Active-β-catenin (ABC) Transcriptional Activity is Associated with and may Promote Invasive Phenotype in Osteosarcoma (OS)

by

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Abstract

Osteosarcoma (OS) is an aggressive primary malignancy of the bone with the greater proportion of affected individuals being in the 10-30 years age range. The 5-year relative survival rates for patients with localized OS is 77% and 27% for patients with metastatic cancer. 15-20% of patients on initial diagnosis present with metastasis of OS. Identifying reliable prognostic biomarkers to facilitate early diagnosis and appropriate treatment strategies is crucial. The canonical Wnt/β-catenin pathway has been shown to be dysregulated in OS. I investigated the role that the Wnt/β-catenin pathway may have in the promotion of the invasive phenotype. Specifically, I focussed on the transcriptionally active form of β-catenin, Active-β-Catenin (ABC) and whether it may have a role in the promotion of invasive phenotype.

I used two sets of paired cell OS cell lines, SaOS2/SaOS2-LM7 and HOS/HOS-143B. I transfected the SaOS2 and HOS cell line with a pEGFP-C2-β-catenin fusion construct plasmid, or the pEGFP-C2-ABC plasmid which mimicked endogenous ABC. Using pEGFP-C2 (empty vector), pEGFP-C2-β-catenin, and pEGFP-C2-ABC I transfected the SaOS2 and HOS cells to measure what affect overexpression of ABC and β-catenin had on the overall invasive phenotype.

My *in vitro* results show that ABC increased the invasive abilities of the SaOS2 and HOS cells, increased migratory abilities in SaOS2 cells, and increased mRNA expression of MMP9 and MMP2 in SaOS2 and HOS cells. Our analysis on the OS pediatric patient tissues showed a positive association between high levels of ABC and metastatic tumors of OS patients at both times of diagnosis and resection. These results suggest that ABC influences the invasive phenotype seen in OS progression and that ABC has the potential to be used as a prognostic biomarker in OS.

Preface

This thesis is an original work by Takaaki Landry. The research project, of which this thesis is a part, received research ethics approval from the University of Alberta Research Ethics Board, "Osteosarcoma (OS) progression: a role for β-Catenin/Active-β-Catenin." No. pro0065705, 9th June 2016.

A dedication to my parents, Tim and Hiroko Landry

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List of abbreviations

β-Trcp - β-Transducin repeats-containing protein

143B – HOS-143B cell line

DAB - 3,3'-Diaminobenzidine

ABC – Active-β-catenin

Akt – Protein kinase B

ALP – Alkaline phosphatase

APC – Adenomatous polyposis coli

BCA - Bicinchoninic acid

BGLAP – Bone gamma-carboxyglutamate protein

BMP-2 – Bone morphogenetic protein-2

BMU – Basic multicellular unit

COL1A – Collagen type I

COX2 – Cyclooxygenase-2

CSC – Cancer stem cell

CSF-1 – Colony stimulating factor 1

Cx43 – Connexin-43

CXCL12 – C-X-C-motif chemokine ligand 12

CXCR4 – C-X-C-motif chemokine receptor 4

DAPI – 4'-6-diamidino-2-phenylindole

ddH2O – Double distilled water

DVL – Disheveled

ECM – Extracellular matrix

EGFP – Enhanced green fluorescent protein

EMT – Epithelial-mesenchymal transition

FasL – Fas ligand

FBS – Fetal bovine serum

FGF23 – Fibroblast growth factor 23

FITC - Fluorescein isothiocyanate

GSK-β - Glycogen synthase kinase 3β

HGF – Hepatocyte growth factor

HIF-1 – Hypoxia-inducible factor

HRP - Horseradish peroxidase

IL-8/CXCR1 – Interleukin-8/C-X-C-motif chemokine receptor 1

IHC – Immunohistochemistry

LM-7 – SaOS2-LM-7 cell line

lncRNA – Long non-coding RNA

LRP-5/6 – Low-density lipoprotein receptor-related protein 5 and 6

MCP-1 – Monocyte chemoattractant protein 1

MEM – Minimal essential media

MEPE – Matrix extracellular phophoglycoprotein

MIOS – Multi-Institutional Osteosarcoma Study

miRNA – MicroRNA

MMP – Matrix metalloproteinase

MSC – Mesenchymal stem cell

MSKCC – Memorial Sloan Kettering Cancer Centre

MSTS – Musculoskeletal Tumor Society

NK – Natural killer

NKD2 – Naked cuticle homolog 2

OPG – Osteoprotegerin

OS – Osteosarcoma

PBS – Phosphate buffered saline

PI3K – Phosphoinositide 3-kinase

PP2A – Protein phosphatase 2

PTH – Parathyroid hormone

PTH1R – Parathyroid hormone 1 receptor

PVDF – Polyvinylidene fluoride

RANKL – Receptor activator of nuclear factor κ-B ligand

RB1 – Retinoblastoma 1

RGD – Arginine-glycine-aspartic acid

RIPA - Radioimmunoprecipitation assay

RT-qPCR – Quantitative real time Reverse Transcriptase polymerase chain reaction or Quantitative real time PCR

RUNX2 – Runt-related transcription factor 2

Src – Proto-oncogene tyrosine-protein kinase

TBS – Tris buffered saline

TBST – Tris buffered saline with Tween-20

TCF/LEF – T-cell factor/Lymphoid enhancer factor

TGF-β - Transforming growth factor β

TP53 – Tumor protein p53

VEGF – Vascular endothelial growth factor

Chapter 1

Introduction

1.0. Introduction

1.1. What is the long bone?

The bones of the human body are pivotal structures that provide mechanical support, levers for our muscles which grant movement, and organ protection (Clarke, 2008). Along with these structural features they provide other functions such as calcium homeostasis and the majority of the bodies hematopoietic function (Clarke, 2008). In the human body, bones are generally categorized in to four types: flat bones, short bones, irregular bones, and long bones (Clarke, 2008).

The long bone is composed of a hollow shaft referred to as the diaphysis, the diaphysis is composed of cortical bone, or also known as compact bone (Clarke, 2008). A fibrous layer of connective tissue called the periosteum surrounds the diaphysis externally (Clarke, 2008). The periosteum can be further dissected into its two layers, the outer and inner layer (Dwek, 2010). The outer layer is important for blood supply to the bone and the inner layer, or also known as the Cambium layer, is composed of cells important to the biology of the long bone: this includes osteogenic progenitor cells, osteoblasts, and fibroblasts (Dwek, 2010). The hollow shaft of the long bone is lined with a layer called the endosteum, more specifically within the diaphysis it is called the cortical endosteum (Nahian & Chauhan, 2021). The endosteum is important for the long bones due to its functions which include appositional bone growth, bone repair, and bone remodelling (Nahian & Chauhan, 2021).

The epiphysis is what constitutes the two ends of the long bone and is composed of mostly trabecular bone, also known as spongy bone. The spongy bone houses the red marrow which is composed mostly of hematopoietic cells which can give rise to cells such as monocytes, macrophages, neutrophils, eosinophils, erythroblasts, erythrocytes, megakaryocytes, platelets, and osteoclasts (Clarke, 2008) (Gurkan & Akkus, 2008). The epiphysis like the diaphysis has an outer layer and an inner layer, the inner layer is called the trabecular endosteum and the outer layer is surrounded by a layer called articular cartilage. The role of the articular cartilage is to absorb shock and reduce friction.

To recap, the long bone consists of a long hollow structure call the diaphysis and two end structures known as the epiphyses, however there is another important structure that is a part of the long bone, the metaphysis. The metaphysis is a particular region that is characterized by its abundance of trabecular bone and thin layer of cortical bone (Clarke, 2008). The metaphysis is an important region in our research because it is a site that undergoes extensive bone remodelling during growth spurts and has been attributed as the primary site for osteosarcoma (OS) (Clarke, 2008) (Lindsey, Markel, & Kleinerman, 2017).

1.2. Bone formation

Bone undergoes adaptations throughout life to help preserve the skeletal structure and maintain mineral homeostasis for the integrity of the skeleton (Raggatt & Partridge, 2010). The formation of bone, or osteogenesis is completed in two separate ways with both requiring mesenchymal tissue transformation into bone tissue (Gilbert, 2000). Intramembranous ossification is when mesenchymal tissue is directly converted into bone and this type of formation is typically seen in the bone types like that of the skull (Gilbert, 2000). The long bones of the body are formed by endochondral ossification spreading from the centre of the diaphysis outwards bidirectionally to the epiphyses where the cartilage models formed from the transformation of mesenchymal cells into cartilage cells acts as a scaffold for the eventual replacement by bone (Gilbert, 2000) (Mackie, Ahmed, Tatarczuch, Chen, & Mirams, 2008). The ossification of bones is completed during fetal development and during postnatal growth and without this developmental mechanism the human body would not grow (Gilbert, 2000) (Mackie et al., 2008). And the ability to grow would not be possible without the epiphyseal growth plates in the metaphysis that gives bones the ability to grow (Gilbert, 2000).

1.3. Bone modelling and remodelling

There are two important processes that work in synchrony that are involved in the maintenance of bone modelling and remodeling (Raggatt & Partridge, 2010). Bone modeling is involved in growth and requires the processes of bone formation and resorption, whereas bone remodeling is in charge of bone removal and repairing of any damaged bone (Raggatt & Partridge, 2010). The process of bone modelling and remodelling involves multiple cell types to ensure that bone mass is preserved (Raggatt & Partridge, 2010).

1.4. Cells involved in bone modelling and remodelling

Osteoclasts are the only known cell type that are adapted to remove mineralized bone while also ensuring remodelling and development of the skeleton (Clarke, 2008) (Jacome-Galarza et al., 2019). They are characterized by their multinucleated cell bodies, tartrate-resistant acid phosphatase, and calcitonin receptor (Raggatt & Partridge, 2010). Survival, expansion, and differentiation of osteoclast precursor cells require the cytokines RANKL (receptor activator of nuclear factor κ-B ligand) and CSF-1 (colony stimulating factor 1) (Raggatt & Partridge, 2010). The regulation of osteoclastogenesis can be inhibited by the OPG (osteoprotegerin) which binds to RANKL and prevents its activity on osteoclasts and so the RANKL:OPG expression ratio is important when determining the function and differentiation of osteoclasts (Raggatt & Partridge, 2010).

While osteoclasts are involved in the removal of bone, osteoblasts have a special role in bone formation due to their ability to synthesize new bone from type I collagen and other matrix proteins they secrete (Clarke, 2008). Osteoblasts are cells derived from pluripotent mesenchymal stem cells, the differentiation of the pluripotent mesenchymal stem cells into osteoblasts is regulated by RUNX2 (runt-related transcription factor 2) and by the activation of the canonical Wnt/β-catenin pathway (Clarke, 2008) (Raggatt & Partridge, 2010). The differentiation pathway for osteoblasts is important because it results in the formation of a heterogenous population of osteoblasts that consists of two cell types, osteoprogenitors that are spindle shaped and differentiated osteoblasts that have a more cuboidal shape (Clarke, 2008). Interestingly enough, the two populations have different roles when it comes to the bone remodelling and modelling process, the osteoprogenitors assist with the process of osteoclastogenesis whereas the differentiated osteoblasts primarily have a role in production of the bone matrix (Raggatt & Partridge, 2010).

Osteocytes are osteoblasts that entrench themselves within the unmineralized osteoid in a space called the lacunae and have a role in supporting bone structure and bone metabolism (Clarke, 2008) (Raggatt & Partridge, 2010). Mechanosensation is an important function provided by the osteocytes, when they recognize a change in bone integrity they release signals to communicate

with osteoblasts on the surface of the bone and with other osteocytes within the lacunae indicating there has been a change in bone integrity and it is time to initiate the bone remodelling and modelling process (Raggatt & Partridge, 2010).

Bone-lining cells are flat cells and are proposed that their origins stem from the osteoblast lineage (Matic et al., 2016). There is no truly accepted function of the bone lining cells but one proposed action is that they help in the removal of matrix on the bone after bone resorption by the osteoclasts prior to the bone formation completed by osteoblasts (Matic et al., 2016).

The specialized bone cells mentioned earlier are important for bone modelling and remodelling, however there is also an important relationship between these cells and the cells of the immune system. T-cells and B-cells which are vital to the immune system also have a role in maintenance of bone, this was shown when mice that lacked T-cells or B-cells were seen to have osteoporosis (Raggatt & Partridge, 2010). To better understand why a skeletal pathology was associated with an immune system deficiency, an investigation into this phenomenon resulted in the discovery that >50% of the total bone marrow derived OPG came from mature B-cells which is vital to the regulation of osteoclastogenesis (Raggatt & Partridge, 2010). The evidence for T-cell involvement has been less clear but it is believed that T-cells work alongside B-cells to enhance OPG production, however future research into this is still required (Raggatt & Partridge, 2010).

Another cell that has a role in bone maintenance are the megakaryocytes, cells that have a role in blood clotting (Raggatt & Partridge, 2010). Megakaryocytes have shown to help with differentiation and proliferation of osteoblasts and also express RANKL and OPG, highlighting that megakaryocyte have the capacity to influence bone resorption and formation (Raggatt & Partridge, 2010).

In summary, it is clear that there are many moving parts involved in the maintenance of bone homeostasis and that it involves a complex series of interactions. Next I will review process of how both bone remodelling and modelling is completed.

1.5. Bone remodelling

Bone remodelling is a process that is completed by a combination of osteoclasts and osteoblasts, the combination of these cells forms a temporary structure called the BMU (basic multicellular unit) (Raggatt & Partridge, 2010). These BMU's allow for the "coupled" effect of osteoclast resorption and osteoblast bone formation and ensure that there is no discrepant net change in the bone during remodelling (Raggatt & Partridge, 2010). The BMU has a leading side where bone resorption occurs, following the bone resorptive activity of the osteoclasts an unknown cell line helps clean up the new surface of the bone which will then be followed by a tail end of osteoblasts secreting osteoid and assisting in the mineralization of the new bone (Raggatt & Partridge, 2010). The bone remodelling process is typically divided into five phases (Raggatt & Partridge, 2010).

1. Activation

For bone remodelling to occur, a signal needs to be released and perceived indicating the need for it. This can come in the form of mechanical strain that causes damage to the bone or the action of a hormone on bone cells in response to a change in homeostasis (Raggatt & Partridge, 2010). The cells that recognize the mechanical strain are the osteocytes, this mechanical strain can cause damage which ultimately leads to the apoptosis of osteocytes (Raggatt & Partridge, 2010). Since osteocytes secrete TGF-β (transforming growth factor β) which is involved in inhibiting osteoclastogenesis the apoptotic event created by the mechanical strain leads to a decrease in TGF-β and an increase in osteoclastogenesis, allowing for the activation phase to begin (Raggatt & Partridge, 2010). Another way to start the activation phase is through hormonal activity which can be seen when the body recognizes a decreased level of serum calcium (Raggatt & Partridge, 2010). Upon recognition of decreased calcium in the serum the parathyroid glands release PTH (parathyroid hormone), an endocrine hormone that can help maintain homeostasis of calcium (Raggatt & Partridge, 2010). The PTH will bind to osteoblasts and will activate intracellular signalling pathways which will lead to the production and secretion of molecules that will both recruit and start the differentiation of osteoclast precursors (Raggatt & Partridge, 2010). This process will help establish the necessary conditions required for bone resorption activity (Raggatt & Partridge, 2010).

2. Resorption

Once osteoblasts have received a signal either by endocrine activity or from osteocytes they will start the process of directing osteoclast precursors to the damaged site, they do this by releasing MCP-1 (monocyte chemoattractant protein 1), CSF-1, RANKL, and reducing the expression of OPG (Raggatt & Partridge, 2010). MCP-1 attracts osteoclast precursors, CSF-1 along with RANKL enhances motility, proliferation, survival, differentiation of osteoclast precursors into mature osteoclasts by allowing for reorganization of the cytoskeleton, extends the life of osteoclasts, and upregulates the resorptive abilities of osteoclasts (Raggatt & Partridge, 2010). MMPs (matrix metalloproteinases) are secreted from osteoblasts which help start the initial break down of osteoid to expose RGD (arginine-glycine-aspartic acid) adhesion sites for osteoclasts to bind to (Raggatt & Partridge, 2010). This attachment will allow the osteoclasts to form a "sealed zone", a microenvironment where they can pump in hydrogen ions, tartrate resistant acid phosphatase, cathepsin K, MMP9, and gelatinase to degrade and digest the organic bone matrix resulting in a cavity known as the Howships' lacunae on trabecular bone and Haversian canals on cortical bone (Clarke, 2008) (Raggatt & Partridge, 2010). Once the multinucleated osteoclasts have completed their resorption, these cells will undergo apoptosis (Clarke, 2008).

3. Reversal

These areas of bone resorption are now a messy collection of undigested collagen matrix and to clean them up the bone lining cells mentioned earlier follow the osteoclasts in the BMU's (Raggatt & Partridge, 2010).Once the area has been cleaned, this allows for the bone forming osteoblasts to lay down new bone (Raggatt & Partridge, 2010).

4. Formation

The formation phase is involved in the formation of bone, this process is comprised of three phases: production of osteoid, maturation of osteoid, and mineralization of the matrix (Hadjidakis & Androulakis, 2006). The osteoblasts are the cells that are in charge of this process due to their ability to produce organic matrix with collagenous origins and regulating the mineralization of it (Clarke, 2008). At the end of bone formation, osteoblasts that are buried within the matrix will undergo a transformation into osteocytes and most of the osteoblasts will undergo apoptosis

leaving behind a small population of osteoblasts, the bone lining cells, and osteocytes (Clarke, 2008).

