penicillin-Streptomycin-Fungicide). The volume was adjusted to a final concentration of 25,000 cells/ml.

A second preparation of assay medium containing 4% matrigel was made and kept chilled. Once the bottom layer had set, the cell-containing suspension and the matrigel-containing suspension were combined in a 1:1 ratio and 500μl added to each well. This was then incubated until set, and the cultures checked for an appropriate concentration and that the cells were suspended singly and not clumped. Cells were then fed with assay media containing 2% matrigel every four days.

2.9 Mouse Xenograft Assay

All animal care was performed in accordance with the "Guidelines of the Canadian Council on Animal Care". Approval for the research was obtained from the Animal Care Committee of the University of British Columbia. This experiment was carried out in the animal facility at the Jack Bell Research Centre, University of British Columbia.

HOS, OST and MNNG osteosarcoma cell lines that had been stably transfected with empty vector MSCV, *HA.HACE1* or *HA.C876S* were grown to 80% confluency in T-75 flasks and then harvested with trypsin, washed and then suspended at 10^6 cells/ml. Nu/Nu Cd-1 male 6 week old mice were anaesthetized with isoflurane and had 100μl of cell suspension injected into each flank, giving four sites per mouse. Three mice were used for each of the cell lines, so there were 12 readings for each cell line and a total of 36 mice used.

The mice were checked every second day and weighed to monitor tumour burden. The tumours were measured in three orthogonal planes and the volumes estimated assuming roughly spherical growth by the formula 0.5236 x length x width x height ((4/3)π)(length/2)(width/2)(height/2)) as described by Bogden[118]. On day 27 the mice were euthanized using a CO_2 chamber and the tumours dissected, weighed, divided and then each half placed into formalin or liquid nitrogen.

Chapter 3: RESULTS

3.1 Immunohistochemistry for HACE1 in Osteosarcoma

3.1.1 Prospectively collected osteosarcoma specimens

Immunohistochemistry was carried out on specimens from 3 patients who presented with osteosarcomas requiring urgent resection prior to any chemotherapy. We ran these pilot specimens to ensure that our technique worked prior to undertaking a more laborious search for specimens.

The first specimen showed significant staining for HACE1 protein in the osteoblasts which can be seen rimming the normal bone, while in the tumour cells on the same slide there was minimal staining for HACE1 protein (Figure 3). The second specimen showed intermediate staining in both the osteoblasts and tumour cells. The third specimen showed no signal in the tumour cells, but there was also no staining in any of the normal cells present in the specimen, leaving no internal control, so this specimen was discarded. Although the profile of expression was what may have been expected from our hypothesis, clearly no definitive conclusions can be made from such a small sample.

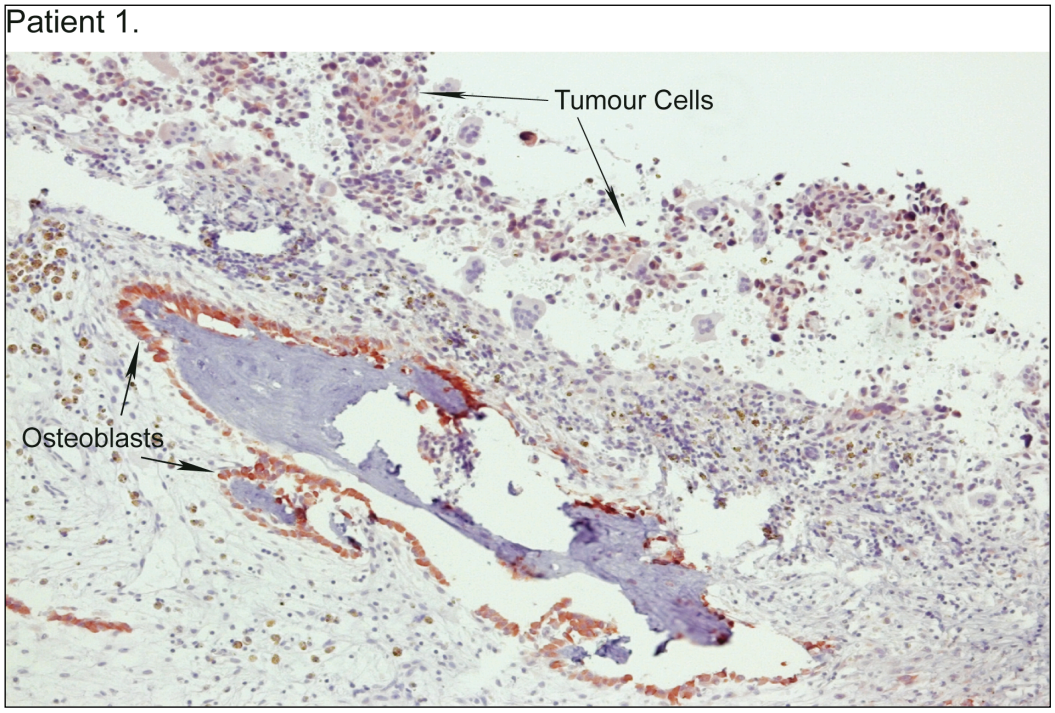


Figure 3. Immunohistochemistry for HACE1, patient 1.

Immunohistochemistry for HACE1 protein in the first specimen is shown above. Plump osteoblasts can be seen rimming the normal bone, and exhibit strong nucleocytoplasmic staining for HACE1 protein. Tumour cells are seen exhibiting much lower levels of staining at the top of the photomicrograph (Immunoperoxidase stain).

3.1.2 Retrospectively collected osteosarcoma specimens

Having established that our immunohistochemistry technique was effective on prospectively collected, non-decalcified osteosarcoma specimens, we performed immunohistochemistry on a group of specimens identified as outlined in the methods section.

Of the 25 specimens, 14 (56%) showed no staining for HACE1 protein at all in the tumour cells, but also no staining in the other cells that were present, similar to the specimen from patient 3 above. Although our positive control did work in this experiment, we were very concerned about the different processing of the specimens, such as decalcification, that could render the individual specimens non-reactive to the HACE1 antibody. We felt it was safest not to include these specimens, and this limits our statistical analysis.

In the remaining 11 specimens the staining for HACE1 amongst the tumour cells was variable, ranging from 5% of the tumour cells up to 90% of the tumour cells, with a median of 40% (Table 1). By splitting the 11 into those above and below the median of 40%, there was a trend towards lower grade tumours with a higher expression of HACE1, but this was not significant ($p=0.19$, Mann Whitney U test).

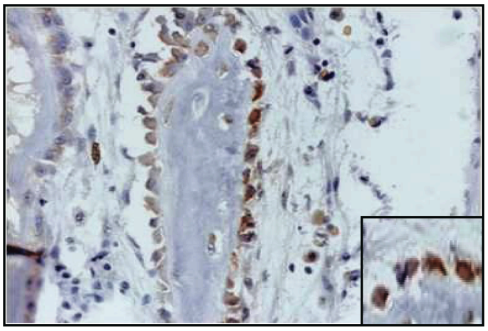
Specimen	%HACE1	Age	Sex	Grade	Size (cm)
1	5			2	5
2	10	50	F	3	8
3	25			1	4
4	30	24	F	1	8
5	30	50	F	3	7.7
6	40			2	3.5
7	50	67	M	1	14.5
8	60	35	M	2	3.5
9	70			1	3
10	70	25	M	1	4.5
11	90	18	M	1	5.9

Table 1. Characteristics of IHC specimens positive for HACE1.

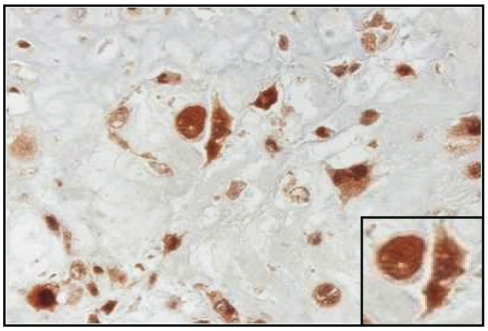
The percentage of tumour cells staining positive for HACE1 is represented in the %HACE1 column. There is a trend towards lower grade tumour with higher percentages of HACE1 positive cells, but the small numbers preclude valid statistical analysis. Grade is the French histological grading system, size is maximum diameter.