5. Quiescence/Termination

The end of bone remodelling is described as the successful and equal level of both formation and resorption (Raggatt & Partridge, 2010). Though how it exactly ends is still speculative, some believe that osteocytes once they have been entrapped will release some sort of signal triggering the end of bone remodelling (Raggatt & Partridge, 2010).

1.6. What is Osteosarcoma?

Sarcomas are malignant tumors that arise in bone, cartilage, and muscles (Taran, Taran, & Malipatil, 2017). OS is the most common and prevalent primary malignancy of the sarcomas (Taran et al., 2017). OS is a type of sarcoma that typically exhibits excess production of osteoid by malignant mesenchymal stem cells (Lindsey et al., 2017) (Taran et al., 2017).The World Health Organization divides the classification of OS is into primary or secondary with primary OS being further divided based on its histological presentation; central/intramedullary and surface tumors (Kundu, 2014). Within the central/intermedullary category conventional central/intramedullary high-grade OS represents approximately 80% of all the total OS cases, whereas the other types of represent less than 5% of OS cases, respectively; telangiectatic, small-cell, and low-grade (Misaghi, Goldin, Awad, & Kulidjian, 2018). Surface OS represents less than 6% of all OS and consists of parosteal, periosteal, and high-grade surface (Misaghi et al., 2018).

1.7. Epidemiology

OS is most common during the rapid growth phase of bones, which is typically when adolescents are going through puberty (de Azevedo, J W V et al., 2020). Greater than 50% of cases originate at the femur, specifically the metaphysis of the distal femur and proximal tibia (de Azevedo, J W V et al., 2020). The epidemiology of OS indicates that the individuals most affected are children, teenagers and young adults from ages 10-30 (de Azevedo, J W V et al., 2020) (Taran et al., 2017).OS is the third most common cancer among adolescents constituting 5.6 cases per million children who are 15 years of age and younger (Misaghi et al., 2018). OS also accounts for 3-5% of all newly diagnosed cancers in the pediatric population (Misaghi et al., 2018) (Durfee,

Mohammed, & Luu, 2016). Although the reasons are unknown, biologically male children are reported to be more affected than biologically female children (Taran et al., 2017). There is also an ethnic disparity between black children and Caucasian children, where OS is slightly higher in the black children than in the Caucasian children (Taran et al., 2017). OS can also present in adults. However, it is important to note that over half of the adult cases of OS are seen in adults over the age of 60, and the majority of them are secondary neoplasms stemming from conditions such as Paget's disease and complications from bone lesions (Mirabello, Troisi, & Savage, 2009).

1.8. Initiation

Understanding the process of how OS initiates is important to understand the full pathology of this disease. There are two hypotheses as to the origin of OS, one being the mesenchymal stem cell (MSC) origin hypothesis and the other the osteoblast origin hypothesis (Lin, Y. H. et al., 2017). The MSC hypothesis claims that OS originates from mutated MSCs while the osteoblast origin hypothesis states that deregulation of the differentiation pathway of osteoblast cells leads to the development of OS (Lin et al., 2017) (Figure 1). These two hypotheses are contested because some believe that the steps of differentiation from MSC to osteoblast cells can be exposed to changes and alterations that would make the MSCs more favorable to undergo a malignant transformation while others believe that OS must be derived from osteoblasts because of the excess osteoid that is produced (de Azevedo, J W V et al., 2020) (Yang, Y. et al., 2017). These mutated cells that arise and gain this ability to produce osteoid reside in the intramedullary space within the bones. However, while it is possible for the sarcoma to grow on the surface of the bone, the periosteum, this only accounts for around 3-6% of all OS cases (Kumar, Barwar, & Khan, 2014) (Gorlick & Khanna, 2010)

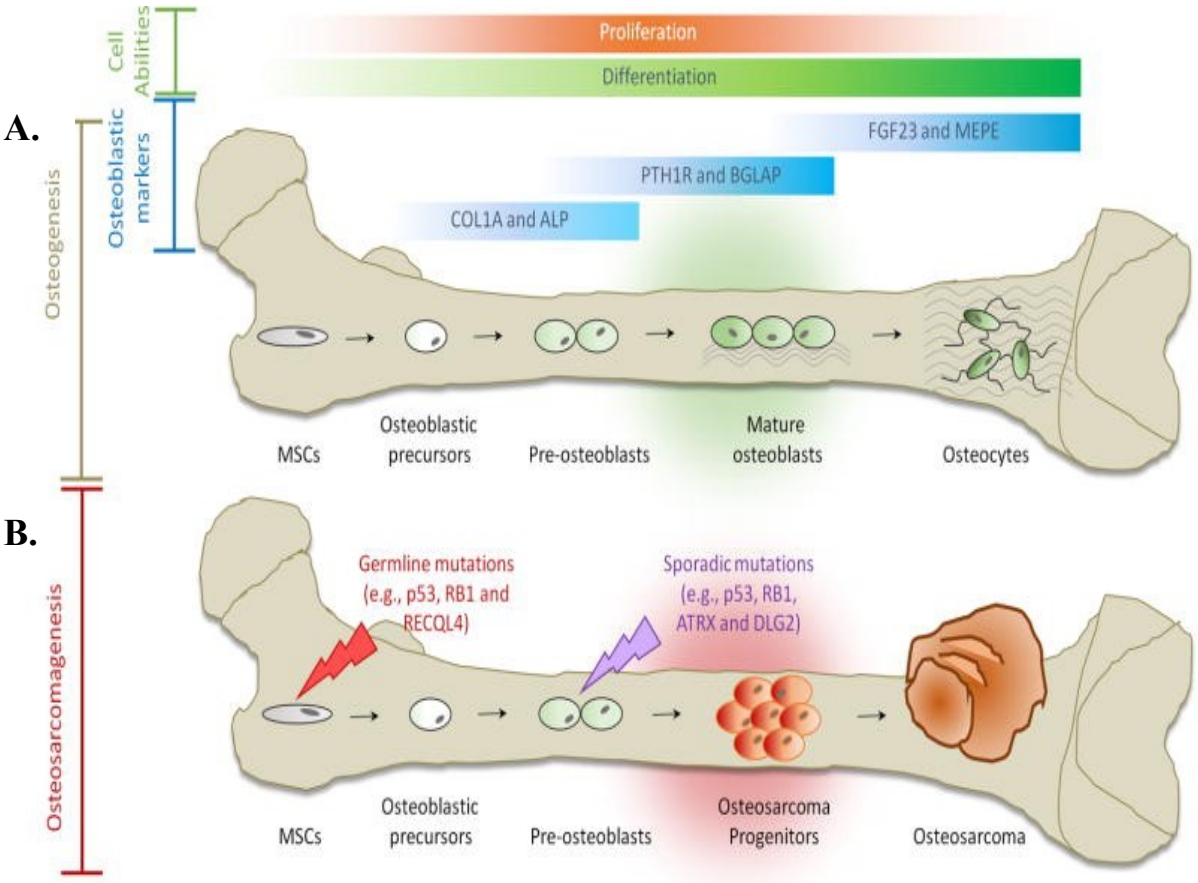


Diagram reprinted with permission from Lin *et al.,* 2017

Figure 1. Key Steps involved in Osteogenesis and Osteosarcomagenesis

A. Osteogenesis. The regulation of osteogenesis involves the differentiation of MSCs into mature osteoblasts. Three factors that have to be highly regulated is the signal transduction pathways, transcriptional regulators, and the genes responsible for cell cycle. Along each step of differentiation of MSCs there are important markers that are involved, osteoblastic precursors and pre-osteoblasts have collagen type I (COL1A) and alkaline phosphatase (ALP) markers, mature osteoblasts have parathyroid hormone 1 receptor (PTH1R) and bone gamma-carboxyglutamate protein (BGLAP), and osteocytes have fibroblast growth factor 23 (FGF23) and matrix extracellular phosphoglycoprotein (MEPE). **B. Osteosarcomagenesis** Any disruption of osteogenesis can lead to osteosarcomagenesis, this includes potential germline mutations that create the opportunity for MSCs to enter a state of incomplete differentiation and uncontrolled cell

proliferations. Such issues within osteogenesis can lead to the potential rise of OS progenitors which can form into a fully formed OS.

Now that there is an understanding of how OS initiates, the next step is to understand what mutations can lead to osteosarcomagenesis. OS has been characterized with having high levels of genomic instability, specifically chromosomal instability (Martin et al., 2012) Chromosomal instability is when sections or entire chromosomes are elevated or lost (Martin, J. W., Squire, & Zielenska, 2012). Studies have shown that individuals that have mutations of the genes tumor protein p53 (*TP53)*, retinoblastoma 1 (*RB1*), and *RECQL4* which are important checkpoints for mitosis are the potential causes for the chromosomal instability seen in OS and individuals with these mutations have higher incidences of OS (Martin et al., 2012). Most studies have focused on conventional OS, this is due to the fact that is the most frequent and that the rarer types typically present with their own distinguishable features not seen in conventional OS (Martin et al., 2012). These three genes that have been implicated in OS usually present in the form of inactivation of *RB1* and *TP53* and a dysregulation of *RECQL4* (Martin et al., 2012). *RB1* inactivation is frequent in OS, around 50% of patients with this inactivation presents as a deletion or mutation at the *RB1* (Martin et al., 2012). The *RB1* gene is involved in tumor suppression by regulating the cell cycle progression from G1/S (Martin et al., 2012). The *TP53* gene codes for the p53 protein which is a tumor suppressor with the role of recognizing DNA damage within cells and works to remove these damaged cells (Martin et al., 2012). More specifically in 29-42% of OS cases it has been shown to have a deletion at the 17p.13 locus of the *TP53* gene (Martin et al., 2012). Dysregulation of the *RECQL4* gene has been associated with the development of OS (Martin et al., 2012). The protein that is encoded by the *RECQL4* gene and that belongs to the RecQ helicase family is a DNA helicase. In individuals with the autosomal recessive Rothmund-Thomson syndrome there is a loss of function at the *RECQL4* gene due to a truncating mutation which predisposes these individuals to a higher risk of OS (Martin et al., 2012). However, it is important to note that in a sporadic OS case the rate of *RECQL4* mutation represents less than 5% of these cases and typically these sporadic cases showed that there was actually an increased level activity of the *RECQL4* gene contrary to that seen in Rothmund-Thomson syndrome affected individuals (Martin et al., 2012). Other syndromes like RAPADILINO can be associated with OS, however it is extremely rare and mainly seen in Finland, but is caused by a RECQL4 mutation (Calvert et al., 2012).

Another syndrome known as Werner syndrome which is primarily seen in Japan is also associated with OS (Calvert et al., 2012) Bloom syndrome which is characterized by a short stature, sun-sensitive rash, and subcutaneous fat that is sparce in a child is associated with OS, although its incidence is low within the population of people affected with Bloom syndrome, it exceeds the expected rate in the general population (Calvert et al., 2012). Lastly another syndrome known as Diamond Blackfan anemia which is a rare condition with an incidence rate of 1 in 100,000 and 1 in 200,000 has also been implicated with and associated with OS (Calvert et al., 2012). With that being said, it is important to note that the genetics of OS are complex and largely inconsistent and although research has an understanding of the roles of the more common genetic anomalies like *TP53*, *RB1*, and *RECQL4* and how the genes play in the pathogenesis of OS, a vast majority of the cases of OS arise from patients with no known germline abnormalities (Taran et al., 2017) (Martin et al., 2012).

Table 1. Osteosarcoma associated syndromes (adapted from Calvert et al., 2012)

Syndrome	Gene	Chromosome	Gene Product
Li-Fraumeni	TP53	17p13.1	Tumor Suppressor
Retinoblastoma	RB1	13q14.2	Tumor Suppressor
Rothmund Thomson II	REQ4	8q24.3	DNA Helicase
RAPADILINO	REQ4	8q24.3	DNA Helicase
Werner	WRN	8p12	DNA Helicase
Bloom	BLM	15q26.1	DNA Helicase
Diamond Blackfan	RPS19, RPL5, RPL11, RPL35A, RPS24, RPS17, RPS7, RPS10, RPS26	Multiple	Ribosomal Protein

Now that a basic understanding of the genomic mutations that are potential causes for osteosarcomagenesis and how they originate has been visited the exploration in how the development of the tumor happens will be done next. In general tumors are characterized by the

uncontrolled proliferation of cells and this process requires a lot of energy. In order to sustain this process, cancerous cells facilitate angiogenesis (Zheng, Wang, Chen, Hua, & Cai, 2018). Without angiogenesis the tumor would fail to develop and grow because it needs to be able to transport nutrients and also remove waste (Martin, T. A., Ye, Sanders, Lane, & Jiang, 2013). These newly formed blood vessels due to angiogenesis allow for an important process of intravasation, allowing the cancerous cells an easy way to enter the circulatory system which is vital for metastasis (Martin et al., 2013). Vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF) are important proteins that have demonstrated this angiogenesis enhancement (Martin et al., 2013). VEGF regulates proliferation and survival of endothelial cells and is a major target for the development of anti-angiogenic therapies (Martin et al., 2013). HGF has been associated with the metastatic traits like motogenesis, morphogenesis, mitogenesis, and angiogenesis (Martin et al., 2013). This association with these metastatic traits is both indirect and direct due to HGF assisting in stimulating morphological changes, increasing motility of endothelial cells, and enhancing the effect of VEGF (Martin et al., 2013). These processes of excess proliferation require the availability of growth factors to allow development of these tumors (Cheng et al., 2020). Growth Hormone receptor (GHR) and Growth Hormone (GH) are important growth factors as they can influence certain processes like aging, metabolism, and nuclear translocation (Cheng et al., 2020). More specifically, studies have shown that the interaction of GHR and GH can play a role in the formation of tumors and in OS (Cheng et al., 2020). Cheng et al., 2020 demonstrated this interaction by showing that when GHR was knocked down in 143B and U2OS cells there was significant inhibition of the cells ability to form colonies and the promotion of apoptosis. These changes in protein expression are important for the initiation of OS, however, it is important to note that although we investigate some of the more invaluable proteins there are others that assist in tumor initiation, invasion, and metastases: serine/threonine kinase receptor-associated protein and GIT1 are involved in growth, invasion and angiogenesis (Cheng et al., 2020).

1.9. Metastases

With the tumor starting to develop and OS progressing, the next and by far the most dangerous feature involved in the pathogenesis of this disease is the metastatic component. Metastases is when cancer cells that are a part of the primary tumor disseminate to the distant sites,

this process can be broken down into a step-by-step process known as the metastatic cascade (Fan, Roberts, & Lizardo, 2020). It involves a series of steps including dissemination of tumor cells from the primary tumor, degradation of the ECM, intravasation and navigation through the vasculature, extravasation and arrest, and lastly colonization of the secondary site, typically the lungs in the case of OS (Gorlick & Khanna, 2010) (Fan et al., 2020) (Broadhead, Clark, Myers, Dass, & Choong, 2011) (PosthumaDeBoer, Witlox, Kaspers, & van Royen, 2011). Although the process of metastases in OS is laid out with a step-by-step manual, it is actually not as easy as it seems because of the natural barriers within the body that are in place to prevent the metastatic cascade. Some of these barriers include the cells have to disseminate from the tumor and from each other, gain the ability to become motile and invasive to leave the initial site and degrade the extracellular matrix (ECM), circulate in the circulatory system while adjusting to the fluid shear stress, avoiding the process of anoikis, evade immune system destruction, and be able to extravasate and colonize at the secondary site (Fan et al., 2020) (Fares, Fares, Khachfe, Salhab, & Fares, 2020). The reason that there is a lot of information about metastases in OS is because typically the secondary site is the lungs and is the main cause of death, even if a possible resection of the metastases in the lungs is done the 5-year survival rate is ~40% due to recurrence (Gorlick & Khanna, 2010).

(i) Degradation of the ECM

In order for metastasis to occur there needs to be detachment of the cell-cell and cell-ECM interactions, these actions are important to allow the OS cells to dissociate and invade into surrounding stroma. One of the major and common adhesion molecules that is important for this eventual detachment from other cells are the integrins (Martin et al., 2013) (Florencio-Silva, Sasso, Sasso-Cerri, Simoes, & Cerri, 2015) (Broadhead et al., 2011). Integrins play a role in cell signalling, cell migration, and cell adhesion by assisting the cells connection to the ECM due to their interaction with fibronectin (Martin et al., 2013) (Broadhead et al., 2011). This interaction has been shown to be crucial in the progression of metastases in OS because cells in general aggregate towards ECM that is more rigid and ligand dense which occurs during OS due to the increased activity of fibroblasts, collagen deposition, and fibronectin(Broadhead et al., 2011) (Cui, Dean, Hornicek, Chen, & Duan, 2020). The stiff ECM results in the formation of integrin cluster, cell signaling pathway activation, stimulation of tumour growth, and most importantly the decrease in cell-cell interactions (Broadhead et al., 2011). As cell-cell interactions start to cease, there is

now one more barrier to overcome, the degradation of the ECM. The OS cells will produce MMPs, specifically MMP2 and MMP9, to break down collagen and facilitate the invasion of the tumour and endothelial cells which are vital for the development of leaky blood vessels and the progression of metastases into intravasation (Broadhead et al., 2011) (Cui et al., 2020). The promotion of the invasive capacities and decrease in cell-cell interactions is assisted by a phenomenon known as the epithelial-mesenchymal transition (EMT), a change from an epithelial phenotype to a mesenchymal phenotype (Danieau, Morice, Rédini, Verrecchia, & Royer, 2019). In EMT we will see the repression of intercellular junctions and an upregulation of proteins like N-cadherin, vimentin, and fibronectin which are important mesenchymal markers (Danieau et al., 2019). This transition from epithelial phenotype to mesenchymal phenotype is also not binary but rather creates a spectrum of cells that express both epithelial and mesenchymal phenotypes, the cells that express a mix are typically more successful due to their efficacy in disseminating, circulating the system effectively, colonizing and the development of metastasis (Fares et al., 2020). Along with the changes to the ECM, the OS cells undergoing EMT they will start to lose those cell-cell adhesions, undergo transcription factor activation, changes to the cell surface proteins, reorganization of the cytoskeletal proteins, promotion of stem-cell-like characteristics which allows for the constant self-renewal involved in tumorigenesis, this phenotype is called a cancer stem cell phenotype (CSC), and as mentioned earlier increased secretion of MMPs (Martin et al., 2013)(Danieau et al., 2019).

(ii) Intravasation and navigation through the vasculature

The development of new blood vessels, angiogenesis, is an important factor involved in the progression of the tumor and metastatic potential (Duffy, Bouchier-Hayes, & Harmey, 2013). Angiogenesis allows the tumor site to be supplied with nutrients, allow for the dissemination of metastatic cells, and a site for intravasation (Duffy et al., 2013). The steps involved in angiogenesis include the endothelial cells proliferating and migrating to form new vessels and the recruitment of pericytes/smooth muscles cells to produce the vessel (Duffy et al., 2013). An important factor for this is VEGF an angiogenic factor which has been seen to be upregulated in tumor development (Duffy et al., 2013). In order for tumor cells to metastasize they have to be able to circulate throughout the body and the increase in blood or lymphatic vessels allows for the OS cells to transit do so (Chiang, Cabrera, & Segall, 2016). Although OS cells were able to undergo the necessary

changes required to disseminate and degrade the ECM the entry into circulatory system is considered an inefficient method because the shear stress from the blood decimates a majority of the cells (Duffy et al., 2013). Even if the cells are able to survive the shear stress another obstacle that presents itself is the ability to escape the surveillance of the immune system, specifically natural killer NK cells and CD8+ T cells (Zheng et al., 2018). One of the mechanisms that is used to evade the immune system is the secretion of cytokines like TGF-β which can dampen the effects of the antitumor response (Zheng et al., 2018). Another mechanism that can be done is to down regulate human leukocyte antigen HLA I and Fas, receptors on the cell surface that hides them from CD8+ T cells and evade cell mediated apoptosis by Fas ligand (FasL) on NK cells, respectively (PosthumaDeBoer et al., 2011). Evading the immune system seems to be an important mechanism, but another way the OS cells cope within the circulatory system is increasing cell cycle progression via the involvement Active β-catenin (ABC) co-regulating transcription of oncogenes within the Wnt/β-catenin pathway to promote survival (PosthumaDeBoer et al., 2011).

There are many factors that can make the travel of cancerous cells in the blood or lymph difficult, an important barrier that cells must circumvent is the action of anoikis. Anoikis is triggered when cells lose their interaction with the ECM and undergo programmed apoptosis, a vital process for maintaining homeostasis and stability in tissues (Diaz-Montero, Wygant, & McIntyre, 2006). In OS, anoikis resistance has been described as the cells ability to grow anchorage independent and has been extensively studied due to its impact in cancer cell survival, migration, and colonization at the secondary site (Diaz-Montero et al., 2006) (Gao et al., 2019). Due to the importance of anoikis resistance in OS, research into understanding how it is regulated has shown that the activation of PI3K/AKT, Src, Wnt/β-catenin, and other molecular pathways play a role in the establishment of anoikis resistance (Gao et al., 2019).

(iii) Extravasation

It has been noted that cancer cells tend to have a specific target where they metastasize and in OS over 80% of all metastases occurs in the lungs (Ando et al., 2012). Because the size of tumor cells is larger than what normal cells are, they tend to be considered trapped in the microcirculation (Ando et al., 2012). The average size of the alveolar capillaries is around 5-8 μm whereas the osteoblastic OS cells are around 10-19 μm in diameter (Fan et al., 2020). The extravasation

process is also mediated through the expression of chemokines and proteinases, the combination and interaction of C-X-C-motif chemokine receptor 4 (CXCR4) and the ligand associated with it C-X-C-motif chemokine ligand 12 (CXCL12) promotes for the extravasation of OS cells in the lungs due to their ability to form a chemotactic gradient allowing for organ specific metastases (Ando et al., 2012).

(iv) Lung colonization

Since the cells had to disseminate from its previous cell-cell adhesions in the initial stage of the metastatic pathway, in order to attach itself to the new secondary site it now has to re-establish those adhesions. This process has to be successful for the tumor cells because the majority of tumor cells do not reach this final stage and those that do are a small population of 1-6% (Fan et al., 2020). The colonization of the cells is considered to be non-linear and newly arrested cells undergo many different options, they can either enter a state of dormancy, undergo apoptosis, or grow (Ando et al., 2012). The dormancy has two possible choices, tumor mass dormancy or cell dormancy (Ando et al., 2012). In tumor mass dormancy, or also referred to as dormant micrometastases, the cells will divide but the growth will be limited. In cell dormancy the cells will become quiet and stay dormant but become very difficult to remove (Ando et al., 2012). This dormancy has been postulated for when cells are unable to bind with the new locations ECM, however binding to the ECM via integrins has been shown to activate the cell from a dormant state into a proliferative state (Ando et al., 2012).

In conclusion, The progression of OS metastasis hinges on many different steps within in the metastatic cascade and that the success of cancer cells for the secondary site is lined with adversities and obstacles. However, regardless of the many natural barriers, cancer cells develop a multitude of abilities to circumvent these roadblocks. However, the average proportion of people diagnosed with metastasis at OS diagnosis is 18% indicating that there is a unfortunate level of success by these OS cells in their ability to successfully maneuver through the human body's natural defences and proceed with the metastatic cascade (Marko, Diessner, & Spector, 2016).

1.10. Clinical manifestations

OS in the early stages typically cannot be palpated and can escape visualization from x-ray diagnosis (Taran et al., 2017). A typical presentation of OS includes the patient experiencing pain and swelling in the affected areas, mainly the limbs. However, while patients may experience pain over several months, OS is normally detected at the time of a recent trauma or worsening of the localized pain for which medical assistance is sought (Taran et al., 2017). Majority of OS cases in children do not have any identifiable risk factors (Harrison, Geller, Gill, Lewis, & Gorlick, 2018).

1.11. Diagnosis

Laboratory findings are typically not correlated with disease extent however physicians will use alkaline phosphatase and lactate dehydrogenase measurements to help discern the prognosis of the current tumor (Taran et al., 2017). A definitive test to confirm the presence of OS is via a biopsy of the lesion (Taran et al., 2017) . However, even with current strategies and treatments 15-20% of patients on their initial diagnosis present with metastasis of the lungs, and 40% of patients will develop metastases within the lungs if the OS is untreated (Taran et al., 2017)

1.12. Staging system

According to the American Cancer Society, once a patient has been positively diagnosed with OS they will begin the process of staging the cancer. The Musculoskeletal Tumor Society (MSTS) staging system, or also known as the Enneking system, is the preferred staging system for bone sarcomas and is based on three key pieces of information. Grade (G) within the MSTS staging system will inform the likelihood that the tumor will grow and spread. This is based on the appearance of the cells under a microscope. They can either be classified as low grade (G1) or high grade (G2); low grade cells can be indistinguishable from normal cells whereas the high-grade cells have a very abnormal look to them. Extent of the primary tumor (T) is used to characterize whether the tumor is intracompartmental (T1) which refers to the tumor remaining within the bone, or extracompartmental (T2) meaning that it has expanded its growth into nearby structures. The last piece of information used for staging is metastasis (M) and is indicated by either the tumor has not spread (M0) or it has (M1). Based on all of this information the OS will then be given a staging ranging from IA, IB, IIA, IIB, and III, the combinations that define each staging can be seen in Table 2.

Table 2. The Musculoskeletal Tumor Society staging system (adapted from the American Cancer Society)

Stage	Grade	Tumor	Metastasis
IA	G1	T1	M0
IB	G1	T2	M0
IIA	G2	T1	M0
IIB	G2	T2	M0
III	G1 or G2	T1 or T2	M1

1.13. Current Treatments

The current strategy to manage a newly diagnosed case of OS involves the use of neoadjuvant chemotherapy before and after the surgical removal of the tumor. If possible, all the evident/accessible metastases are removed as well (Isakoff, Bielack, Meltzer, & Gorlick, 2015). This treatment strategy was developed over several years of studies, the Multi-Institutional Osteosarcoma Study (MIOS) has defined the importance of jointly implementing chemotherapy and surgical removal of the tumor (Isakoff et al., 2015). Completed in 1982-1984 this new process showed that the 6-year-survival with just surgery which was around 11% could be drastically increased to 61% when patients received adjuvant chemotherapy along with surgery (Isakoff et al., 2015). During the time of this study another study done at Memorial Sloan Kettering Cancer Centre (MSKCC) introduced neoadjuvant chemotherapy in their protocol (Isakoff et al., 2015). This trial showed that the 5-year survival rate could be increased to 65% (Isakoff et al., 2015). A study from 2018 reviewing the current approach to treating OS explains that the 5-year-event-free survival of patients who do not have metastatic disease is ~60-70% (Harrison et al., 2018). There has also been implications of radiation use in treating cancer but according to the American Cancer Society OS cells are notorious for not being easily irradicated by radiation, so it is not part of the conventional treatment strategy. It is evident that over the last 30-40 years there has not been much of a change in the outcome for patients with OS.

1.14. Etiology

The etiology of OS is largely unknown. However, there are some known risk factors that have been associated with OS development: environmental factors, Paget's disease, genetic conditions, and other bone abnormalities (Ottaviani & Jaffe, 2009). Environmental factors that could affect the risk of developing OS include ionizing radiation (which is indicated in ~3% of OS cases), alkylating agents, and trauma which both account for a very small proportion of OS cases (Ottaviani & Jaffe, 2009). The effects of viruses and perinatal factors are suggested but has been complicated and contradictory (Ottaviani & Jaffe, 2009). Risk factors that are specific to the patient's characteristics are considered host factors and these include age, gender, ethnicity, and growth spurts (Ottaviani & Jaffe, 2009). Genetic conditions such as the inactivation of p53 and RB1 genes has been seen in a significant amount of OS cases (Ottaviani & Jaffe, 2009). Li-Fraumeni syndrome has been seen in around 3% of OS cases, Rothmund-Thomson, Bloom, and Werner syndrome positive patients have been seen to develop OS and all three are diseases where there is a mutation in the RECQ-family DNA helicase (Ottaviani & Jaffe, 2009). 1% of patients with Paget's disease has been seen to develop OS and is typically seen in patients who are 40 and older (Ottaviani & Jaffe, 2009). Other bone abnormalities that can lead to potential risk of OS are changes that occur in the bone from radiation, osteochondromas, Ollier disease, and sites of bone infarcts to just name a few of the bone abnormalities that could increase the risk of developing OS (Ottaviani & Jaffe, 2009). However, as mentioned earlier the vast majority of OS cases in children are sporadic and most do not have any known hereditary complications (Taran et al., 2017).

1.15 Histology

Histology in cancer is the description of the tumor based on how the cancer tissue and cells present and the likelihood of growth and spread (Lin, P. P. & Patel, 2013). The histology of the cells can be used to determine prognosis and the initiation of a treatment strategy (Lin, P. P. & Patel, 2013). OS can have many histological variants, the most common is the conventional OS which has three histologic subtypes: fibroblastic, chondroblastic and osteoblastic (Lin, P. P. & Patel, 2013). There are several rare histological variants which are telangiectatic, small cell, epithelioid, and giant cell-rich (Lin, P. P. & Patel, 2013). These rare variants typically produce lytic lesions, and the histology of these tumors is based on their name (Lin, P. P. & Patel, 2013). There are two types of OS called well-differentiated and are typically low-grade, the two types are well-differentiated

intramedullary and parosteal (Lin, P. P. & Patel, 2013). Periosteal OS is similar to parosteal however their difference is that periosteal tends to have a chondroblastic histological appearance (Lin, P. P. & Patel, 2013).

1.16. Prognosis

The American Cancer Society with information obtained by SEER which is then maintained by the National Cancer Institute stated that the 5-year relative survival rates for OS between 2008 and 2014 for localized OS was 77%, regional (spread outside the bone and into nearby structures or has reached lymph nodes) was 65%, and metastatic cancer was 27%. However, another study from 2017 reported that the metastatic 5-year survival could be as low as 13% (Lindsey et al., 2017).

1.17. Biomarkers and other pathways involved that are affected by osteosarcoma

The fact that little has changed in the survival rate over the last three decades highlights the importance of identifying novel biomarkers that can assist in the early diagnosis and prognosis of patients with OS, more specifically for patients where OS has metastasized (Zamborsky, Kokavec, Harsanyi, & Danisovic, 2019). Biomarkers, according to the World Health Organization are "any substance, structure or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease". These structures can be anything from DNA, RNA, proteins, and peptides (Goossens, Nakagawa, Sun, & Hoshida, 2015). There have been many prognostic biofactors investigated such as MMP9, hypoxia-inducible factor (HIF-1), cyclooxygenase-2 (COX2), miR-195, miR-21, TGF-β, proto-oncogene tyrosine-protein kinase (Src), and Ezrin but unfortunately the research on them has not improved outcomes for patients with OS (Zamborsky et al., 2019). Therefor there is dire need for the discovery of a novel biomarker that will assist in early diagnosis in patients with OS and the possibility of it being a potential therapeutic target (Zamborsky et al., 2019). To do this a better understanding of the biological mechanisms driving this malignancy is paramount for identifying prognostic markers for aggressive/metastatic disease and if we are able to discover more reliable prognostic markers it could allow us to discern an early diagnosis of the aggressiveness of the disease and promote the early initiation of appropriate treatment strategies (Zamborsky et al., 2019). Looking at different pathways affected in OS can allow us to hopefully discover a novel and more reliable prognostic

biomarker. The Wnt/β-catenin pathway has been implicated as an affected pathway in OS and my research focuses on the biomarker that is ABC (Saraf, Fenger, & Roberts, 2018).

1.18. The Wnt/β-catenin pathway

The Wnt/β-catenin pathway, also known as the Wnt canonical pathway has been shown to be dysregulated in OS (Ali et al., 2019). However, to understand the effect of the Wnt/β-catenin pathway it is important to understand what happens when there is the presence or absence of the Wnt protein. In the event of Wnt absence or inhibition the cytoplasmic β-catenin is recruited by the Axin complex (destruction complex) (Ali et al., 2019) (Lin, C. H., Ji, Chen, & Hoang, 2014). The Axin complex consists of the proteins Axin, adenomatous polyposis coli gene product (APC), Casein Kinase 1 (CK1), and Glycogen synthase kinase 3 (GSK3-β) (Krishnamurthy & Kurzrock, 2018) (MacDonald, Tamai, & He, 2009). The Axin complex regulates β-catenin by sequential phosphorylation by CK1 and GSK3-β at the destruction domain located at the amino terminal region of β-catenin (Ali et al., 2019) (Lin et al., 2014). This phosphorylation of β-catenin allows the E3 ubiquitin ligase subunit β-transducin repeats-containing protein (β-Trcp) to target β-catenin for ubiquitination and proteasomal degradation (MacDonald et al., 2009). When Wnt is present it will bind to low-density lipoprotein receptor-related protein 5 and 6 (LRP-5/6) and Frizzled receptors and recruit the scaffolding protein Disheveled (DVL) which in turn recruits the proteins that constitute the Axin complex, phosphorylating it, which ultimately leads to an inhibition of GSK3-β (Lindsey et al., 2017) (Ali et al., 2019) (Lin et al., 2014) (MacDonald et al., 2009). This process allows β-catenin not to be targeted for degradation leading to an accumulation of β-catenin in the cytoplasm and its stabilization (Lin et al., 2014) (Persad et al., 2016). The accumulating β-catenin then undergoes a partial dephosphorylation at serine 37 and threonine 41 by the phosphoinositide 3-kinase (PI3K) pathway via the activity of protein phosphatase 2 (PP2A) (Persad et al., 2016). This post translational modification helps create a pool of β-catenin known as ABC which has been shown to preferentially localize to the nucleus (Ali et al., 2019) (Lin et al., 2014) (Persad et al., 2016) (Pai et al., 2017).ABC translocates to the nucleus and will act as a co-activator for the T-cell factor/lymphoid enhancer factor TCF/LEF family of DNA binding proteins to activate transcription of its target genes which involve oncogenes like *c-myc*, *Cyclin D1*, and *Axin-2* which are important for the enhancement of cell proliferation, differentiation, and invasiveness (Ali et al., 2019) (Lin et al., 2014) (Persad et al., 2016) (Pai et al., 2017). I am

investigating the effect the Wnt/β-catenin pathway, more specifically ABC has on OS due to its association with metastatic dissemination, recurrence of the tumor, and tumorigenicity (Danieau et al., 2019)

The dysregulation of the Wnt/β-catenin pathway has been shown to deregulate homeostasis and the natural cycle of bone remodeling so that it benefits the development of OS (Ali et al., 2019) (Danieau et al., 2019). The Wnt/β-catenin pathway plays a role by changing the differentiation and activity of both osteoblasts and osteoclasts (Danieau et al., 2019). Osteoblast's bone forming ability and differentiation is increased by the induction of Wnt ligands activating the Wnt/β-catenin pathway which affects osteoclasts due to the increased OPG produced by the osteoblasts and osteocytes (Danieau et al., 2019). However, osteoblasts also secrete Wnt ligands which then enhances the proliferation of osteoclast precursors via the Wnt/β-catenin pathway (Danieau et al., 2019). This ultimately leads to increased osteoclast resorption that releases pro-tumoral factors and Wnt ligands. This cycle of bone resorption and osteoblast bone formation is known as the "vicious cycle" (Danieau et al., 2019).