The localization of HACE1 protein within the cell varied. In osteoblasts and tumours where greater than 40% of the tumour cells expressed HACE1 the protein was nucleo-cytoplasmic and usually stained with high intensity. In tumours where less than 40% of the tumour cells stained positive for HACE1 the protein was located in the cytoplasm and not present in the nucleus, and usually stained with mild to moderate intensity. This is shown in Figure 4, where several examples of the staining are shown.

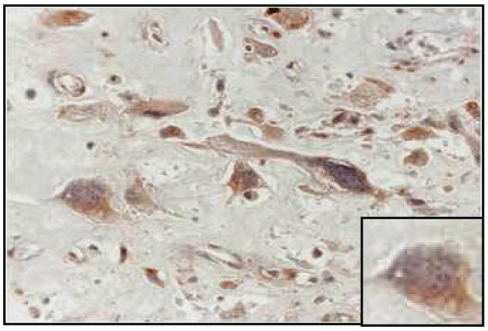
As noted earlier, there was a trend to lower grade tumours with higher rates of HACE1 positivity in the tumour cells. This result suggests that lower grade tumours have similar cellular localization of HACE1 protein to normal osteoblasts, while higher grade tumours tend to exclude HACE1 from the nucleus, unlike differentiated osteoblasts. In addition the intensity of staining appears to be higher in the lower grade tumours, similar to osteoblasts.



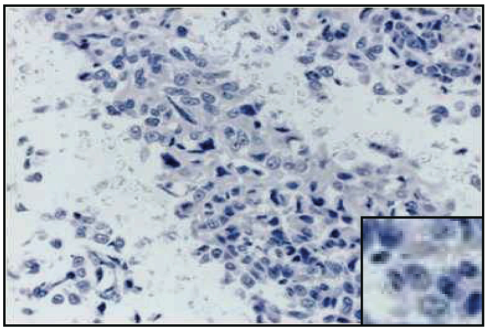
Osteoblasts showing strong nucleo-cytoplasmic staining for HACE1 protein



Low grade osteosarcoma showing strong nucleo-cytoplasmic staining for HACE1 protein



High grade osteosarcoma showing moderate cytoplasmic staining for HACE1 protein



High grade osteosarcoma showing no reactivity for HACE1 protein

Figure 4. Immunohistochemistry of osteosarcoma examples.

Examples of HACE1 immunostaining of osteosarcoma specimens. Images prepared by Dr Amal elNaggar (Immunoperoxidase stain).

3.2 Fluorescent In Situ Hybridization

In the osteosarcoma set from the Children's Oncology Group analysed by Dr Squire 33 of 44 samples had sufficient tissue for analysis. Of these, 15 (45%) showed significant loss of *HACE1* signal, in four of those there was a distinct clone with *HACE1* loss. 13 (39%) showed no change and 4 (12%) showed gain or complex alterations.

In the group of 16 osteosarcomas from BC Children's Hospital one case showed the two BAC signals were split, indicating a chromosomal disruption at 6q21 in the region of *HACE1*, but this was only present in 10% of cells in the touch prep of this specimen. Two cases were monosomic for chromosome 6 in some of the cells present and 5 out of 16 (31%) showed a normal chromosome 6 pattern. The rest of the cases had abnormal chromosomes, but no definitive evidence of *HACE1* loss by FISH.

3.3 Human Osteoblast Culture

The intimate relationship between the molecular pathology of osteosarcoma and the normal developmental physiology of human osteoblasts has been outlined in the introduction. The predominant current theory is that osteosarcoma arises from disordered osteoblast differentiation. In order to fully investigate the role of *HACE1* in osteosarcoma it was essential that we had a model of normal osteoblast development to act as a control.

We investigated several different models, and attempted to implement them with varying degrees of success. Ultimately we ended up developing our own novel model involving culture of osteoblasts from discarded bone obtained during scoliosis surgery. This section will briefly discuss the models that we trialled, and then outline the model that we developed.

3.3.1 Human foetal osteoblasts

Human foetal osteoblasts were obtained from the ATCC (designation hFOB 1.19, Catalog No. CRL-11372). The cell line was established from a spontaneous miscarriage and subsequently transfected with SV40 T antigen. This antigen promotes cell growth and division[119]. In this cell line the T antigen is temperature sensitive; it is active and promotes rapid cell proliferation at 33°C but becomes unstable and inactive at 39°C, resulting in a cessation of cell division and a more osteoblastic phenotype. The properties of this cell line have been characterized, and found to somewhat approximate a normal osteoblast phenotype.[120].

Our experience with these lines was unsatisfactory. After a few days at 39°C the cells would simply die, presumably due to heat shock. With shorter exposures or lower temperatures the expression of osteoblastic markers or staining for calcium was inconsistent.

3.3.2 Differentiation of osteoblasts from human mesenchymal stem cells

We obtained human mesenchymal stem cells (MSCs) from Stem Cell Technologies, Vancouver, Canada, and used their proprietary osteogenic medium to differentiate these into osteoblasts. However, again, the expression of osteoblastic markers assessed by RT-PCR was inconsistent. Even in osteogenic medium, MSCs are still able to differentiate into other cell types such as lipoblasts[121]. We had lingering concerns that we were not achieving a very good population of mature, fully differentiated osteoblasts from this model that would be satisfactory for our purposes.

3.3.3 Human osteoblasts from joint replacement patients

Dr Marianne Sadar of the British Columbia Cancer Research Centre and the University of British Columbia has developed a model of human osteoblast culture from joint replacement offcuts, and she kindly donated four lines to us for initial investigation. After washing and rinsing with PBS, the bone fragments were mechanically broken down and then placed in T-75 tissue culture flasks with MEM with Earle's salts, supplemented with non essential amino acids, fetal bovine serum and L-glutamine. Once the osteoblasts had migrated from the bone they were

harvested with trypsin and split into flasks for culture, at which point they were supplied to us. We designated these cell lines OBA, OBB, OBC and OBD.

We found that these cell lines did not culture for very many passages before dying, and the lines that did keep growing changed morphology to resemble fibroblasts and lost expression of osteoblastic markers. Only OBB generated consistent evidence of osteoblastic markers, but these survived only for 8 or 9 passages. Presumably the osteoblasts in these cell lines had limited population expansion capacity, and were soon outgrown by the more rapidly dividing fibroblasts. As most of these patients were all over 55 years of age, it might be expected that their osteoblasts would be nearer to terminal differentiation with fewer cell divisions available than the young patients who typically get osteosarcoma.

3.3.4 Human osteoblasts from scoliosis patients

We decided to develop our own human osteoblast model. Each of the models above had its drawbacks. The most promising were the osteoblasts from the joint replacements, in particular cell line OBB, but these seemed to have a short lifespan and limited ability to expand their osteoblast population. The age group of these patients is also quite different to that of osteosarcoma patients, and the osteoblast biology and *HACE1* expression may or may not be comparable. In addition, there was a limit to the amount of material Dr Sadar could provide.

Scoliosis is a spine disorder that typically affects adolescents of a similar age distribution to adolecesent osteosarcoma. The mainstay of treatment for progressive scoliosis is surgical stabilization of the spine using bone graft and internal fixation devices. At the time of surgery for scoliosis there is frequently excess bone resected that is not required for subsequent grafting, and this would potentially provide cells that could survive for more passages and represent a better osteoblast model than the others we had tried.

Ethics approval was obtained from the Research Ethics Board of British Columbia, and all patients provided informed consent along with parental consent as required. At the time of surgery if there was any bone graft that was not required for clinical purposes it was placed into a sterile container and transferred to the lab. The grafting material consisted of bone from the posterior spinous processes that had been

meticulously stripped of soft tissue and morcellised into small fragments prior to leaving the surgical field.

We ultimately established the following protocol for osteoblast culture from the bone fragments. Under sterile conditions the bone fragments were washed three times with PBS, and broken up further if necessary. They were then placed in T-75 flasks with culture medium (MEM with Earle's salts supplememted with 20% FBS and 1%PSF) and placed in an incubator for 4 weeks. The media was changed weekly, and care was taken not to disturb the bone fragments during the media change as this can affect osteoblast migration from the bone fragments.