A key component of the metastasis of OS is the EMT like process (Danieau et al., 2019). A recent study showed the involvement of the Wnt/β-catenin pathway in the EMT like process in OS cells by demonstrating the increase in the expression of bone morphogenetic protein-2 (BMP-2) which ultimately led to an enhancement of motility and invasive capacity (Tian et al., 2019). Other molecules like Fibulin-3, N-terminal truncated form of carboxypeptidase, ubiquitin-specific protease 7 have been also associated with helping these OS cells undergo EMT due to activation via the Wnt/β-catenin pathway (Danieau et al., 2019).

Long non-coding RNA (lncRNA) and microRNA (miR) have recently starting to gain interest in their ability to promote OS progression (Danieau et al., 2019). Studies have shown that overexpression of many miRs in OS cells have shown the ability to increase growth and metastasis (Danieau et al., 2019) The most likely cause is associated with the miRs down regulating Wnt/β-catenin pathway antagonists (Danieau et al., 2019). One example of this is that OS tissue and cells had overexpression of miR-552-5p when compared to healthy tissue and osteoblasts, furthermore, when miR-552-5p was knocked down there was a significant decrease in proliferation and

metastasis (Danieau et al., 2019). miRs like miR-377, miR-873, mir758, and miR-885-5p which target intermediates and regulators that inhibit the Wnt/β-catenin pathway have been shown to be downregulated leading to increased activation of the Wnt/β-catenin (Danieau et al., 2019).

The Wnt/β-catenin pathway has been implicated in both tumorigenesis and dissemination of cancer cells from the primary tumor, but also plays a role in the process of intravasation (Danieau et al., 2019). Intravasation is vital for the cells to enter the blood stream and travel to the secondary site and is well documented that an increase in MMPs are linked to the increased invasive capacities in OS (Danieau et al., 2019) Two MMPs that have been closely studied are MMP9 and MMP2 due to increased expression and their association with metastases, poor prognosis, and chemotherapeutic resistance (Danieau et al., 2019). As discussed earlier it has been observed in OS that patients with high VEGF levels are typically associated with low overall survival (Danieau et al., 2019). This association most likely comes from the VEGF gene promoter having TCF binding sites that allow ABC to preferentially bind to it (Danieau et al., 2019). One study showed that when the negative regulator of Wnt, naked cuticle homolog 2 (NKD2) was present it significantly decreased the expression of vascular developmental genes, which then suggest that the Wnt/β-catenin pathway has a pro-angiogenic role in OS (Danieau et al., 2019).

In summary the evidence of the Wnt/β-catenin pathway playing an important role in the development of tumors and metastases in OS is substantial. With this being said the importance of studying the Wnt/β-catenin pathway and more specifically ABC could have strong implications on determining how it regulates OS progression in hopes to better understand this disease.

1.19. Hypothesis

The Wnt/β-catenin pathway and ABC play a role in OS progression. I investigated the association between ABC and the OS progression. The hope for our lab's research is that in the future it will contribute to the understanding of ABC and its role and whether it can be used as a potential prognostic biomarker for metastatic OS. **I hypothesize that there is a positive association between the Wnt/β-catenin pathway, more specifically ABC and the promotion of the invasive phenotype observed in OS progression**. This investigation was done studying two OS cell lines coupled with the metastatic cell lines and studying the effect of overexpressing ABC and β-catenin. I expect that the overexpression of ABC in the parental cell lines will cause these parental cell lines (SaOS2 and HOS) to elicit invasive characteristics similar to that of the metastatic cell lines (LM7 and 143B).

Chapter 2

Materials and methods

2.0. Materials and Methods

2.1. Immunohistochemistry (IHC)

For deparaffinization and rehydration of tissue slides, slides were baked at 60^{o}C for 2 hours and then immersed in xylene twice for 10 minutes, 2 minutes, and another 2 minutes followed by graded ethanol washing; 100% (twice), 95%, 85%, 75% and 50% for 2 minutes each and then in double distilled water (ddH_2O) for 5 minutes. The next step is called antigen retrieval, and this was done by placing slides in boiling sodium citrate (10 mM) for 30 minutes. Once the slides cooled down at room temperature a hydrophobic pen was used to draw around the tissue samples which was then followed by a wash with the Wash Solution three times (phosphate buffered saline (PBS) with 0.05% Triton X-100) 5 minutes each. Slides were then blocked for 2 hours in Blocking Buffer (10 mL of 10X PBS, 5 mL goat serum, 200 µL Triton X-100, 100 mg BSA and ddH_2O added up to 100 mL) which was followed by an overnight incubation with the primary antibody: Anti-Active-β-catenin (Catalog no. 05-665, Millipore) and Anti-β-catenin Clone 14/β-catenin (Catalog no. 610154, BD Biosciences) (1:200 dilution) in the Blocking Buffer at 4^{o}C in a humidity chamber. The following day slides were washed three times with the Wash Solution 5 minutes each. Slides were then incubated with 0.3% H_2O_2 for 30 minutes. After this step the slides were washed three times with the Wash Solution 5 minutes each. Slides were then incubated with a horseradish peroxidase labelled (HRP) secondary antibody (1:200 dilution) (Catalog no. NEF822001EA, Perkin Elmer) in Blocking Buffer for 2 hours. After the incubation with the secondary antibody the slides were washed three times with the Wash Solution 5 minutes each. This next step was important to amplify the signal, so I used Tyramide Signal Amplification reagent (Catalog no. NEL700A001KT, Perkin Elmer) and Avidin-Biotin Complex (Catalog No. PK-6100, Vector Laboratories) solution for 7 and 30 minutes, respectively. Between the Tyramide Signal Amplification step and the Avidin-Biotin Complex step the slides were washed 3X with the Wash Solution 5 minutes each. After this step 3,3'-Diaminobenzidine (DAB) chromogenic substrate (Catalog no. SK-4105, Vector Laboratories) was added to the tissue slides for 1-10 minutes until a brown stain was detected, once the stain was detected slides were then washed under a running tap for 5 minutes. After amplifying the signal, I counterstained the slides in hematoxylin (Catalog no. SH26-500D, Fisher Scientific) for 30 seconds. After this step I incubated the slides with Scott's Top water (3.5 g sodium bicarbonate, 20 g magnesium sulphate and 1 L ddH_2O) then rinsed them

under running tap water for 2 minutes. The slides were then dehydrated in graded ethanol; 50%, 75%, 85%, 95% and 100% for 2 minutes each, followed by xylene for 2 minutes. Coverslips for viewing of the tissue were mounted on slides with the use of Permount (Catalog no. SP15-100, Fisher Scientific).

Antibodies used for staining included Anti-Active-β-catenin clone 8E7 (Catalog no. 05-665, Millipore) and Anti-β-Catenin Clone 14/β-Catenin (Catalog no. 610154, BD Biosciences). 30 Formalin-fixed, paraffin embedded OS tissue slides were obtained from British Colombia Children's Hospital Depository.

This project continues to be carried out as a multicenter initiative between the Universities of Alberta, British Columbia, and Manitoba. ABC levels were correlated to the clinic-pathologic features of the patients (stage of tumor: necrosis and metastasis at both resection and diagnosis), this stage was completed by our University of British Columbia colleagues.

2.2. Culture conditions

The SaOS2-LM-7 (LM-7) and its parental cell line SaOS2 along with HOS-143B (143B) and its parental cell line HOS were cultured in our lab. SaOS2 and LM-7 cells were a gift to our lab from Dr. Eugenie Kleinerman from the University of Texas M.D. Anderson Cancer Center, USA. HOS (Catalog no. CRL-1543™) and 143B (Catalog no. CRL-8303™) were purchased from ATCC. Further information about the cell lines can be found at **2.13.**

All cell lines were grown in Minimal Essential Medium (MEM) (Catalog no. 10320-021, Gibco), which contained 50 mg/mL fetal bovine serum (FBS) (Catalog no. 12483-020, Gibco), 5 mL of 100 mM penicillin-streptomycin (Catalog no. 15140-122, Gibco), 5 mL of 100 mM sodium pyruvate (Catalog no. 11360-070, Gibco) and 5 mL of 100 mM L-Glutamine (Catalog no. 25030-081, Gibco) and incubated at 37ºC under the conditions of 95% O_2 and 5% CO_2. To passage the cells, I would add trypsin (0.25% trypsin EDTA Solution, Catalog No. 25200-072, Gibco) to the plates and then after 5 minutes I added the FBS medium to stop the activity of the trypsin. I collected these cells, centrifuged them at 1500RPM for 5 minutes and then removed the

supernatant containing the trypsin. The pellet was resuspended in 1mL of FBS medium, counted and then re-seeded.

2.3. Transient transfection

SaOS2 or HOS cells were plated at 50% confluence one day before transfection then transfected with either pEGFP-C2-β-catenin: β-catenin gene inserted into the pEGFP-C2 plasmid (this plasmid has been discontinued by Clontech) or pEGFP-C2-ABC: a modified β-catenin fusion construct which simulates Active-β-catenin (ABC) (pEGFP-ABC: S33D, S37A, T41A, S45D) [GeneArt, Invitrogen] which was also inserted into the pEGFP-C2 plasmid (Figure 2). The Lipofectamine LTX and Plus^{TM} reagent kit (Catalog No. 15338-100, Invitrogen) was used for transfection. OptiMEM (Catalog No. 31985-070, Gibco) was added to two microfuge tubes with one for Lipofectamine LTX reagent and the other for the Plus^{TM} reagent plus DNA (plasmid). After both microfuge tubes were prepared the two mixes were combined, lightly vortexed, and allowed to mix for five minutes. The seeded SaOS2 or HOS cells were then supplemented with OptiMEM to cover the base of the plate. After five minutes, the transfectant mix was added to cells for four-five hours. After that time the OptiMEM and transfectant mix were removed and the cells were supplemented with 10% FBS Medium and incubated at 37ºC and 95% O_2 and 5% CO_2 overnight. In Tables 3, 4, and 5 the optimized quantities for transfection in the three plate types I used is listed.

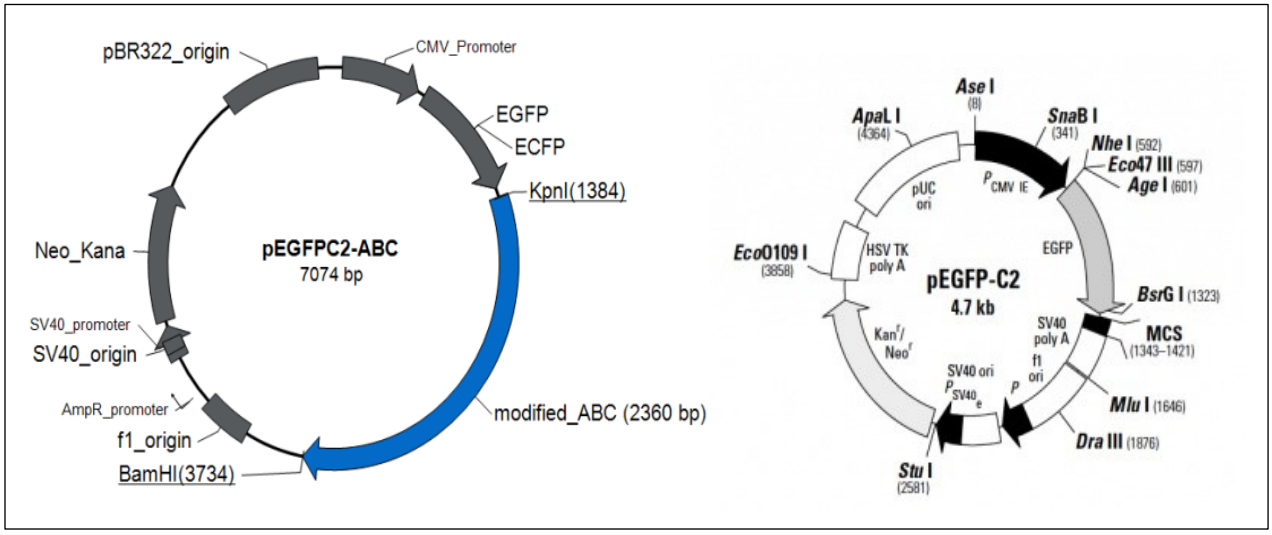


Figure 2. pEGFP-C2-ABC construct and pEGFP-C2 construct

The plasmid map shows all the restriction enzyme sites unique to these two plasmids. KpnI and BamHI restriction enzymes were important in the pEGFP-C2-ABC plasmid because these were the sites that were cut in the pEGFP-C2 plasmid to allow insertion of our modified ABC gene. It is important to note that the arrows show the coding regions of the plasmid. Where the modified ABC promoter site was added there is no stop codon for the EGFP (enhanced green fluorescent protein) promoter before it, this allows for the modified ABC and EGFP reporter gene to fuse and become one modified ABC protein that fluoresces green. Antibiotic resistance genes present include Kanamycin and Neomycin. CMV: Cytomegalovirus promoter, SV40: Simian virus 40. pEGFP-C2 plasmid map image obtained from Clontech.

Table 3. Optimized quantities of reagents for 10 cm plate

Reagents	Total Amount added
OptiMEM in one microfuge tube	500 μL
OptiMEM to cover base of plate	4 mL
Lipofectamine LTX	25 μL
Plus™ reagent	25 μL
DNA (plasmid)	10 μg

Table 4. Optimized quantities of reagents for one well in a 6-well plate

Reagents	Total Amount added
OptiMEM in one microfuge tube	100 μL
OptiMEM to cover base of plate	800 μL
Lipofectamine LTX	7 μL
Plus™ reagent	2.5 μL
DNA (plasmid)	2.5μg

Table 5. Optimized quantities of reagents for one well in a 96-well plate

Reagents	Total Amount added

OptiMEM in one microfuge tube	5 μL
OptiMEM to cover base of plate	40 μL
Lipofectamine LTX	0.5 μL
Plus™ reagent	0.1 μL
DNA (plasmid)	0.1 μg

2.4. Western blot

SaOS2 and HOS cells were transfected with either pEGFP-C2-ABC, pEGFP-C2-β-catenin, pEGFP-C2 using LTX and Plus™ reagent kit protocol. Cells were checked by a microscope (EVOS) to detect fluorescence the following day to ensure sufficient transfection. Upon sufficient transfection the cells were trypsinized and transferred to a Corning centrifuge tube (Catalog No. 430052) and centrifuged at 1000 RPM for five minutes. 1X PBS was used to wash the cells and they were centrifuged again. The 1X PBS was removed and cells were flash frozen with the use of liquid nitrogen and then stored at -80 ºC.

When ready to run the Western blots, the cells were thawed and prepared for loading. 1X radioimmunoprecipitation assay (RIPA) lysis buffer (Catalog No. 20-188, Millipore), containing: 10 μL 100 mM Halt Phosphatase inhibitor cocktail (Catalog No. 78420 Thermo Scientific), and 10 μL 100 mM protease inhibitor (Catalog No. 11836153001, Roche) were mixed together to make 1 L of the RIPA lysis solution, which was then added to the cells, vortexed at max speed for fifteen seconds and placed on ice in a 4ºC room for twelve minutes. Cells were then centrifuged for twelve minutes at max speed at 4ºC and after completion I would then remove the supernatant and discard the pellet.

Protein quantification of samples was carried out using Pierce™ bicinchoninic acid (BCA) protein assay kit (Catalog no. 23227, Thermo Scientific). The protein standards (Catalog No. P0914-10AMP, Sigma Aldrich) were loaded onto a 96 well plate with concentrations from 0 μg/uL, 0.05 μg/uL, 0.10 μg/uL, 0.15 μg/uL, 0.20 μg/uL, 0.25 μg/uL, 0.30 μg/uL, 0.35 μg/uL in triplicate in 10 μL amounts. Once cells were centrifuged for twelve minutes, I diluted the samples 1:10 (4 μL of the supernatant and 36 μL of Hyclone™ Water (Catalog No. SH3052902, Fisher

Scientific) and 10 μL of each sample was added to the 96 well plate in triplicate. After addition of 190 μL of Pierce™ BCA solution to each well the 96 well plate it was placed into a 37ºC incubator for thirty minutes. Analysis on the protein concentration was done by using FLUOstar Omega Plate Reader (BMG LABTECH).

The whole cell lysate samples and our positive control Non-Stimulated A431 Cell Lysate (Catalog No. 12-301, EMD Millipore) was prepared by boiling 50 μg and 20 μg of total protein, respectively, in 100 μL of 1X loading buffer with 0.031252 μg β-mercaptoethanol for five minutes for the samples and 2.5 μg of β-mercaptoethanol for the A431 cell lysate positive control. 40 μL of the sample was loaded into each lane and run on a 7.5% SDS-PAGE. After completion of electrophoresis proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (Catalog no. 1620177, Bio-Rad) at 110 V for seventy minutes. The membrane was blocked for one hour in 5 g of non-fat dry milk powder in 100 mL TBS (Tris buffered saline) containing 1 mL Tween-20 (TBST), or also known as Blocking Solution. This step was then followed by incubation of the membrane in primary antibody diluted in the Blocking Solution overnight at 4ºC. The membranes were rinsed three times in TBST, and then secondary antibody diluted in Blocking Solution was added to the membrane for one hour at room temperature. The blots were then rinsed three times in TBST for 10 minutes each and visualized using Western Lighting Plus ECL (Catalog no. NEL104001, Perkin Elmer).

The primary antibodies that were used: β-catenin antibody (amino-terminal antigen) Catalog no. 9581S, Cell Signalling) 1:1000, Anti-Active-β-catenin Clone 8E7 (Catalog no. 05-665, Millipore) 1:250, anti-GFP (Catalog No. 2555, Cell Signaling) 1:1000, and anti-β-actin (sc-69879, Santa Cruz Biotechnology) 1:10,000. The secondary antibodies used were Amersham ECL Anti-Rabbit IgG Horseradish Peroxidase linked whole antibody (Catalog No. NA934, Cytiva) 1:1000, and Anti-Mouse IgG (goat) HRP-labeled (Catalog No. NEF822001EA, Perkin Elmer) 1:1000. The HRP-secondary antibody signal was detected using the Western Lighting Plus ECL detector reagent and the bands were visualized on Fuji medical X-Ray films. The bands were analysed and quantified using the ImageJ software.

Anti-Active-β-catenin Clone 8E7 antibody is specific to the amino terminal of the protein and would only bind to the ABC because it specifically targets the site where it is dephosphorylated at Serine 37 and Threonine 41. This was important not only for detection of endogenous ABC but also for our construct because our ABC construct simulated this lack of phosphorylation by having a neutral charge at the Serine 37 and Threonine 41 location due to these sites being modified into an Alanine. Our ABC construct simulated the phosphorylated sites of Serine 33 and Serine 45 by having negative charges with the use of an Aspartic Acid.

Originally, I used the Anti-β-catenin carboxy terminal antigen antibody which would detect both ABC, β-catenin, pEGFP-C2-ABC, and pEGFP-C2-β-catenin, this was problematic. To rectify this issue and make sure that binding was specific I used the β-catenin antibody (amino-terminal antigen) (Figure 3).

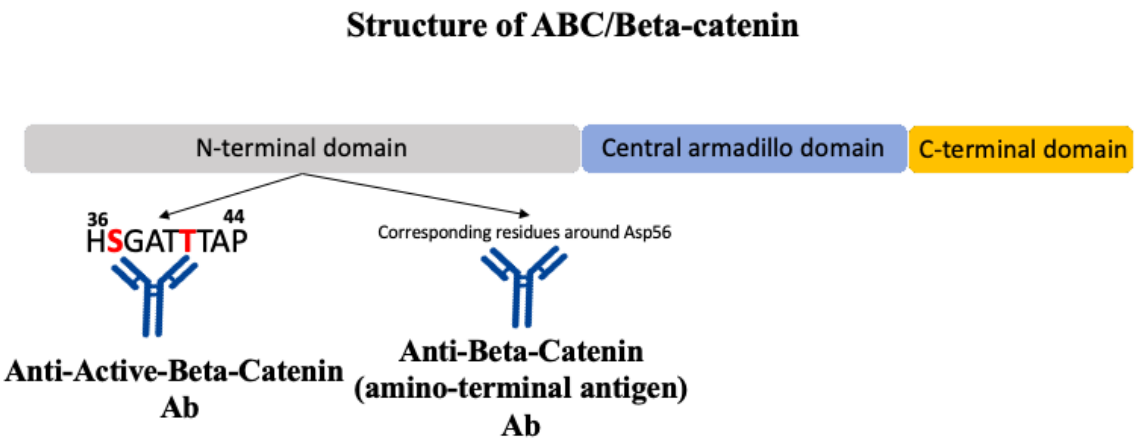


Figure 3. Anti-Active-β-catenin and Anti-β-catenin amino terminal antigen binding sites for Western blot

This figure shows a simplified diagram of the structure of ABC/β-catenin. The Anti-ABC antibody binds to a specific epitope range between amino acid residue 36 to 44. Within this epitope it binds specifically to two amino acid residues which need to be non-phosphorylated, serine 37 and threonine 41. Whereas the anti-β-catenin antibody binds to corresponding residues around aspartic acid amino acid residue 56.

2.5. Immunofluorescence

Cells were grown to 60% confluence on coverslips and then transfected with either pEGFP-C2, pEGFP-C2-ABC, or pEGFP-C2-β-catenin using the Lipofectamine LTX and Plus^{TM} reagent kit. The following day after 18 hours cells were washed with PBS twice and fixed with 4% paraformaldehyde (Catalog No. 15710, Electron Microscopy Sciences) for 10 minutes. After which, the cells were permeabilized with 100% methanol at -20°C for 10 minutes and blocked for 1 hour with 2 g bovine serum albumin (Fraction V, Catalog no. BP1600-100, Fisher Bioreagent), 100 mL 1X PBS, and 5 mL 100mM goat serum, this reagent mix was called Blocking Buffer. SaOS2 and HOS transfectants were incubated with the primary antibodies 1:500 anti-β-catenin antibody (Catalog no. 2677, Cell Signaling) or 1:200 Anti-Active-β-catenin Clone 8E7 diluted in 0.2 g bovine serum albumin in 100 mL Dilution Buffer overnight at 4ºC. The following day incubation using the secondary antibody AlexaFluor® 555 goat anti-mouse antibody 1:1000 (Catalog no. A-21422, Invitrogen) along with Fluorescein isothiocyanate (FITC) Anti-GFP antibody (Catalog no. ab6662, abcam) was used. However, the addition of the FITC Anti-GFP antibody was used just for the HOS transfectants to increase the GFP signal. Secondary antibodies were added for 1.5 hours after two washes with PBS and one wash with PBS-Triton. Once slides had been incubated with the secondary antibody, coverslips were mounted onto microscope slides with Mounting Medium containing 4′,6-diamidino-2-phenylindole (DAPI) (provided by the Cell Imaging Facility at Cross Cancer Institute, Edmonton, Alberta). Imaging of the slides was carried out at 40X magnification (oil immersion) using the Carl Zeiss Laser Scanning Microscope (Cross Cancer Institute, Edmonton, Alberta) and image processing was carried out using LSM image browser software.

This experiment allowed me to be able to visualize and confirm that localization of both the endogenous ABC and β-catenin and GFP tagged ABC or β-catenin were localizing together. The primary antibodies and secondary antibody AlexaFluor® 555 would show us our endogenous ABC and β-catenin stained in red (AlexaFluor® 555) and since our ABC or β-catenin construct was tagged with GFP they would fluoresce green. Colocalization of endogenous protein and our constructs were visualized via merge image (green/red=orange). Localization would be confirmed if both the endogenous protein and our construct were in the same plane of view and created an orange merged image.

2.6. alamarBlue™ cell viability assay

In a 6-well plate SaOS2 and HOS cells were seeded and left to grow overnight. The following day SaOS2 and HOS cells were transfected with either pEGFP-C2, pEGFP-C2-ABC, pEGFP-C2- β-catenin using the Lipofectamine LTX and Plus™ reagent kit. After transfection, the transfected cells were left to recover in FBS medium overnight. The following day 10,000 cells were added to each well in a 96-well plate and left to grow over night. 1 hour before hours 8,18,24, and 48 11µL of alamarBlue™ Cell Viability Reagent (Catalog No. DAL1025, Invitrogen™) was added to each well and incubated for 1 hour and then were analyzed using FLUOstar Omega Plate Reader (BMG LABTECH) measuring fluorescence intensity at 544 ηm Excitation and 590 ηm Emission. A blank with no cells, Complete Media, and alamarBlue™ Cell Viability Reagent was used for this experiment and all samples were done in triplicate.

When alamarBlue™ Cell Viability Reagent or Resazurin encounters cells with active metabolism the Resazurin is reduced into a product called Resorufin which gives it a pink color (Riss et al., 2004). The quantity of Resorufin in the well is measured and is known to be proportionate to the number of cells (Riss et al, 2004).

2.7. Invasion assay and migration assay

In a 6-well plate SaOS2 and HOS cells were seeded and left to grow overnight. The following day SaOS2 and HOS cells were transfected with either pEGFP-C2, pEGFP-C2-ABC, or pEGFP-C2-β-catenin using the Lipofectamine LTX and Plus™ reagent kit. After transfection, the transfected cells were left to recover in 10% FBS medium overnight. Both the Migration Assay and Invasion Assay protocols are similar with the exception that the Invasion Assay Matrigel Invasion Chambers (Catalog No. 354480, Corning) have ECM proteins that coat the bottom of the wells and have to be warmed for an hour in MEM, with 5 mL of 100 mM penicillin-streptomycin, 5 mL 100 mM sodium pyruvate and 5 mL 100 mM L-Glutamine before using them. This medium was called No FBS medium. Once the invasion chambers were thawed the No FBS medium was removed. SaOS-2/transfectants/LM-7 cells were trypsinized, centrifuged and resuspended into 1 mL of No FBS Medium and the HOS/transfectants/143B cells were trypsinized, centrifuged, and resuspended into 1 mL of 0.1% FBS Medium (Medium with 0.1% FBS). 50,000 cells in 300 µL

(SaOS-2/transfectants/LM-7) or 0.1% FBS Medium (HOS/transfectants/143B) were added inside of each chamber. For SaOS-2/transfectants/LM-7 cells I added 750 µL of FBS medium outside of the chambers. For HOS/transfectants/143B the outside of the chambers were filled with 5% FBS medium. Chambers were incubated at 37ºC, 95% O_2 and 5% CO_2 overnight. After incubation, medium was removed, the inside and outside of the chambers were washed in 1X PBS twice, then 500 µL of 3.7% PFA was added to the inside and outside of the chambers for 2 minutes. After two minutes the inside and outside of the chambers were rinsed with PBS twice. Chambers were rinsed inside and outside with 500 µL ice cold 100% methanol for 20 minutes. After 20 minutes the inside and outside of the chambers were washed with PBS twice. The inside and outside of the chambers were washed with 500 µL of 0.05 g Crystal Violet (Catalog No. C6158-50G, Sigma-Aldrich) diluted in 7.5 mL ddH_2O and 2.5 mL 100% methanol for 15 minutes. After 15 minutes each chamber was rinsed in water and then PBS. With a cotton swab I removed the excess Matrigel. The chambers were left to dry and when ready were imaged with the Axio-Color microscope (Cross Cancer Institute, Edmonton, Alberta) and analyzed by the High Content System at 10X and MetaExpress software (Katz Group Center for Pharmacy and Health Research, University of Alberta).

2.8. Gelatin zymography

Cells were grown in 10 cm plates and after transfection of the cells with either pEGFP-C2, pEGFP-C2-ABC, or pEGFP-C2-β-catenin, following the Lipofectamine LTX and Plus™ reagent kit protocol. After 4-5 hours the cells were left to recover in 20% FBS Medium (100 mL FBS in FBS medium) for another 4-5 hours. After the allotted time the plate was washed with PBS lightly and 4 mL of No FBS Medium was added to the plates overnight. The following day all 4 mL of medium was collected and concentrated to 50 µL using the Amicon ® Ultra-4 Centrifugal Filter Unit (Catalog No. UFC801024, Millipore). The supernatant that was collected were then flash frozen in liquid nitrogen and stored at -80ºC.

Protein quantification of the supernatant was carried out using Pierce™ BCA protein assay kit (Catalog no. 23227, Thermo Scientific). The protein standards (Catalog No. P0914-10AMP, Sigma Aldrich) were loaded onto a 96 well plate with concentrations from 0 µg/uL, 0.05 µg/uL, 0.10 µg/uL, 0.15 µg/uL, 0.20 µg/uL, 0.25 µg/uL, 0.30 µg/uL, 0.35 µg/uL in triplicate in 10µL amounts. Once cells were centrifuged for twelve minutes, I diluted the supernatant 1:10 (4 µL of

the supernatant and 36 μL of Hyclone™ Water (Catalog No. SH3052902, Fisher Scientific) and 10 μL of each sample was added to the 96 well plate in triplicate. After addition of 190 μL of Pierce™ BCA solution to each well the 96 well plate it was placed into a 37°C incubator for thirty minutes. Analysis on the protein concentration was done by using FLUOstar Omega Plate Reader (BMG LABTECH)

Stock solutions of reagents required for Gelatin Zymography. Separating Gel: For an 8% separating gel, 3 mL 40% acrylamide/3.3% bis-acrylamide, 3.75 mL 4X Tris-HCl, pH 8.8 (Tris base, Invitrogen #15504-020), 1.50 mL 20 mg/mL gelatin (Gelatin sigma G8150) and 10% w/v SDS, 0.05 mL 10% Ammonium Persulfate (APS) (Bio-Rad #161-0700) (0.05 g APS and 0.5 mL ddH_2O), and 0.01 mL TEMED. Stacking Gel: For a 5% stacking gel, 0.975 mL 40% acrylamide/3.3% bis-acrylamide, 4X Tris-HCl/SDS, pH 6.8, 6,525 mL ddH_2O, 0.05 mL 10% APS, and 0.01 mL TEMED. Once reagents were prepared I would make our separating and stacking gels following the Bio-Rad tera Cell Gel electrophoresis systems guidelines. For sample preparation I prepared 6X Loading Buffer (5 mL 0.5M Tris-HCl, pH 6.8, 3 mL glycerol,1 g SDS, and 1.2 mg bromophenol blue) and RIPA buffer (150 mM sodium chloride, 1.0% IGEPAL$^{R)}$ CA-630, 0.5% Sodium deoxycholate Sigma D6750, 0.1% SDS, 50mM Tris base pH 8.0, and ddH2O). To prepare the samples I loaded 25 ug worth of protein from our samples, 6X loading buffer and RIPA buffer added to reach the final total of 30 uL. 10 uL of BLUeye pre-stained protein ladder (Frogga-Bio PM007-0500), MMP9 and MMP2 controls (conditioned serum-free medium from HT1080 cells was used as a standard for MMP2 and medium from HT1080 cells stimulated with 100 ng/ml phorbol myristate acetate was used as a standard for MMP-9) and samples were loaded. Electrophoresis was run for 2 hours at 110 V. After completion gels were transferred to plastic containers and washed in 2.5 % TritonX-100 solution three times for 20 minutes. Gels were then incubated overnight with 1X incubation buffer (8.766 g Sodium Chloride, 0.7351 g Calcium Chloride, 6.057 g Tris base pH 7.6, 0.5 g Sodium Azide, and ddH2O to 1 L). The following day the Incubation Buffer was removed and 0.05 % Coomassie stain (250 mg Coomasie Brilliant Blue G-250 Sigma B1131, prepared in 125 mL methanol, 50 mL glacial Acetic acid and ddH2O to 325 mL) was added for the next three hours. After three hours 1X de-staining solution (40 mL methanol, 80 mL glacial acetic acid, ddH2O to 1 L) was added to remove the stain and then the gel is analyzed under light. The bands were analysed and quantified using the ImageJ software.

This study is known for its ability to measure the gelatinase activity of MMP9 and MMP2, the reason that we specifically chose this assay and the measuring of MMP9 and MMP2 is because they are shown to be the most overexpressed in malignancies and are associated with tumor aggressiveness, metastasis, and poor prognosis (Bjornland et al., 2005). Ultimately a study done by Bjornland et al., (2005) came to the conclusion that MMP9 and MMP2 are important in the invasive process of human OS. This is important because our invasion assays require the activity of MMPs, and our RT-qPCR results also look at the mRNA transcription of MMP9 and MMP2.

2.9. Soft agar colony forming assay

Preparation of stock solutions: 2X MEM was prepared by adding MEM (Catalog No. 11700077, Gibco^{TM}) in 1L of ddH2O to create a 10X stock, then diluted to a 5X MEM stock with the addition of 5X of L-Glutamine, Sodium Pyruvate, and Penicillin Streptomycin. 5X MEM was diluted to 2X with the addition of 5% FBS and 1X Amphotericin B (Catalog No. 15290018 Gibco^{TM}). Then I prepared and autoclaved 1.6% Bottom agar and 0.7% Top agar using Bacto^{TM} Agar (Catalog No. 214010, BD). Before adding the agar to the plates, I added 4 mL of sodium bicarbonate (Catalog No. 792519, Sigma-Aldrich) and 2X of HEPES (Catalog No. 15630080, Gibco^{TM}) to the 2X MEM and mixed. 25 mL of the Bottom agar was added to 25 mL 2X MEM and 0.5 mL was added to the bottom of a 24-well plate. 1250 cells were counted for addition to 0.75 mL Top agar along with 0.75 mL of 2X MEM. 0.5 mL of the mixture was added on top of the Bottom agar, this was done in triplicate. Once solidified the plates were incubated at 37ºC, 90% O_2 and 5% CO_2 for three weeks with 250 uL of Complete Medium added every 4 days to prevent the cells from drying out and providing them with nutrients. Analysis of the soft agar colony forming assay was completed by imaging the colonies and counting the colonies using the High Content System and MetaExpress software (Katz Group Center for Pharmacy and Health Research, University of Alberta). It is important to note that this protocol was put together during the pandemic so a lot of my work was done alone and I believe that this protocol does need to be reviewed to improve upon concentration accuracy and overall efficacy of the experiment.