At four weeks, if the culturing had been successful, the cells were harvested using trypsin and the bone fragments discarded. If there were no signs of cells at 4 weeks, the culture was continued in the T175 flask until 8 weeks, as often the osteoblasts would appear only very slowly and very late. The cells then underwent serial passaging and were differentiated using bone morphogenic protein (BMP2/BMP7 heterodimer, R and D Systems, catalog number 3229-BM). They were then characterized to determine if they contained cells with osteogenic potential, either osteoprogenitors or osteoblasts. They were assigned the labels OB1, OB2, OB3, etc.

We were able to establish four primary human osteoblast cell lines (OB1, OB2, OB3 and OB5). Figure 5 shows the bone being harvested intra-operatively, the morphology of the cells and representative Alizarin Red and Von Kossa staining. ALP expression was confirmed by staining (Figure 6), which showed that almost all of the

cells were expressing ALP after exposure to BMP 2/7 for one week indicating that we had a relatively pure culture of human osteoblasts.

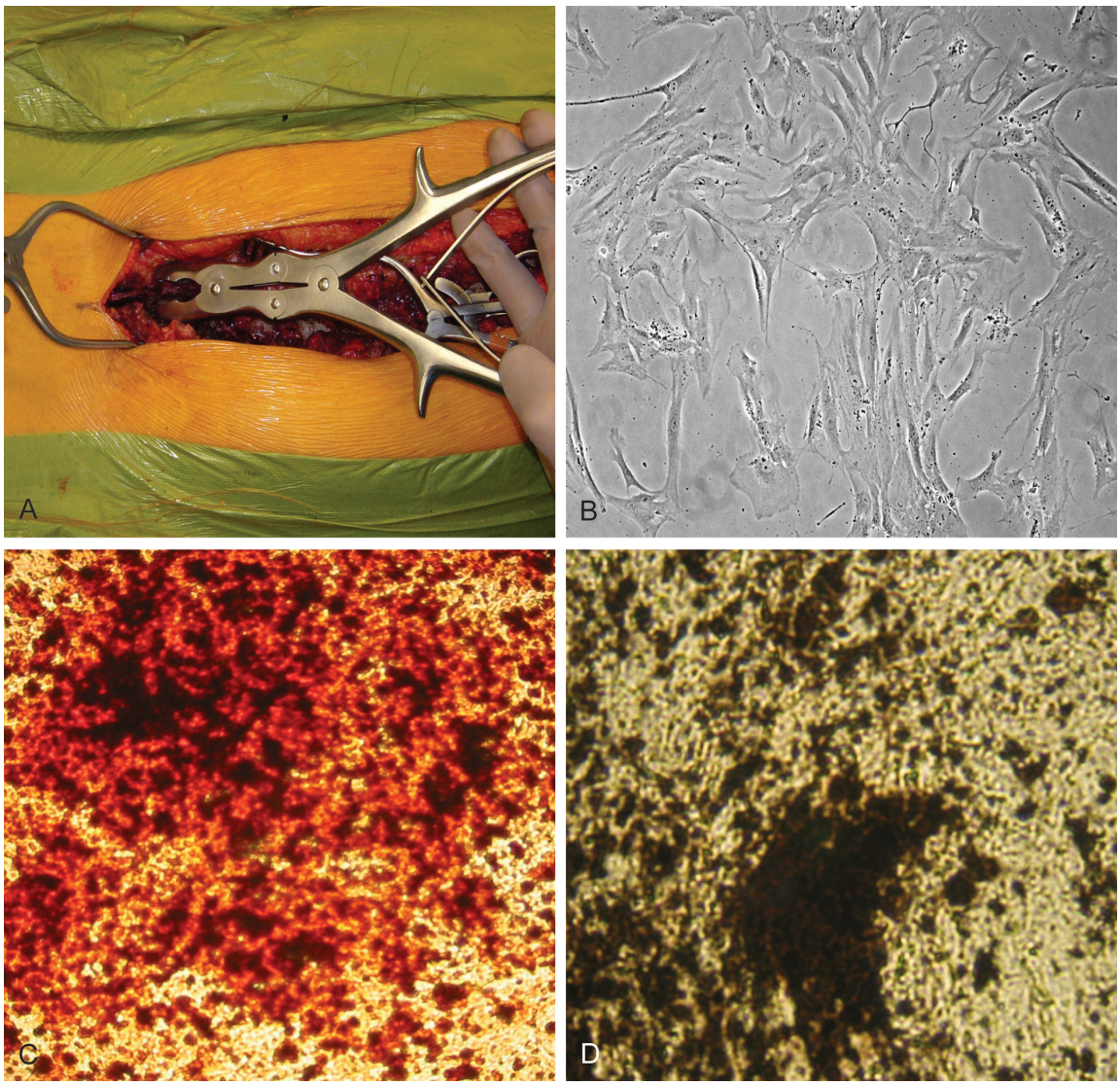
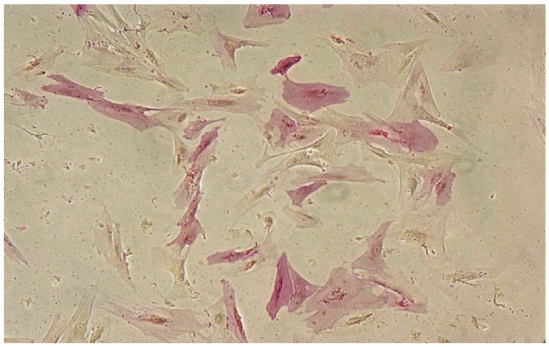


Figure 5. Human osteoblast harvesting and culture.

Clockwise from top left: A: Harvesting of bone from posterior spinous process, B: Osteoblasts in monolayer culture, C: Alizarin staining showing extracellular calcium deposition, D: Von Kossa staining showing extracellular phosphate deposition.

OB3



OB5

Figure 6. ALP staining in primary osteoblast cell lines.

These slides show representative ALP staining in primary human osteoblast cultures isolated from scoliosis patients (x400) after exposure to BMP 2/7 for one week. OB3 is the third line isolated and OB5 is the fifth line isolated.

RT-PCR for osteoblastic factors was performed after administration of bone morphogenic protein, with the results shown in Figure 7. HF-1 represents immortalized human fibroblasts as a negative control. Note that the OBD cell line from Dr Sadar's lab shows a similar expression profile to the human fibroblast controls, while OBB has strong expression of all markers.

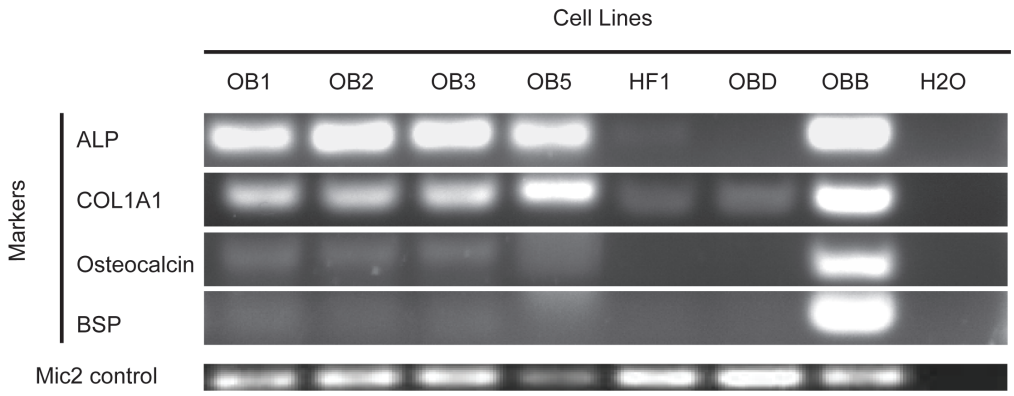


Figure 7. RT-PCR for markers of osteogenic differentiation.