2.10. Quantitative Real Time PCR

600,000 cells were seeded in 6-well plates, transfected, incubated for times indicated, trypsinized, and centrifuged. Collection of total RNA was isolated using RNeasy Plus Mini Kit (Catalog No. 74134, Qiagen). After collection of the total RNA, the concentration was measured using the Nanodrop 2000 (Thermo Scientific™). 1.5 µg of total RNA was collected and then was filled up to 10 µL with Hyclone™ Water. Using the High-Capacity cDNA Reverse Transcription Kit (Catalog No. 4368814, Applied Biosystems™) I added Hyclone™ Water, 10X RT Buffer, 25X dNTP, 10X Random Primers, and Reverse Transcriptase, in this order, and mixed it all together to achieve a final 1X concentration for a total amount of 200 µL per sample. Transcription mix was loaded into PCR plates (Catalog No. MLL4851, Bio-Rad) and placed in the MJ Mini™ Thermal Cycler and MiniOpticon™ PCR System to undergo PCR (25ºC for 10 minutes, 37ºC for 2 hours, and 85ºC for 5 minutes, according to manufacturer conditions).

In a MicroAmp™ Fast Optical 96-well reaction plate (Catalog No. 4346906, Applied Biosystems™) I added Fast SYBR™ Green Master Mix (Catalog No. 4385612, Applied Biosystems™). I added primers (300 nM) for MMP9, MMP2 and our control GAPDH primers: MMP9: Forward (5' -CAC TGT CCA CCC CTC AGA GC- 3') Reverse (5' -GCC ACT TGT CGG CGA TAA GG- 3'), MMP2: Forward (5' -GGC CCT GTC ACT CCT GAG AT- 3') Reverse (5' -GGC ATC CAG GTT ATG GGG GA- 3'), GAPDH: Forward (5' -ACC ACA GTC CAT GCC ATC AC- 3') Reverse (5' -TCC ACC ACC CTG TTG CTG TA- 3'). This was followed by addition of 1.25 uL of the cDNA. and HycloneTM Water up to 25 ul total volume to each well. The analysis was done using the LightCycler®96 (ROCHE) and LightCycler®96 Software. For MMP9, MMP2, and GAPDH I used: amplification 95ºC for 10 minutes, and the denaturation and annealing/extension was at 95ºC for 15 seconds followed by 60ºC for 1 minute for 40 cycles. For OPG and RANKL each well was loaded with either (300nM): PrimePCR Assay TNFRSF11B (OPG) Hsa (Catalog No. 10025636, Bio-Rad), PrimePCR Template TNFRSF11B (OPG) Hsa (Catalog No. 10025716, Bio-Rad), PrimePCR Assay TNFSF11 (RANKL) Hsa (Catalog No. 10025636, Bio-Rad), and PrimePCR Template TNFSF11 (RANKL) Hsa (Catalog No. 10025716, Bio-Rad). 1 µL of PrimePCR Assay (OPG and RANKL) or PrimePCR Template (OPG and RANKL) as a control, 10 µL of SsoAdvanced™ Universal SYBR® Green Supermix (Catalog No. 1725270, Bio-Rad), 1.25 µL of cDNA and HycloneTM Water and to reach a final amount of 20

μL per well. The analysis was done the same with different PCR conditions: 95ºC for 2 minutes, denaturation and annealing/extension was at 95ºC for 5 seconds followed by 60ºC for 30 seconds for 40 cycles. Each experiment was done in triplicate and gene expression was calculated using LightCycler®96 Software.

2.11. Stable cell line formation

SaOS2 cells were grown to 70% confluence. Once confluency was achieved cells were transfected with the Lipofectamine LTX and Plus™ reagent kit and plasmids (pEGFP-C2-ABC, pEGFP-C2-β-catenin and pEGFP-C2) and then left to recover overnight in FBS medium. The following day FBS medium was removed, cells were trypsinized, centrifuged, and then resuspended in 10 mL FBS medium with the addition of and 1.0 mg/mL Geneticin™ (Catalog No. 10131035, Gibco). This medium was called Stable Media. 2 mL of the cells mixed with Stable medium was added to a 10 cm plate along with 8 mL of Stable medium (1:5 dilution). Cells were supplemented with Stable medium every 2 days for 2 weeks while the cells were monitored for growth and fluorescence. After the growth of colonies showing fluorescence was seen, these specific colonies were selected with a glass cylinder and trypsinized, collected and placed into new 10 cm plates to grow with 10 mL of Stable Media. Passaging of the Stable cell lines was done the same way as passaging of the SaOS2, LM7, HOS, and 143B cells.

2.12. Statistical analysis

One-way ANOVA with Dunnett's Test (GraphPad PRISM Software; GraphPad Software, Inc., CA, USA) was used to compare differences within groups while using our parental cell line (HOS/SaOS2) as our control. Results are presented as Mean $\pm$ SEM and values with *$p<0.05$, **$p<0.005$, and ***$p<0.001$ were considered statistically significant. Statistical analysis using Fisher's Exact Test (two tailed) was done on IHC analysis to compare whether high or low levels of ABC correlated with necrosis and metastasis at both diagnosis and resection.

2.13. Cell Lines

The four cell lines that were used for this project were SaOS2, LM-7, HOS, and 143B. The SaOS2 cell line was established in 1973 from an 11-year-old Caucasian female with osteogenic sarcoma. This cell line displays osteoblastic features such as the ability to produce a mineralized

matrix and has been important for research into human osteoblast-like cells and human osteosarcoma (Rodan et al., 1987). The LM-7 cell line was derived from SaOS2 cells that were injected into mice *in vivo* and after the cells metastasized to the lungs after 6 months these cells were collected, isolated, and reinjected 6 more times (Flores et al., 2012) (Jia, Worth, & Kleinerman, 1999). HOS cells are derived from a 13-year-old female Caucasian. 143B cells are derived from HOS due to a transfection with the Ki-RAS oncogene, they are considered to have more metastatic features when in comparison to HOS (Flores et al., 2012) (Ren et al., 2015). To better understand some general characteristics about the cells being used and to ensure my results were similar I read a study that completed characterization studies on multiple OS lines, fortunately it contained three of the four cell lines that I was going to use in my study, SaOS2, HOS, and 143B (Lauvrak et al., 2013). The four categories they used to characterize these cell lines were tumorigenicity, colony forming ability, invasive capacity and migration capacity, and proliferation (Lauvrak et al., 2013). Tumorigenicity was determined by injecting cells into nude mice subcutaneously then measuring the tumor after a set amount of time and depending on the speed of tumor formation each cell line was given a grade from 0-4, with 4 being the most tumorigenic (Lauvrak et al., 2013). Colony forming ability was measured on their ability to form colonies in anchorage-independent conditions (Lauvrak et al., 2013). Invasive and migratory abilities was determined by utilizing invasion and migration assays (Lauvrak et al., 2013). Proliferation rate was determined by live-cell imaging and presented as the increase in confluence over a set time period, each cell line was given grades from 1 to 3 (Lauvrak et al., 2013). HOS cells were considered to be very tumorigenic and received a grade of 4, it was given a grade of 3 for its proliferative abilities, and it was in the top five out of twenty-two cell lines for colony formation, invasion, and migration (Lauvrak et al., 2013). The 143B cell line received similar grades to that of the HOS cell line: a 4 for its tumorigenicity, a grade of 3 for its proliferative abilities and was in the top five for colony formation, invasion, and migration as well (Lauvrak et al., 2013). These results suggest that 143B and HOS are similar in terms of invasion, motility and colony forming ability. However, (Mohseny et al., 2011) and (Ren et al., 2015) agree that HOS is still considered the less metastatic parental cell line when compared to 143B. The SaOS2 cell line was given a grade of 2 for its tumorigenicity, a grade of 2 for its proliferative abilities and was close to the end when looking at its colony forming ability, invasion and migration potential. The Lauvrak et al., 2013 study did not evaluate LM-7, however, LM-7 is considered to be more metastatic than SaOS2

because it has been reported to have significantly higher rates of metastasis to the lungs compared to SaOS2 *in vivo*, and also has been reported to elicit higher migration and invasion compared to SaOS2 *in vitro* (Lyons et al., 2016) (Lisle, Choi, Horton, Allen, & Damron, 2008). Furthermore, both the LM-7 and HOS-143B cell line when injected orthotopically result in the generation of tumors and in a short time period produce high frequency of lung metastases (Yuan et al., 2009). Both of these pairs of cell lines (SaOS2/LM-7 and HOS/HOS-143B) were important for the goal of our study to evaluate the potential impact ABC has in promoting an invasive phenotype by comparing ABC and β-catenin overexpressing SaOS2 and HOS cells to the more aggressive cell lines LM-7 and 143B.

Chapter 3

Results

3.0. Results

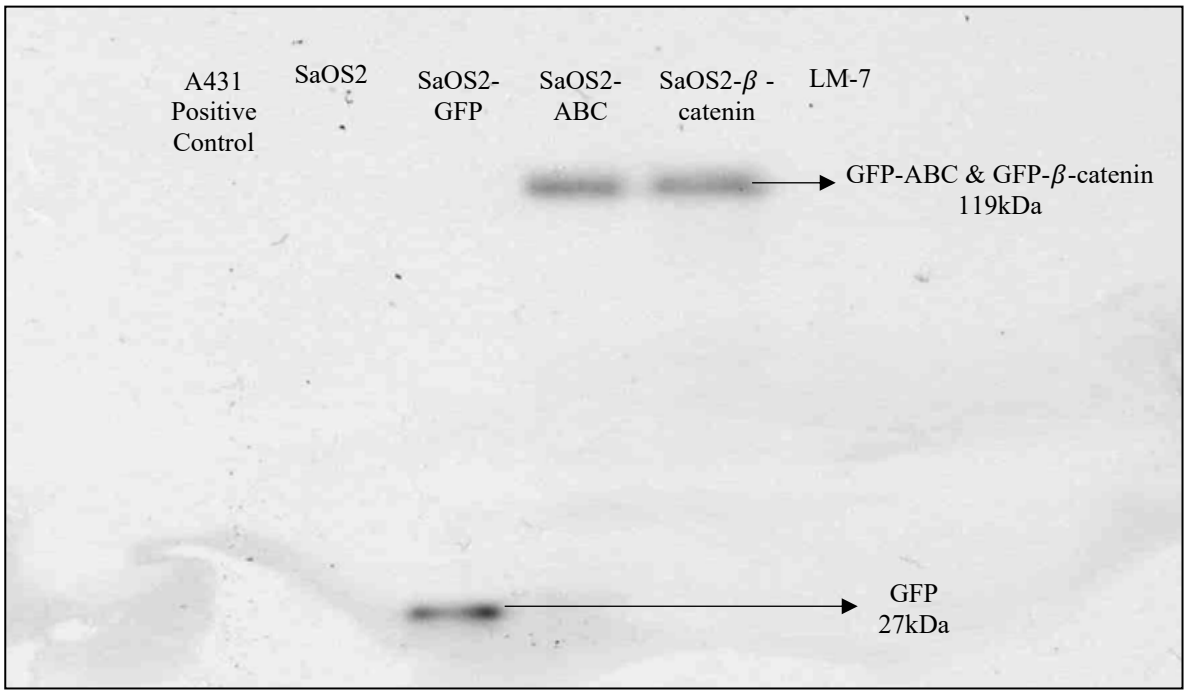
3.1. Plasmid transfection was successful in both SaOS2 and HOS cells

To ensure that the plasmids pEGFP-C2 (empty vector), pEGFP-C2-ABC, and pEGFP-C2-β-catenin were transfected into SaOS2 and HOS cells with sufficient efficiency and to also verify the presence of the modified proteins of interest, GFP-tagged β-catenin and GFP-tagged ABC, I used Western blotting. Since endogenous ABC is a post translational modified β-catenin their sizes do not differ and are both 92kDa. The size of our GFP-tagged β-catenin and ABC was 119kDa, this was due to the added on GFP tag. β-actin was used as the loading control.

i. SaOS2 and HOS Transfectants

My results suggest that the plasmids were successfully transfected into the SaOS2 and HOS cells and were able to confirm the presence of GFP-ABC and GFP-β-catenin (Figure 4). Unfortunately, there are issues with the gels with respect to over exposure and presentation of the blots, these issues and how to address them will be explored in further detail later in "Future Directions". However, these preliminary blots show when incubated with an anti-GFP antibody I was able to see three distinct bands, one at 27kDa for the SaOS2 cells transfected with pEGFP-C2, and two bands at 119kDa for the SaOS2 cells transfected with pEGFP-C2-ABC, and pEGFP-C2-β-catenin (Figure 4A). The anti-β-catenin antibody showed a positive band at 119kDa for SaOS2 and HOS cells transfected with pEGFP-C2-β-catenin, this band would confirm the presence of GFP-β-catenin (Figure 4B) (Figure 4C). When using the anti-ABC antibody, I saw a positive band at 119kDa at the SaOS2 and HOS cells transfected with pEGFP-C2-ABC, this band confirms the presence of GFP-ABC (Figure 4B) (Figure 4C).

A.


A431 Positive Control
SaOS2
SaOS2-GFP
SaOS2-ABC
SaOS2-β -catenin
LM-7
GFP-ABC & GFP-β-catenin 119kDa
GFP 27kDa

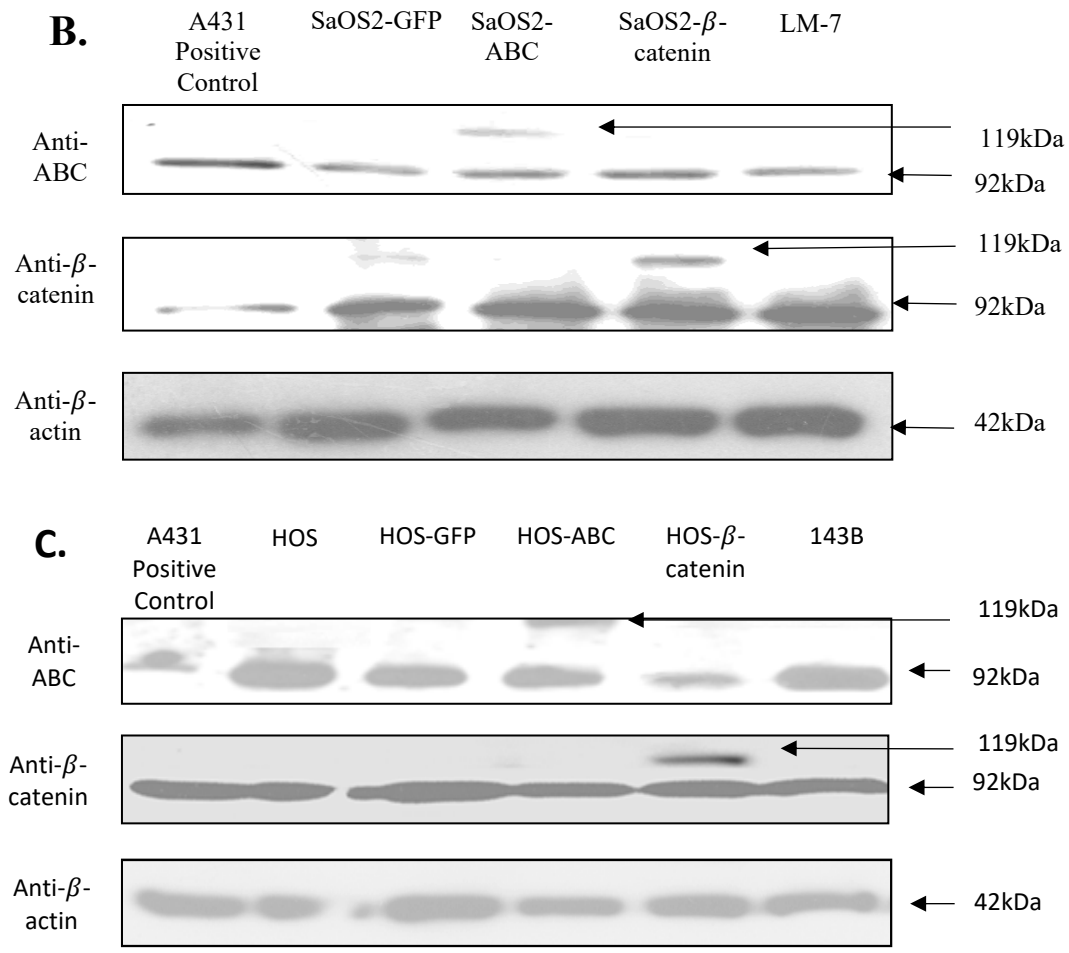


Figure 4. Western blots show successful transfection and expression of GFP, GFP-ABC, and GFP-β-catenin

Western blot analysis was completed the following day 18-24 hours after transfection. **A. Immunoblotting SaOS2 transfectants with anti-GFP antibody**. The results show the size of GFP, GFP-ABC, and GFP-β-catenin is consistent with where the proteins would show up at their predicted sizes, 27kDa, 119kDa, and 119kDa, respectively. **B. SaOS2 transfectants.** Anti-ABC antibody shows endogenous ABC levels along with protein expression of GFP-ABC transgene at 92kDA and 119kDA, respectively. Anti-β-catenin antibody shows endogenous β-catenin and protein expression of GFP-β-catenin transgene at 92kDa and 119kDA, respectively. **C. HOS transfectants.** Anti-ABC antibody shows endogenous ABC levels along with protein expression of the GFP-ABC transgene at 92kDA and 119kDA, respectively. Anti-β-catenin

antibody shows endogenous β-catenin and protein expression of GFP-β-catenin transgene at 92kDA and 119kDA, respectively.

3.2. GFP-ABC and GFP-β-catenin plasmid constructs colocalize with endogenous ABC and β-catenin

Previous results from our lab have shown that ABC and β-catenin localize differently (Ali et al., 2019). ABC localizes mainly to the nucleus and previous work from our lab also showed that there was significantly higher levels of ABC in the nuclei of the more metastatic cell lines, LM-7 and 143B when compared to SaOS2 and HOS, respectively. β-catenin is membrane bound and under microscopy will appear throughout the cell and previous work from our lab showed there was no significant difference in β-catenin levels and localization between the metastatic cell lines and the parental cell lines. The present experiment that I carried out was to validate that GFP-ABC and GFP-β-catenin act similarly to the endogenous proteins in terms of their intra-cellular localization.

i. SaOS2 and HOS transfectants

My results demonstrate that when SaOS2 cells were transfected with pEGFP-C2-ABC both the endogenous and GFP-ABC colocalized in the nucleus (Figure 5A). When SaOS2 cells were transfected with pEGFP-C2-β-catenin both the endogenous and GFP-β-catenin colocalized throughout the cell (Figure 5A). Similar results were seen with the HOS transfectant cell lines (Figure 5B).

These results suggest that the localization of GFP-ABC and GFP-β-catenin are similar to that of the endogenous proteins (Figure 5A) (Figure 5B). With the IF results and Western blot results, I was able to confirm that transfection of pEGFP-C2-ABC and pEGFP-C2-β-catenin was successful and that the SaOS2 and HOS cells would now overexpress both ABC and β-catenin. Proceeding forward any difference seen between the different transfected groups (pEGFP-C2, pEGFP-C2-ABC, and pEGFP-C2-β-catenin) in future assays could be due to the overexpression of both ABC and β-catenin.

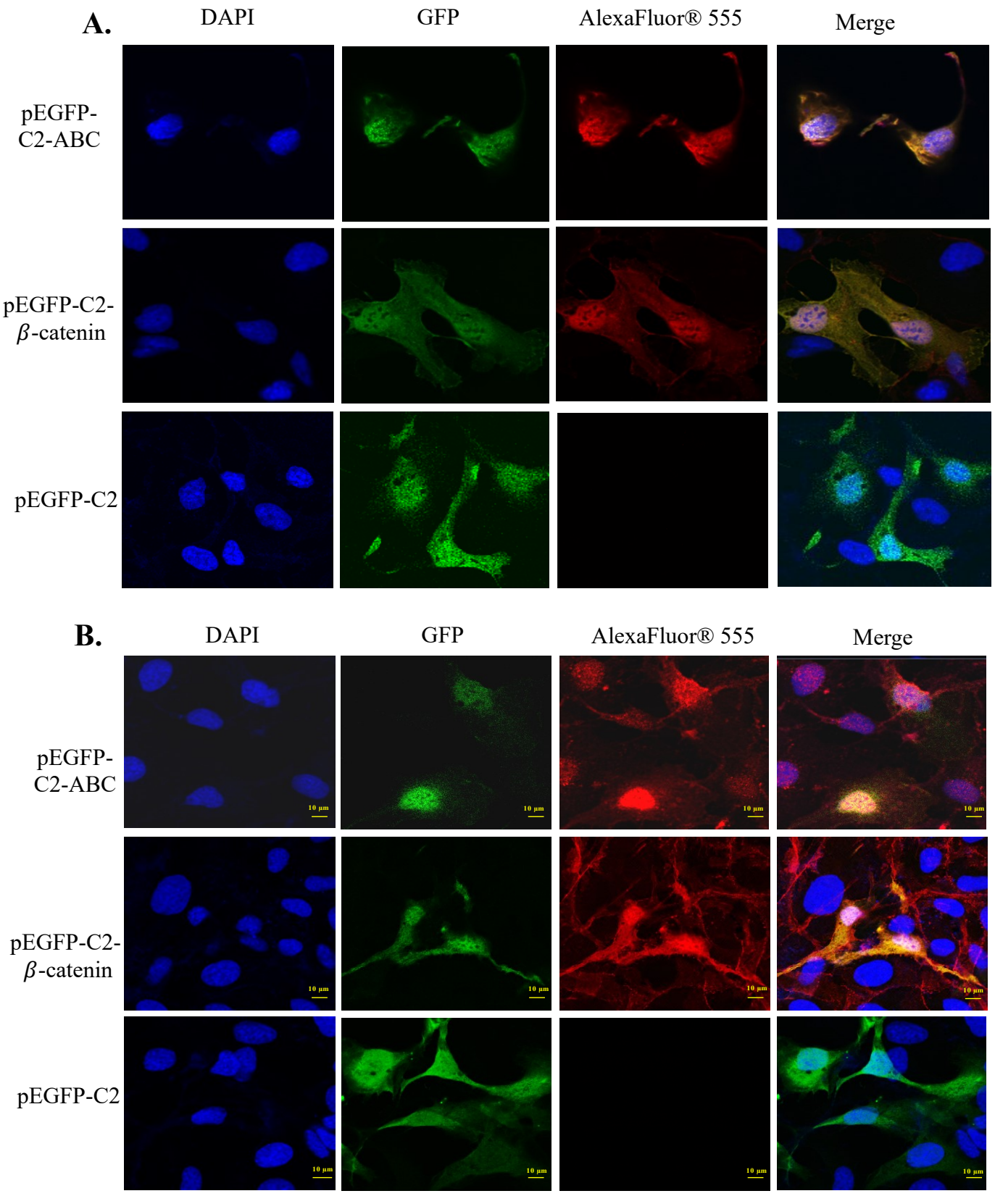

A.
DAPI
GFP
AlexaFluor® 555
Merge
pEGFP-C2-ABC
pEGFP-C2-β-catenin
pEGFP-C2
B.
DAPI
GFP
AlexaFluor® 555
Merge
pEGFP-C2-ABC
pEGFP-C2-β-catenin
pEGFP-C2
10 μm

Figure 5. Confocal microscopy of SaOS2 and HOS cells transfected with pEGFP-C2-ABC and pEGFP-C2-β-catenin show similar colocalization with endogenous ABC and β-catenin and GFP-ABC and GFP-β-catenin

All cells underwent transfection via either pEGFP-C2-ABC, pEGFP-C2-β-catenin, or pEGFP-C2. After the transfection process the cells were prepared for immunofluorescence staining. The cells were incubated with primary antibodies for ABC and β-catenin. Then a secondary antibody was added to the cells to detect the primary antibodies that were bound to ABC and β-catenin, this secondary antibody AlexaFluor® 555 would fluoresce red. Since the cells that were transfected with one of the three plasmids and since they had a GFP-tag, they would fluoresce green. All cells were stained with DAPI which would cause the nucleus to fluoresce blue. Lastly, the merge window indicates all three channels that were looking at DAPI, GFP, and AlexaFluor® 555. The pEGFP-C2 cells did not have a primary antibody and the secondary antibody showed no non-specific staining. **A. SaOS2 transfectants.** SaOS2 cells transfected with pEGFP-C2-ABC showed us that the GFP-ABC and endogenous protein both localized in the nucleus. SaOS2 cells transfected with pEGFP-C2-β-catenin showed us that both GFP-β-catenin and endogenous protein β-catenin localized throughout the cell. SaOS2 cells transfected with pEGFP-C2 were used as a control. **B. HOS transfectants.** Similar results were seen when HOS cells were transfected with pEGFP-C2-ABC, pEGFP-C2-β-catenin, and pEGFP-C2.

3.3. ABC increases invasive ability of OS cells

An important feature involved in OS progression is the ability of the cancer cells to invade. Invasion by cancerous cells is seen as the ability of the cancerous cells to gain motility and degrade the ECM to cleave a pathway for their movement. To determine whether ABC plays a role in promoting the invasive phenotype of OS I measured the *in vitro* invasive capacity of the SaOS2 and HOS transfectants using the Boyden chamber invasion assay. The LM-7 and 143B cells were used as a measure to see if overexpression of ABC or β-catenin would result in a similar invasive ability as the more metastatic cell lines.

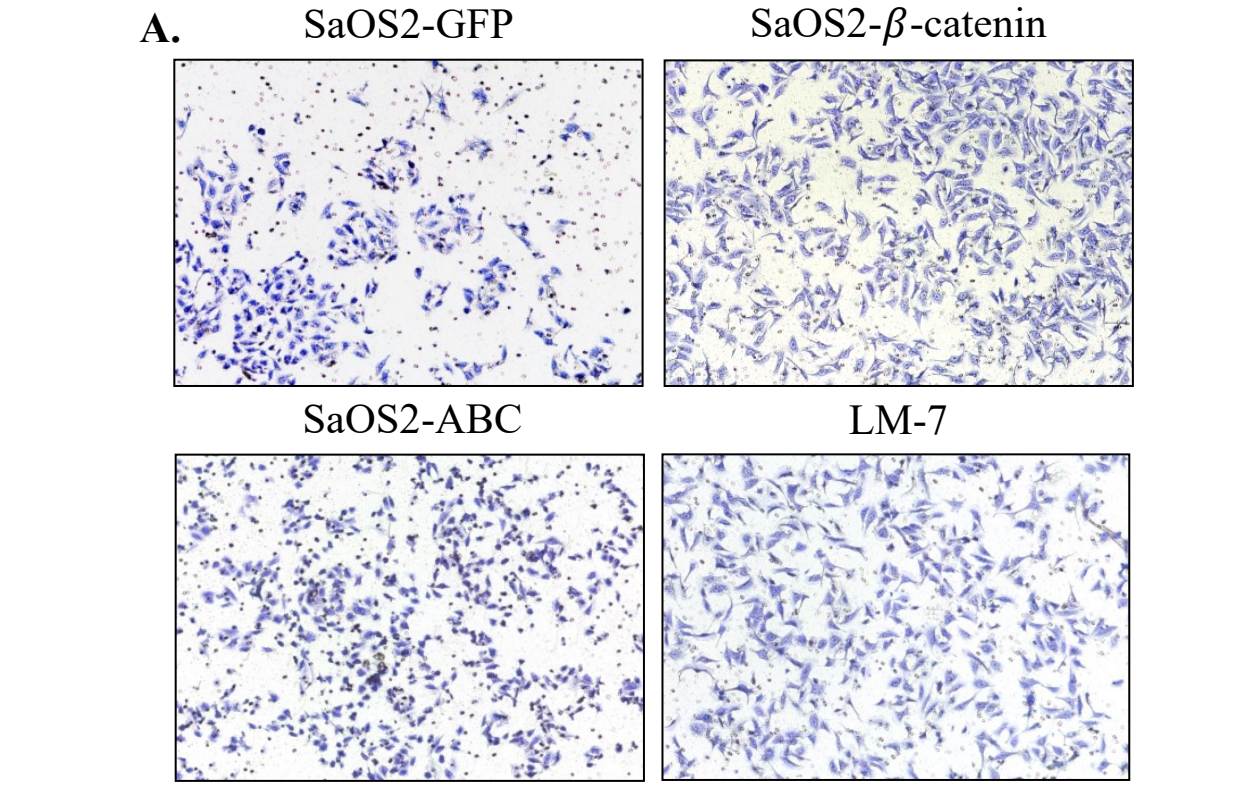
i. SaOS2 transfectants and LM-7

My results suggest that LM-7 cells have a significantly higher invasive potential through Matrigel compared to the control SaOS2-GFP transfected cell line (*$p<0.05$) (Figure 6B). SaOS2 cells overexpressing ABC showed a significant increase in their invasive capacity that was comparable to the LM7 cells (*$p<0.05$) (Figure 6B). However, there was no significant difference in invasive potential of the β-catenin overexpressing cells compared to control (Figure 6B).

ii. HOS transfectants and 143B

My results suggest that when HOS cells overexpress ABC, there is a statistically significant increase in invasion (*$p<0.05$) (Figure 7B). Similar to the SaOS2 results, when HOS cells were transfected and overexpressed β-catenin there was no statistical difference in the invasive capacity (Figure 7B). However, the more metastatic cell line of 143B did not have a statistical difference in invasive capacity (Figure 7B).

These results suggest that the overexpression of ABC in the OS cell lines causes a statistically significant increase in the cells ability to invade and digest the ECM (Figure 6B) (Figure 7B)


A.
SaOS2-GFP
SaOS2-β-catenin
SaOS2-ABC
LM-7

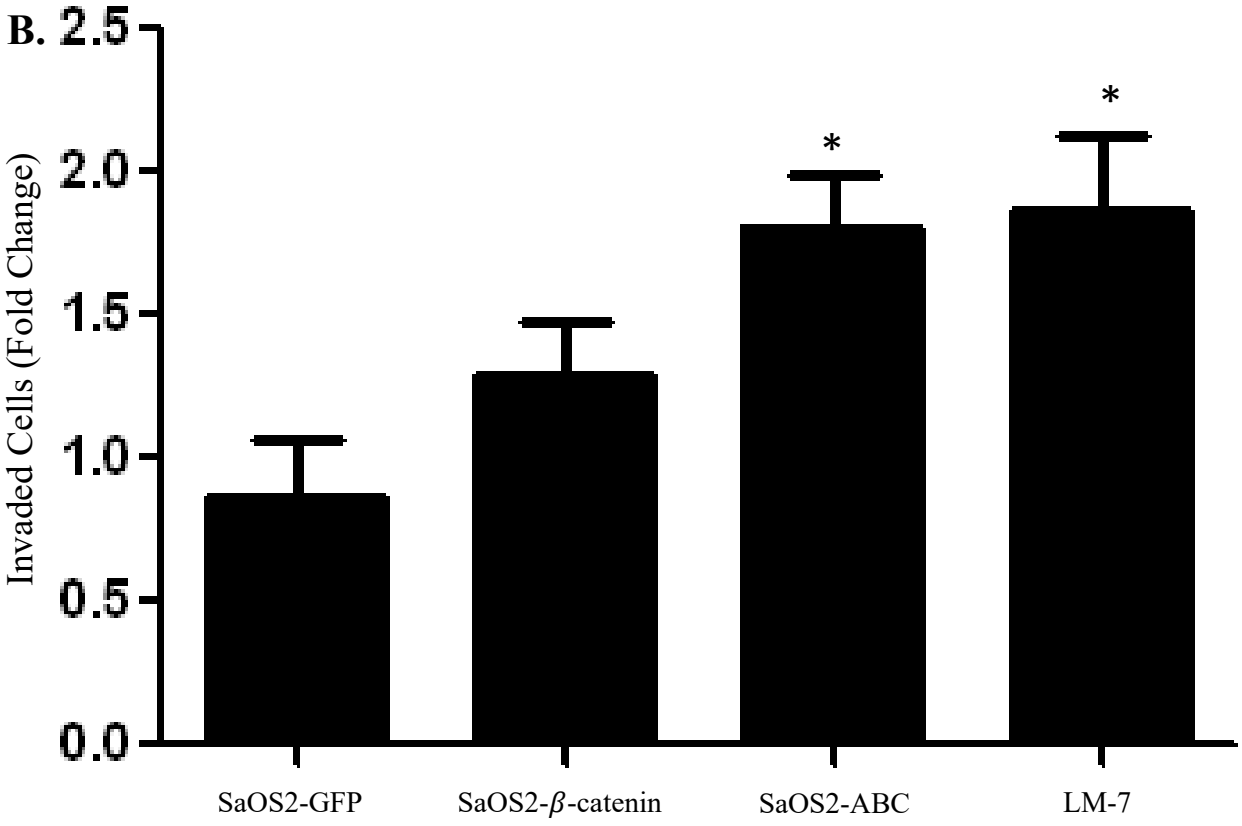

B.
Invaded Cells (Fold Change)
2.5
2.0
1.5
1.0
0.5
0.0
*
*
SaOS2-GFP
SaOS2-β-catenin
SaOS2-ABC
LM-7

Figure 6. Invasion assay analysis of SaOS2 transfectants show increased invasive ability when cells overexpress ABC

A. SaOS2 transfectants and LM-7 representative images. Images taken with Axio-Colour microscope represent invasive ability of the different experimental conditions. **B) SaOS2 transfectants and LM-7.** LM-7 cells show an increase in invasive ability when compared to the control; n=4 *p<0.05. SaOS2 cells overexpressing ABC show a statistically significant increase in invasive ability when compared to the control; n=4, *p<0.05. SaOS2 cells overexpressing β-catenin did not show a significant difference; n=5.