OB1-OB5: Human osteoblasts from scoliosis patients, HF1: Immortalised human fibroblasts, OBD-OBB: Human osteoblasts from joint replacement patients, ALP: Alkaline Phosphatase, COL1A1: Collagen type 1A1, BSP: Bone Specific Protein.

3.4 qRT-PCR for HACE1 mRNA Expression

HACE1 mRNA expression levels as detected by qRT-PCR were significantly lower in osteosarcoma cell lines than in human osteoblasts when grown in monolayer culture. The expression levels in Figure 8 are normalized to the human osteoblast line OBB. This shows that the mRNA expression levels of *HACE1* were significantly lower in the osteosarcoma cell lines than in the osteoblast cell lines, analysis of variance testing showed a p value of <0.01.

This qRT-PCR was run prior to the development of our own osteoblast model, using OBB and hFOB as model osteoblasts, both of which were expressing osteoblastic phenotypes at the time of this experiment. As this experiment gave us enough evidence to continue with our other aim such as *HACE1* rescue and mouse models, we had not repeated it with our own osteoblast model.

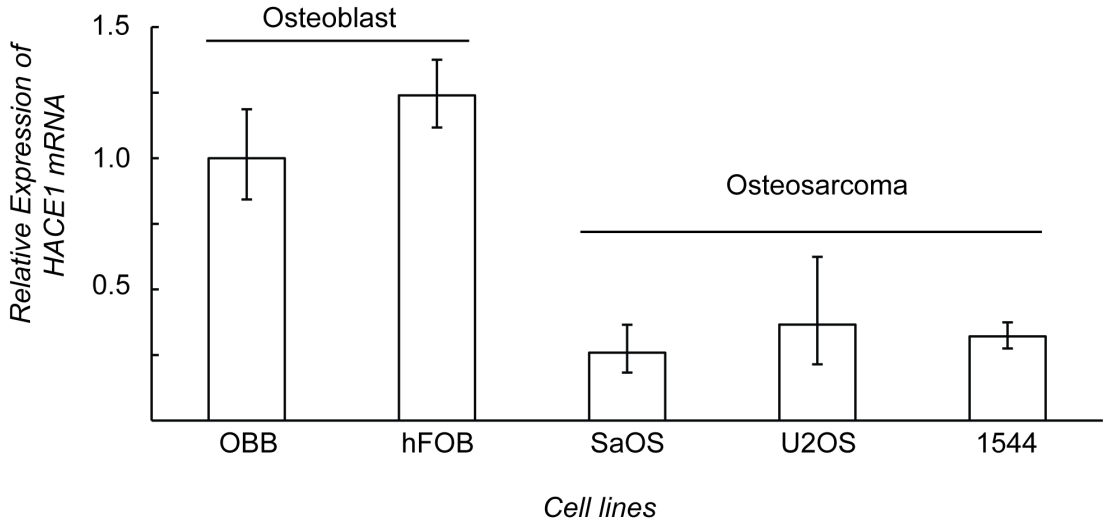


Figure 8. qRT-PCR for HACE1.

Using human osteoblasts and osteosarcoma cell lines grown in monolayer culture. OBB: Human osteoblast from joint replacement patient, hFOB: human foetal osteoblast, SaOS, U2OS, 1544: human osteosarcoma cell lines. *HACE1* expression in osteosarcoma cell lines was significantly lower than in osteoblast lines ($p<0.01$).

3.5 Western Blot Analysis for HACE1

Western blot analysis for HACE1 was performed and showed similar levels of HACE1 protein in the osteoblast as in the osteosarcoma cell lines, possibly even slightly lower levels (Figure 9). SKNEP cell line was used a negative control, and HEK293 as a positive control. In order to obtain a signal from the protein using the weak HACE1 antibody the wells were loaded with maximal amounts of protein, and this is reflected in the β-Actin control. Biorad protein quantification and staining with Ponceau Red were both performed to ensure equal loading.

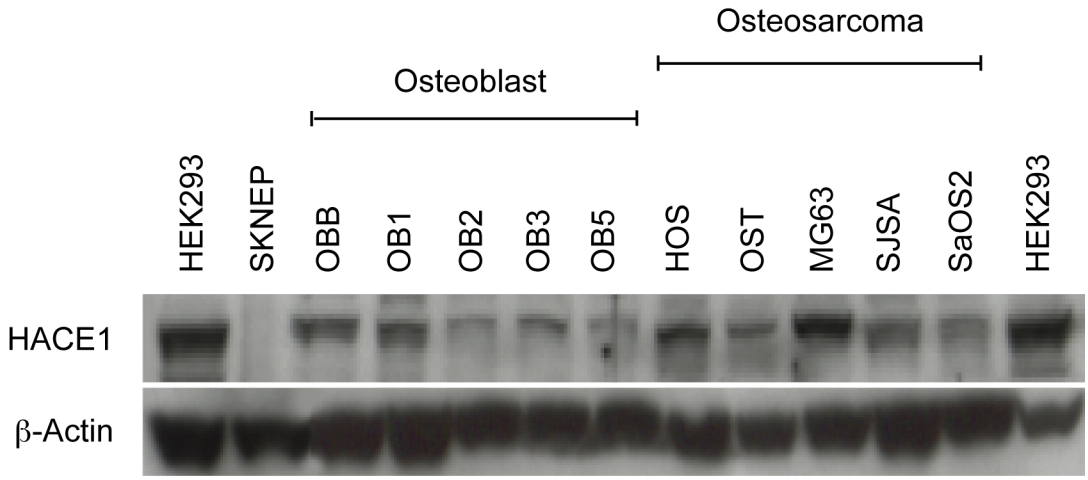


Figure 9. Western blot analysis for HACE1.

All cell lines grown in monolayer cell culture. OBB: Human osteoblasts from joint replacement patient, OB1-5: Human osteoblasts from scoliosis patients, HOS, OST, MG63, SJSA and SaOS2: Human osteosarcoma cell lines, HEK293: Positive contol, SKNEP: Negative Control.

3.6 Soft Agar 3-dimensional Colony Assay

The results of the soft agar assays performed on the HOS and OST osteosarcoma cell lines are shown in Figure 10. The colonies were subjectively smaller and less well organized when transfected with *HA.HACE1* cell line than with the empty vector MSCV cell line.

There was a significant decrease in the number and size of colonies produced by osteosarcoma cells transfected with *HA.HACE1* than those transfected with empty vector (MSCV) or the *HA.C876S* inactivated mutant *HACE1* when the cell lines were grown in soft agar suspension. This effect was seen to a greater or lesser extent across all the osteosarcoma cell lines tested, and was robust on repeated testing. Statistical significance was $p<0.01$ using the Mann-Whitney U test.

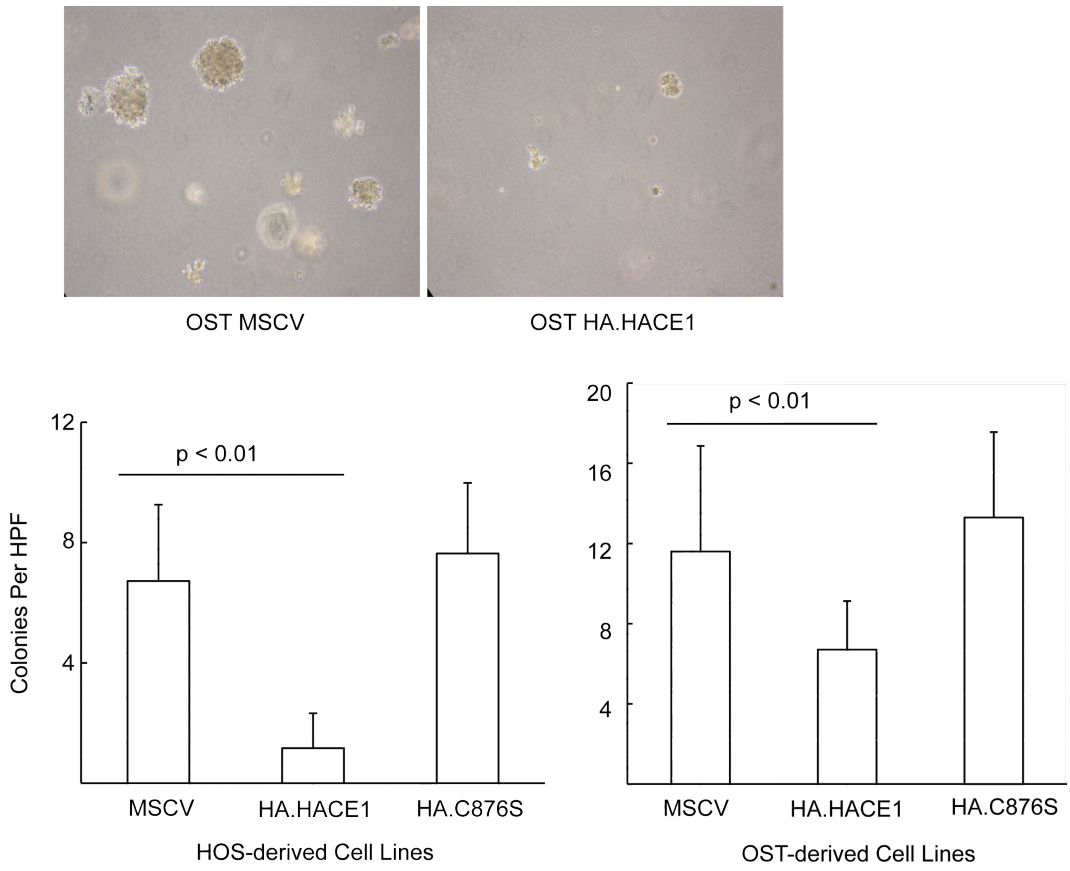


Figure 10. Soft agar colony formation in human osteosarcoma cell lines.

Top: Images of OST cell line showing different colony morphology when transfected with empty vector MSCV or HA.HACE1.

Bottom: Cell lines derived from human osteosarcoma cell lines HOS and OST showed fewer colonies when transfected with *HA.HACE1* than with the empty vector control MSCV. Comparing the MSCV with *HA.HACE1* group resulted in a $p<0.01$ for both cell lines, Mann-Whitney U test.

MSCV: empty vector control, HA.HACE1: cells transfected with *HA.HACE1*, HA.C876S: cells transfected with inactivated mutant *HA.C876S*.

3.7 Matrigel 3-dimensional Colony Assay

There was a significant decrease in the number of colonies and an extremely different colony morphology in osteosarcoma cells transfected with *HA.HACE1* than those transfected with empty vector (MSCV) or the HA.C876S inactivated mutant when the cell lines were grown in Matrigel. The *HA.HACE1* transfected cells mostly died, and some formed very small, round colonies. The empty vector control cells showed a rapidly growing, branching and connecting phenotype with many healthy cells. The cells transfected with the *HA.C876S* inactivated mutant showed an intermediate phenotype, indicating partial loss of function of the protein. The results for the HOS osteosarcoma cell line are shown in Figure 11, and these were reproduced on repeated experiments.

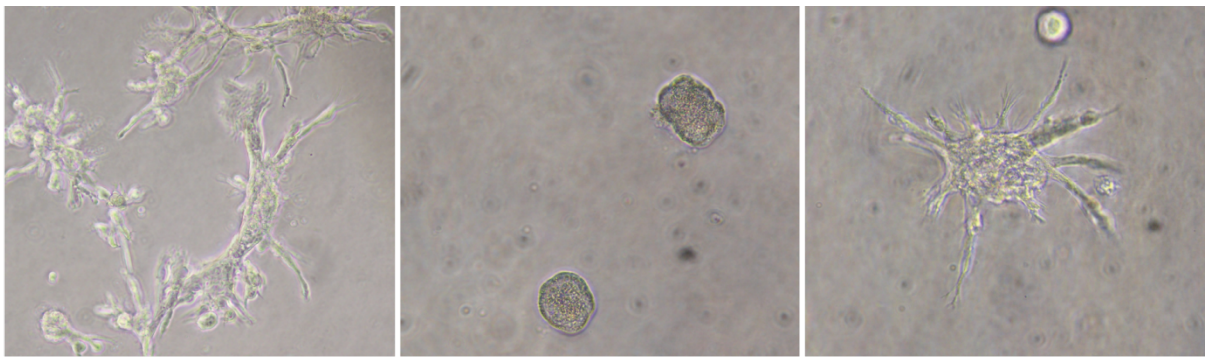


Figure 11. HOS-derived human osteosarcoma cells cultured in Matrigel.

Note the intermediate appearance of the HA.C876S cells suggesting partial loss of function. MSCV: empty vector control, HA.HACE1: transfected with *HA.HACE1*, HA.C876S: transfected with inactivated mutant *HA.C876S*.

3.8 Mouse Xenograft Assay

The mouse xenograft model did not show as marked a difference in growth as was seen in the soft agar and matrigel models. The MNNG osteosarcoma cell lines all showed rapid tumour growth and no detectable differences in size regardless of whether the cells had been transfected with empty vector MSCV, *HA.HACE1* or the non-functional *HA.C876S* mutant. The OST cell lines did not show any tumour growth in any of the mice.

Western Blot analysis of tumour tissue from the HOS cell line mice showed that the *HA.HACE1* transfected cell lines had maintained their expression of the *HA.HACE1* construct, but the expression of the inactivated *HA.C876S* mutant was lost (Figure 12a).

The HOS cells did show some reduction in tumour size in the cells transfected with *HA.HACE1* than those transfected with empty vector (MSCV) as shown in Figure 12b. There was no statistically significant difference in the mean size of the tumorus produced ($p = 0.36$). However, there was a smaller range of tumours sizes produced in the mice transfected with the *HA.HACE1* cell lines, with fewer of the very large tumours seen. Representative mice are shown in Figure 12c.

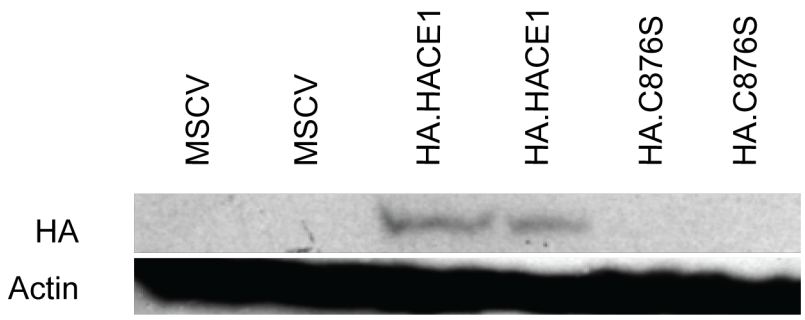


Figure 12a. Western blot analysis for HA in xenograft tumours

Western blot performed on tissue harvested from the mouse xenograft tumours, two sample tumours shown. Note the lack of a band in the HA.C876S tumours. These cells had presumably lost their expression at some point between the western performed prior to cell preparation for this experiment and subsequent harvest of the tumours. MSCV: empty vector, HA.HACE1: *HA.HACE1* transected cells, HA.C876S: inactivated mutant *HA.C876S*, HA: hemagglutinin.

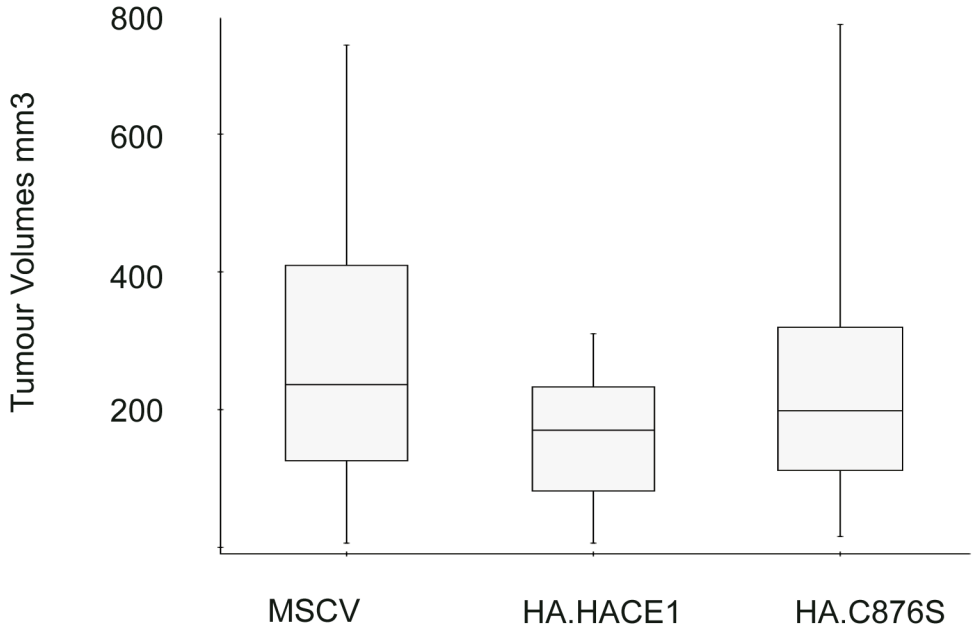


Figure 12b. Mice xenograft model tumour volumes.

Tumour sizes after 27 days in mice inoculated with cells derived from HOS human osteosarcoma cell line. MSCV: empty vector control, HA.HACE1: cells transfected with *HA.HACE1*, HA.C876S: cells transfected with inactivated mutant *HA.C876S*. Although there was no statistically significant difference between the cell lines, note the reduction in the range of tumour sizes produced.

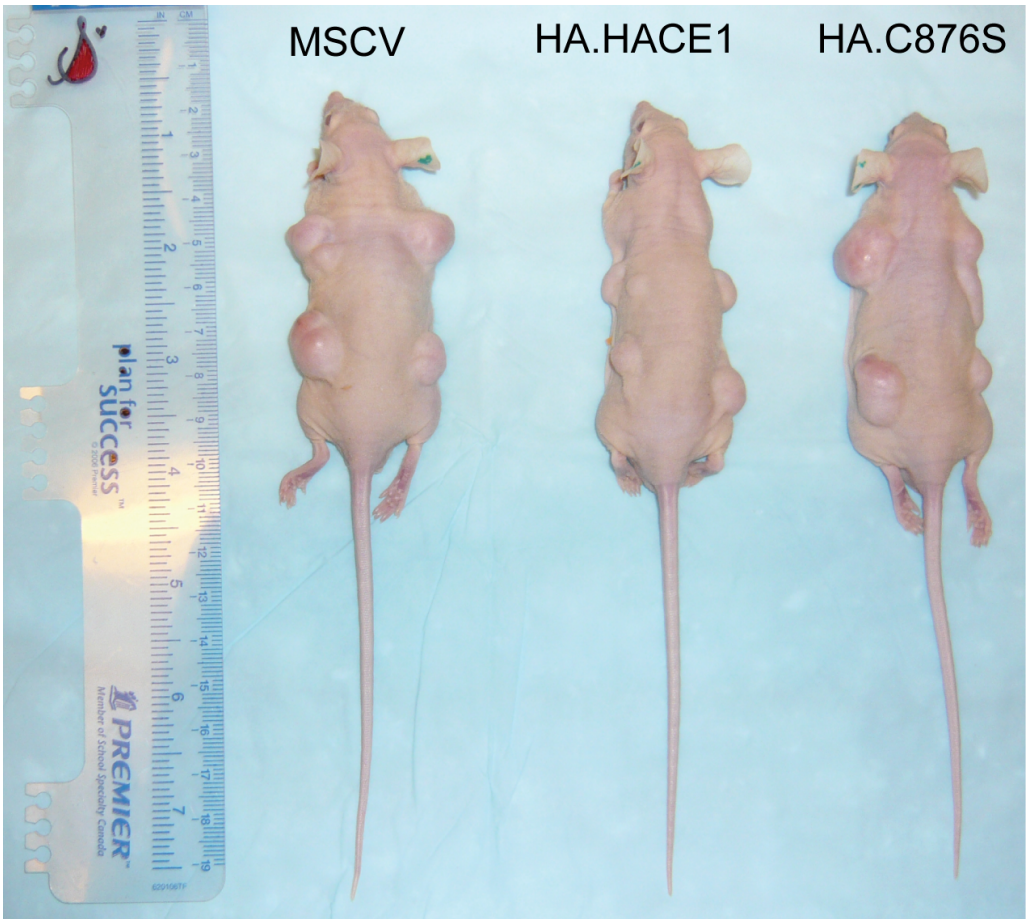


Figure 12c. Representative mice from xenograft experiment.

Mice shown following death at 27 days. Mice were inoculated with cells derived from HOS human osteosarcoma cell line. MSCV: empty vector control, HA.HACE1: cells transfected with *HA.HACE1*, HA.C876S: cells transfected with inactivated mutant *HA.C876S*.

Chapter 4: DISCUSSION

Osteosarcoma is a complex disease that often affects young people. The genomic instability observed in osteosarcomas makes understanding the molecular pathology very challenging. However, there have been some advances in understanding and an association between osteosarcoma and disordered osteoblast maturation is an emerging concept[24].

We were led to the investigation of *HACE1* in osteosarcoma initially by findings in mouse genetic models that were deficient for both *Hace1* and *p53*. Mice that were $p53^{+/-}$ alone developed thymic lymphomas and other malignancies, but did not develop osteosarcoma in this mouse strain. However, the compund model with $Hace1^{-/-}$ on the $p53^{+/-}$ background resulted in the development of osteosarcomas, and mice that were nullizygous for *Hace1* and *p53* developed a wide range of tumours, including metastatic osteosarcoma.

p53 has previously been implicated in osteoblast maturation and osteosarcoma development in human tumours and mouse models. Mutation of *TP53* is reported in up to 60% of human osteosarcomas[43,44,122]. Loss of *p53* in mice has been shown to promote differentiation of mesenchymal stem cells into an osteogenic pathway, but to block terminal differentiation. This fits with the model of osteosarcomagenesis outlined by Kansara[24,123-125]. Mice carrying mutant alleles of *p53* develop osteosarcoma, among other tumour types[126]. Using conditional knockout of both *RB*-

1 and *p53* one group was able to generate a mouse model that develops early onset osteosarcoma with almost complete penetrance[127].

It is important to note, in the context of this thesis, that osteosarcoma was not observed in $p53^{+/-}$ mice. The pattern of tumour development varies depending on the background of the mouse being used, and these mice did not develop osteosarcomas until they had also lost *HACE1* expression. In addition, *HACE1* may be acting in synergy with *TP53* to produce or promote osteosarcomas in humans, similar to the synergy of retinoblastoma protein in the *RB-1, p53* double knockout mouse produced by Berman, et al[127].

A review of the literature[9,70] and analysis of the online Mitelman database suggested that deletions in the 6q21 region where *HACE1* is located are a relatively common finding in osteosarcoma. A chromosomal defect was not found universally as osteosarcoma is a malignancy with complex cytogenetic findings. However, other mechanisms of loss of function of a gene or its protein product, including epigenetic silencing and point mutations would not be detected in standard cytogenetic protocols. Microarray analysis from the GEO and oncomine datasets was non-conclusive, but the only large dataset did show overall low expression of *HACE1*, although this was not compared to a normal control. However, other mechanisms of loss of function of a gene or its protein product, including epigenetic silencing and point mutations would not be detected in standard cytogenetic protocols. There may also be losses of other genes affecting the same as yet undefined pathway, resulting in the same functional impairment to the cell as loss of *HACE1* itself.

Given the near ubiquitous of loss of *HACE1* in the NCI-60 cell line set, evidence of 6q21 as the most common site of loss in some series of osteosarcoma cytogenetic reports and the osteosarcomas produced in the knockout mice, we felt that there was enough evidence to investigate further whether *HACE1* played a role in human osteosarcoma.

In order to assess *HACE1* at the protein level we performed immunohistochemistry on osteosarcoma specimens. This showed heterogeneity, as would be expected, however a significant number showed reduced signal compared to other normal cells in the slide such as osteoblasts. In addition, when there was a low number of cells with HACE1, the staining was of intermediate or low strength, with higher numbers of positive cells the staining was much stronger.

Of interest is the different localization of the HACE1 protein when the cases are grouped according to expression level. Those with high expression showed signal throughout the cell, similar to that seen in normal osteoblasts, while those with low expression showed the protein solely in the cytoplasm and excluded from the nucleus. This strongly suggests that in low expression cases HACE1 is not only at a low level but is not functioning in a normal manner.

HACE1 protein is an E3 ubiquitin ligase, and it is reasonable to assume that it is responsible for the identification, tagging and possibly transport of some as yet unknown target protein or proteins to the proteasome for degradation. If *HACE1* is no

longer entering the nucleus, and its target protein is in the nucleus, then it will not be able to complete its function. This is an area that is being investigated by other researchers in our lab.

We attempted to determine if there was a correlation between the percentage of cells staining for HACE1 protein and the grade of the tumour. While there was a trend to lower grades with higher levels of protein and higher percentages of cells stained, this did not reach significance in this small sample. There were an unusually large number of lower grade tumours in this sample, probably reflecting a selection bias either due to lower grade tumours being less likely to have been aggressively decalcified or possibly as these patients had not received chemotherapy and so had less necrotic tissue, and so these tumours were more likely to be suitable for analysis.

FISH analysis was undertaken to further localize translocations or chromosomal disruptions in the region of 6q21. Dr Squire's group in Toronto showed encouraging results with 45% of specimens appearing to show reduced signal for *HACE1*, supporting our hypothesis. Performing FISH on a series of fresh tissue specimens in our own lab unfortunately was not as encouraging, with only two samples showing chromosome six monosomy. However, one specimen in our series did show a split signal suggestive of re-arrangement at 6q21, apparently at the *HACE1* locus.

We proceeded with investigation using cell lines. In order to develop a normal control and to be able to correlate findings in osteosarcoma we spent quite some time

establishing a reliable model of normal human osteoblast culture. Several options were tried and discarded. ATCC provides a cell line of human foetal osteoblasts that have been immortalised with a temperature-sensitive T antigen, which allows the cells to proliferate at 33°C, and then return to a more normal phenotype at 39°C. We found these cells to be very difficult to work with. At temperatures over 38°C the cells died after a few days, presumably due to heat sensitivity. Mesenchymal stem cells can be differentiated into osteoblasts, but they tend to produce a somewhat mixed population and our results with this technique using cells commercially sourced from Stem Cell Technologies, Vancouver, Canada were unreliable.

Human osteoblasts derived from offcuts of bone at the time of joint replacement were obtained by kind donation from Dr Marianne Sadar, who gave us four lines that she had developed for the investigation of osteoblastic modulation of metastasis. This allowed us to undertake our early investigations and optimize our own culture methods. These cells had originated from patients in their 6^{th} or 7^{th} decade undergoing total knee joint replacement. We were able to establish reliable cell culture, although the expression of osteoblast markers was variable between cell lines, and they were often overrun by fibroblasts, as demonstrated in Figure 7.

Other concerns about these osteoblasts were that they were from diseased tissue, harvested from a joint that had deteriorated due to inflammatory or traumatically induced osteoarthritis. Osteoarthritic joints characteristically have sclerotic bone and bony overgrowths (osteophytes), so the bone physiology is clearly disrupted in the area where the osteoblasts are being harvested from. In addition, the age distribution

of this patient group does not match the typical age distribution for osteosarcoma. For these reasons, as well as the limited supply that Dr Sadar had access to, we decided to develop our own osteoblast model.

The method which we optimized is outlined in the results section. To drive differentiation we first employed a combination of dexamethasone, vitamin C and β-glycerophosphate. While this achieved excellent expression of osteoblastic markers, we later used bone morphogenic proteins as a more physiologic stimulus. These cells seemed to represent an almost pure population of osteoblasts when stained for alkaline phosphatase after exposure to differentiation stimulus. Although ALP is expressed by other tissues such as kidney or liver, within this model the only cells likely to be present and expressing ALP are mature osteoblasts.

Interestingly, these cells did not show markers for osteoblast differentiation until they were exposed to differentiation factors. That we were able to take cells that were not expressing specific differentiated markers and drive almost all of them to do so also suggests that we were initially starting with osteoprogenitors and/or mesenchymal stem cells, and able to take them through the various stages to osteoblastic differentiation. This model certainly deserves further investigation and should be an excellent resource for future research.

Most of the osteoblast cell lines from Dr Sadar were shown to eventually represent fibroblasts, although they often started out expressing osteoblastic factors. We did not have this problem in our model, our only failure was a culture attempt (OB4) that

had only a few very small pieces of bone with extensive electrocautery necrosis that did not grow out any cells at all. We believe our better yield is probably due to harvesting technique. Meticulous surgical removal of any soft tissue that is performed during the preparation of bone graft intra-operatively rather than post-operatively as done by Dr Sadar's team resulted in no soft tissue entering the culture flasks in our model.

We compared the expression of *HACE1* mRNA in osteoblast and osteosarcoma cell lines grown in monolayer culture by using qRT-PCR. The expression of *HACE1* mRNA was less in the osteosarcoma cell lines than in the osteoblast cell lines, and this was statistically siginificant. We have not been back to repeat this with the osteoblast models we developed later, and this is an experiment that is currently being completed.

Western blots of osteoblast and osteosarcoma cell lines grown in monolayer culture appear to show no difference in the levels of HACE1 protein between osteoblast and osteosarcoma cells. However, this protein may not be functional, and as the immunohistochemistry showed, the localisation of the HACE1 protein may be important. It may be that cells with higher doubling rates have HACE1 protein excluded from the nucleus. Analysing malignant cell lines for the localization of HACE1 protein would certainly be part of further investigations. (Western Blot analysis is included here in this logical progression, but was not in fact available until much later in the project).

In order to assess the functional effects of *HACE1* we transfected several of the osteosarcoma cell lines using lentiviral constructs containing either empty vector, functional *HACE1* or *HACE1* that had undergone mutation of the cysteine residue at 876 to serine to de-activate the enzymatic site. This gave us two negative controls; the empty vector with no protein, and the inactivated *HACE1*. Both the active and de-activated mutant constructs were linked to a hemagglutinin tag to aid detection as these experiments were performed prior to the development of a reliable antibody to HACE1 protein.

We tested these cell lines in several different environments. One of the hallmarks of tumour cell behavior is the ability to form independent colonies when grown in an environment free from contact with other cells or a solid surface[128]. The use of contact-free cell culture to assess the proliferative properties of tumour cells is well established[129].

Using soft agar assays we showed a difference in the ability of the transfected cell lines to produce colonies in a contact-independent environment. The osteosarcoma cells that had functional *HA.HACE1* transfected showed lower rates of colony formation. This was quite reproducible at different time points, consistent across different cell lines, and was statistically significant. In addition to the number of colonies, there was a clear difference in colony morphology.

We further investigated this with a Matrigel assay, which is similar in concept but the plating substance contains various growth factors as well as extracellular matrix

proteins. Matrigel is routinely used for 3D cell culturing of normal and transformed breast epithelial cell lines and results in the production of acini or gland-like structures. We also included factors that are important to osteoblast physiology and were able to show a striking difference in colony morphology between the transfected cell types. Those that had functional *HA.HACE1* transfected showed small, round colonies while the control cells showed aggressive, branching colonies.

We proceeded to a mouse model. After subcutaneous injection of the transfected cells, the OST cell line showed smaller tumours in the *HA.HACE1* transfected cells than with the control cells, in particular a reduction in the very large tumours. However, this trend did not reach statistical significance. The only cell line that grew satisfactorily in the mice was the OST cell line. Osteosarcoma cell lines are known to be difficult to grow in subcutaneous injection models[130].

The behavior of the cells transfected with the theoretically inactivated *HACE1* mutant (*HA.C876S*) is worth comment. In the contact-free culture models the cells exhibited an intermediate phenotype on a very consistent basis, although they most closely resembled the empty vector cells. They formed more colonies than the *HA.HACE1* cells, but less than the empty vector control cells. This is consistent with investigations in other, non-sarcomatous, cell types[109]. It may be that *HACE1* exerts some effects by a mechanism independent of this enzymatic site, or it may simply be due to the non-specific effects of a cell expressing extra protein, for example GFP expression has been shown to alter the behaviour of osteosarcoma cells[131].

In the mouse studies the *HA.C876S* mutant cell lines showed similar tumour formation to the empty vector cell lines. Given the intermediate behavior in the contact-free culture models we found this surprising and so we performed western blot analysis on tumour tissue for the hemagglutinin tag. This showed that the cells in the tumours were not expressing the mutant protein at all, suggesting that they had lost expression of the vector and returned to wild-type either immediately prior to or soon after injection.

Given the findings to date, we have satisfied our objective of establishing that *HACE1* does play a role in osteosarcoma, however the exact nature of that role remains unclear. Osteosarcoma is a complex tumour, with significant loss of known tumour suppressors such as *TP53* and *RB1*. It is possible that *HACE1* is playing a tumour suppressor role but that this role is minor compared to other factors, making it difficult to detect reliably.

It is also possible that *HACE1* plays a tumour suppressor role in some individual osteosarcomas, but that in other osteosarcomas additional effects in the same or similar biochemical pathways produce the same functional result as loss of *HACE1* even though it remains intact. This may be equivalent to the interaction of other tumour suppressors and promoters such as deletion of *CDKN2A* (in 10% of osteosarcomas) being equivalent to *MDM2* amplification[58], and is suggested by the striking differences in cellular localization of HACE1 protein in some tumours but not others.

Further routes of investigation can be divided into those directed at osteosarcoma, and those at normal osteoblast physiology.

Further work on the immunohistochemistry would be rewarding, with re-analysis of the specimens that showed no HACE1 protein signal at all. If other sections of these samples can be identified with internal positive controls, it will improve our dataset and allow further analysis. We also plan to correlate the high and low expressing osteosarcomas with clinical outcomes, and Research Ethics Board review of this proposal is pending to allow us to access matched clinical information.

Sequencing of the *HACE1* locus in osteosarcoma specimens would be useful if adequate samples can be obtained from paraffin blocks, although there are significant technical difficulties such as microdissection. With the BC Bone and Soft Tissue Bank established and funding procured by this author, sample collection is now reliable and efficient and projects that require quantities of fresh tissue should be achievable, although this would still be technically challenging. There are also novel osteosarcoma cell lines available through the Children's Oncology Group that have been established by tissue donation from this author's clinical practice.

If cell lines or specimens can be identified that reliably show low *HACE1* expression then the mechanism of that loss could be investigated. Anglesio[104] showed that the mechanism in Wilm's tumour was due to epigenetic silencing of the gene, and methylation of the HACE1 promoter region has also been shown in gastric and colorectal cancers[113,114]. Methylation-specific PCR could be performed on tissue from

those specimens with very low expression of HACE1 protein on immunohistochemistry.

However, perhaps the most exciting avenue for future research is investigation into the role of *HACE1* in normal human osteoblast development. Most genes that are implicated in tumorigenesis, whether oncogenes or tumour suppressor genes, are involved somehow in normal development, and the parallels between osteosarcoma and normal osteoblast maturation are highlighted extensively in the introduction.

The establishment of a reliable, novel model for normal human osteoblast maturation as part of this thesis leaves a great opportunity to investigate the role of *HACE1* in normal tissue. Possibilities include *HACE1* acting to drive cells into an osteogenic pathway, being required for terminal differentiation to osteocytes, or being part of the mechanism that turns off the immortality of mesenchymal stem cells, etc. Examining *HACE1* in a normal model, and establishing its normal functions would undoubtedly shed light on its role in malignancy.

In summary, *HACE1* does appear to play a role in osteosarcoma. We have shown that while the expression of *HACE1* does not reliably differ between osteoblasts and osteosarcomas, there are changes in the localization of *HACE1* in osteosarcoma cells that may explain a loss of function. Expression of *HACE1* in osteosarcoma cell lines changes their behaviour in cell culture and in mice xenografts. Further clarification of the role of *HACE1* in osteosarcoma and establishing the normal role of *HACE1* in cell development await further investigation.

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APPENDIX 1 Ethics Certificates

THE UNIVERSITY OF BRITISH COLUMBIA

ANIMAL CARE CERTIFICATE

Application Number: A04-0282

Investigator or Course Director: Poul H.B. Sorensen

Department: Pathology & Laboratory Medicine

Animals:

Mice 73

Start Date: **July 1, 2003** **Approval Date:** **December 19, 2008**

Funding Sources:

Funding Agency:	National Cancer Institute of Canada
Funding Title:	Altered Expression of a novel E3 Ubiquitin protein ligase gene, WT-Ank, in sporadic Wilms' tumour
Funding Agency:	National Cancer Institute of Canada
Funding Title:	Studies into the tumour suppressor activity of the HACE1 E3 ubiquitin-protein ligase
Funding Agency:	National Cancer Institute of Canada
Funding Title:	Altered expression of a novel E3 ubiquitin protein ligase, WT-ANK, In sporadic Wilms' tumour
Unfunded title:	N/A

The Animal Care Committee has examined and approved the use of animals for the above experimental project.

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

A copy of this certificate must be displayed in your animal facility.

Office of Research Services and Administration
102, 6190 Agronomy Road, Vancouver, BC V6T 1Z3
Phone: 604-827-5111 Fax: 604-822-5093

CHILDREN'S & WOMEN'S HEALTH CENTRE OF BRITISH COLUMBIA
AN AGENCY OF THE PROVINCIAL HEALTH SERVICES AUTHORITY

UBC C&W Research Ethics Board
A2-136, 950 West 28th Avenue
Vancouver, BC V5Z 4H4
Tel: (604) 875-3103 Fax: (604) 875-2496
Email: cwreb@cw.bc.ca
Website: http://www.cfri.ca/research_support > Research Ethics

ETHICS CERTIFICATE OF MINIMAL RISK APPROVAL: RENEWAL

PRINCIPAL INVESTIGATOR:	DEPARTMENT:	UBC C&W NUMBER:
Poul H.B. Sorensen	UBC	H03-70332

INSTITUTION(S) WHERE RESEARCH WILL BE CARRIED OUT:

Institution	Site
Children's and Women's Health Centre of BC (incl. Sunny Hill)	Child & Family Research Institute

Other locations where the research will be conducted:
N/A

CO-INVESTIGATOR(S):

Fan Zhang
Valentina Evdokimova
Michael S. Anglesio
Maureen J. O'Sullivan
Kenneth L. B. Brown
Paul W. Clarkson

SPONSORING AGENCIES:

- Canadian Cancer Society Research Institute - "Altered Expression of a Novel E3 Ubiquitin Protein Ligase, WT-ANK in a Sporadic Wilm's Tumour"
- Canadian Cancer Society Research Institute - "Altered Expression of a Novel E3 Ubiquitin-Protein Ligase, HACE1 in Sporadic Wilms' Tumour"
- Canadian Cancer Society Research Institute - "Studies into the Tumour Suppressor Activity of the HACE1 E3 Ubiquitin Ligase"

PROJECT TITLE:
Studies into the Tumour Suppressor Activity of the HACE1 E3 Ubiquitin Ligase

REMINDER: The current UBC Children's and Women's approval for this study expires: July 21, 2011

APPROVAL DATE: July 21, 2010

CERTIFICATION:
In respect of clinical trials:
1. The membership of this Research Ethics Board complies with the membership requirements for Research Ethics Boards defined in Division 5 of the Food and Drug Regulations.
2. The Research Ethics Board carries out its functions in a manner consistent with Good Clinical Practices.

3. This Research Ethics Board has reviewed and approved the clinical trial protocol and informed consent form for the trial which is to be conducted by the qualified investigator named above at the specified clinical trial site. This approval and the views of this Research Ethics Board have been documented in writing.

The Chair of the UBC Children's and Women's Research Ethics Board has reviewed the documentation for the above named project. The research study, as presented in the documentation, was found to be acceptable on ethical grounds for research involving human subjects and was approved for renewal by the UBC Children's and Women's Research Ethics Board.

Approved by one of:

Dr. Marc Levine, Chair **Dr. Caron Strahlendorf, Associate Chair**