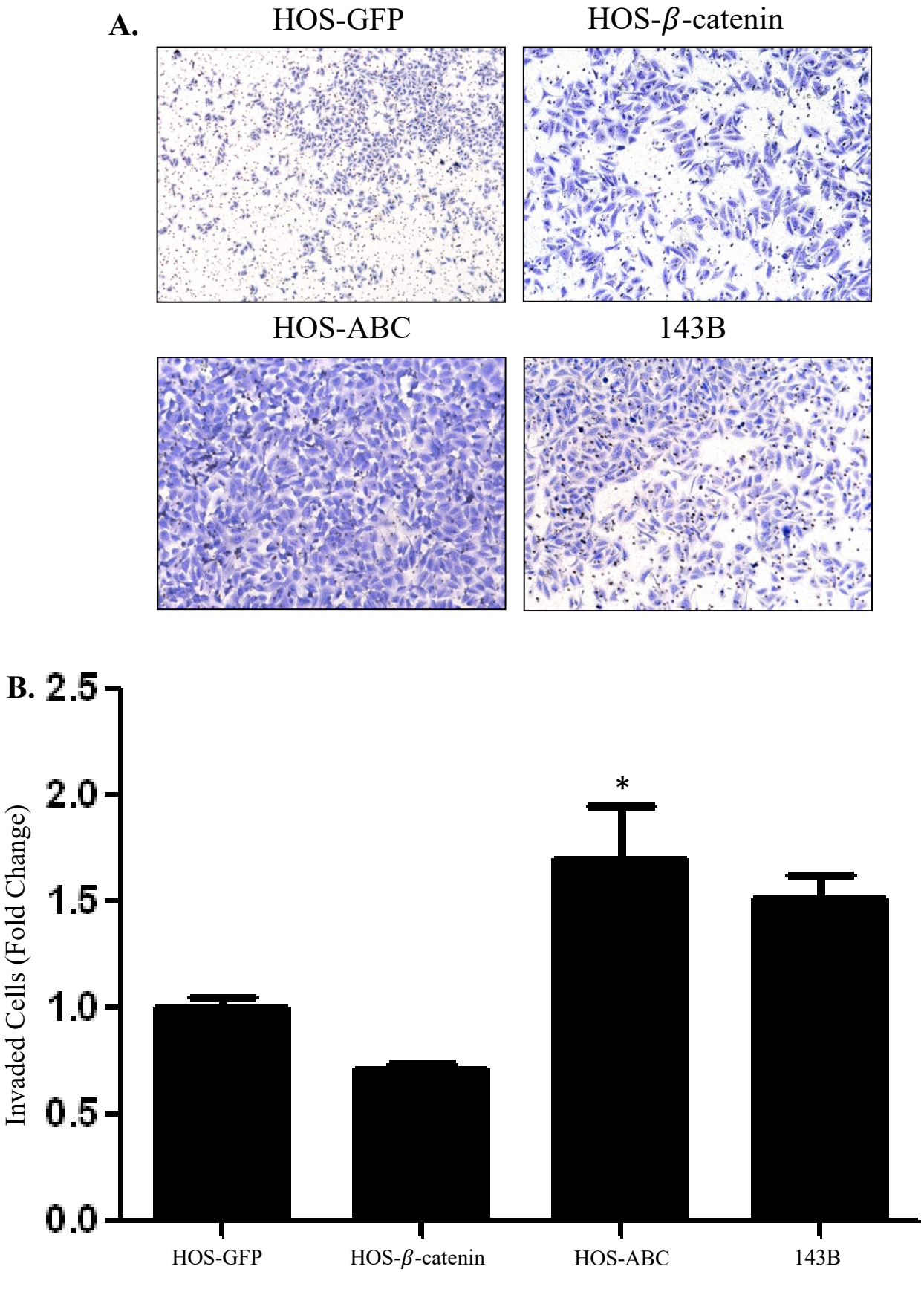

A.
HOS-GFP
HOS-β-catenin
HOS-ABC
143B
B.
Invaded Cells (Fold Change)
2.5
2.0
1.5
1.0
0.5
0.0
*
HOS-GFP
HOS-β-catenin
HOS-ABC
143B

Figure 7. Invasion assay analysis of HOS transfectants show increased invasive ability when HOS cells overexpress ABC

A. HOS transfectants and 143B representative images. Images taken with Axio-Colour microscope represent invasive ability of the different experimental conditions. **B. HOS transfectants and 143B.** 143B cells did not show a statistical difference in invasive ability when compared to HOS-GFP; n=3. HOS cells overexpressing β-catenin also did not show statistical difference in invasive ability; n=5. HOS cells overexpressing ABC showed an increase in invasive ability; n=4 *$p<0.05$.

3.4. ABC overexpression may play a role in migratory abilities in OS cells

Another important feature for OS to invade is the ability of the cells to migrate. Using Boyden chamber assay in the absence of Matrigel, I measured the migratory ability of SaOS2 and HOS transfectants and LM-7/143B.

i. SaOS2 transfectants and LM-7

My results show that there was a statistically significant increase in the SaOS2 cells ability to migrate when overexpressing ABC compared to control cells (*$p<0.05$) (Figure 8B). However, the increase in the migratory ability of the SaOS2-ABC cells was not as high as that of the LM-7 cells which exhibited significantly higher migratory capacity compared to control. (***$p<0.001$) (Figure 8B). SaOS2 cells overexpressing β-catenin did not show any significant difference in their migration compared to control (Figure 8B).

ii. HOS, transfectants, and 143B

My results show there was no statistically significant difference in migratory capacity with overexpression of ABC or β-catenin in HOS cells. However, 143B cells exhibited significantly higher migration compared to control. (***$p<0.001$) (Figure 9B).

These results suggest that ABC may have an effect on the cells overall ability to migrate in SaOS2 cells, but this observation cannot be associated with HOS cells since there is no difference in migratory ability when overexpressing ABC (Figure 8B) (Figure 9B).

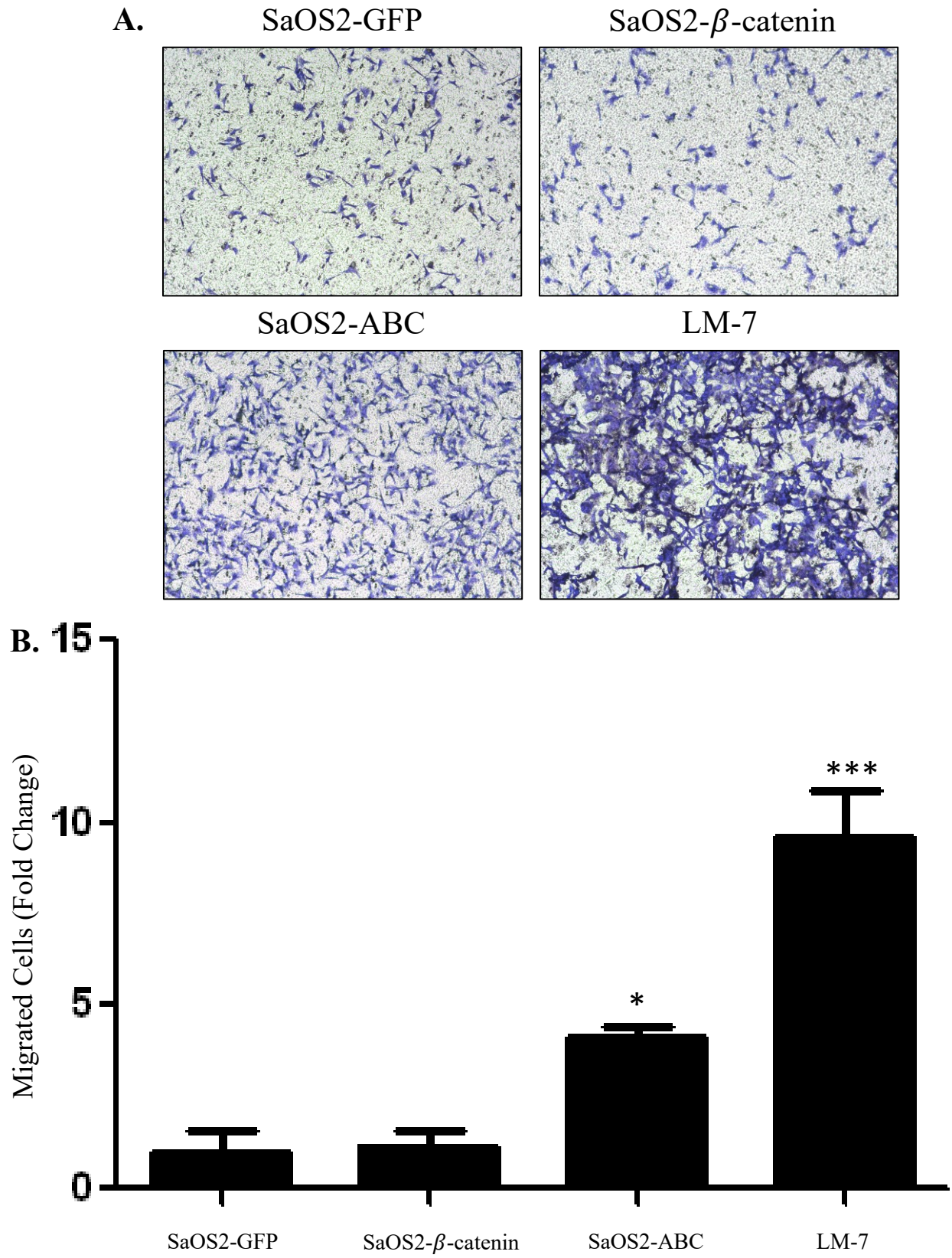

A.
SaOS2-GFP
SaOS2-β-catenin
SaOS2-ABC
LM-7
B.
Migrated Cells (Fold Change)
15
10
5
0
*
***
SaOS2-GFP
SaOS2-β-catenin
SaOS2-ABC
LM-7

Figure 8. Migration Assay analysis of SaOS2 transfectants show increased migration when SaOS2 cells overexpress ABC

A. SaOS2 transfectants and LM-7 representative images. Images taken with Axio-Colour microscope represent migratory ability of the different experimental conditions. **B. SaOS2 transfectants and LM-7.** LM-7 cells show a statistical increase in migratory ability when compared to SaOS2-GFP cells; n=4, ***$p<0.001$. SaOS2 cells overexpressing ABC show a statistical increase in migratory ability; n=3, *$p<0.05$. However, SaOS2 cells overexpressing β-catenin did not show any difference.

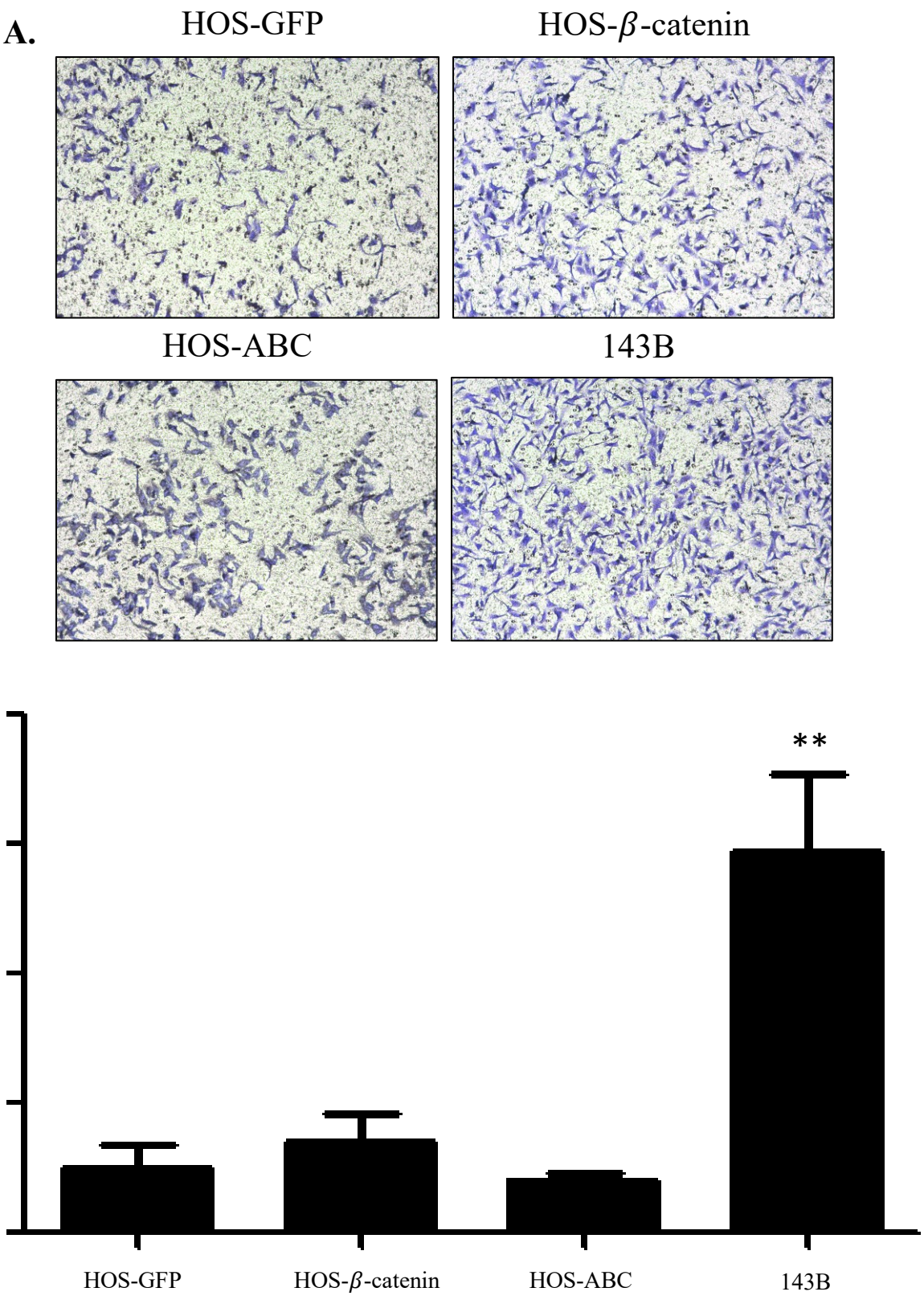

A.
HOS-GFP
HOS-β-catenin
HOS-ABC
143B
B.
Migrated Cells (Fold Change)
0
2
4
6
8
**
HOS-GFP
HOS-β-catenin
HOS-ABC
143B

Figure 9. Migration assay analysis of HOS transfectants show no change in migration

A. HOS transfectants and 143B representative images Images taken with Axio-Colour microscope represent migratory ability of the different experimental conditions. **B. HOS transfectants and 143B.** 143B cells show a statistically significant increase in migratory ability; n=5, **p<0.005. HOS cells overexpressing ABC and β-catenin did not show any statistical difference in migratory ability; n=4, n=4, respectively.

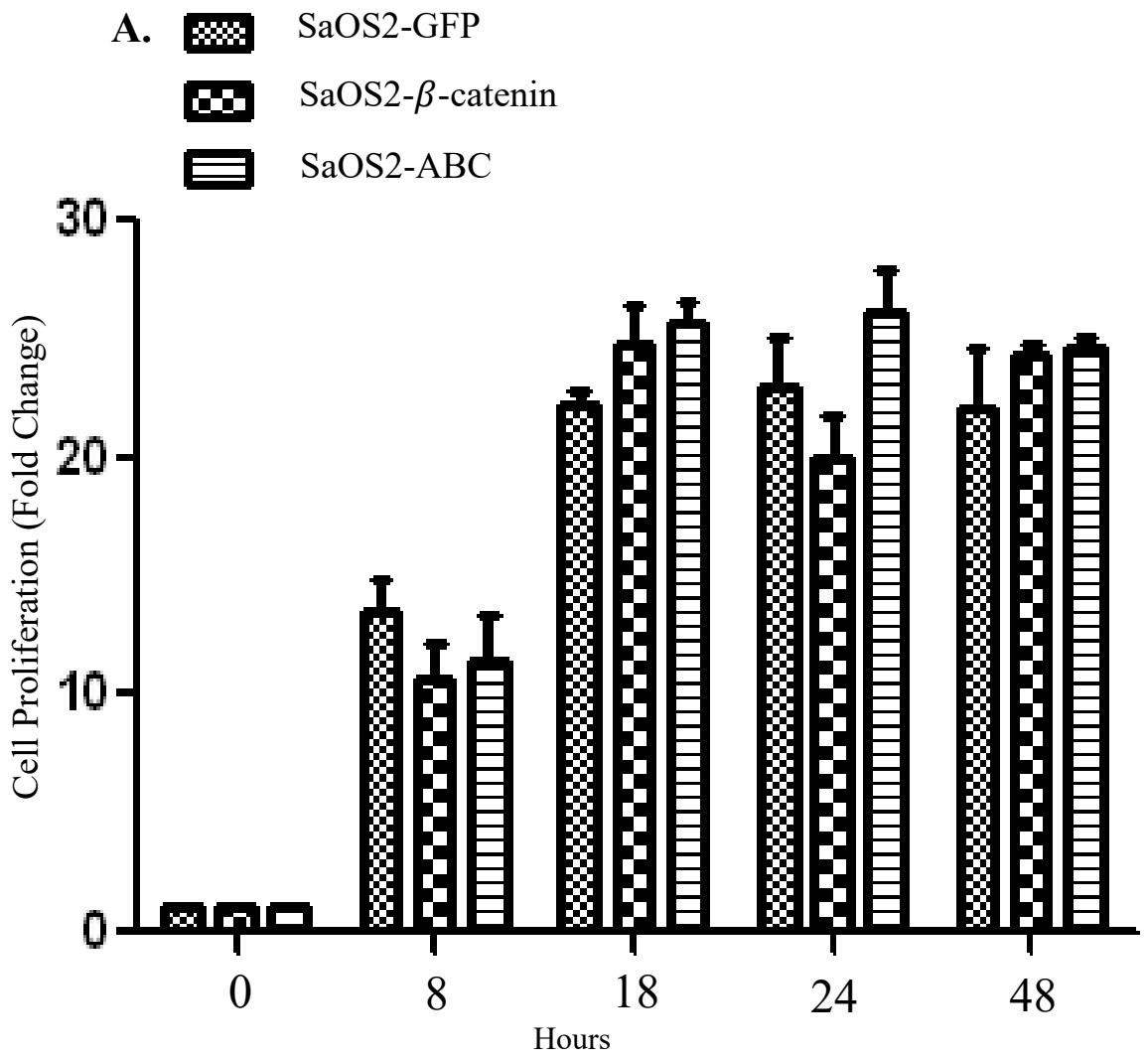
3.5. Overexpression of ABC and β-catenin has no effect on cell proliferation

It is possible that the increased invasion through Matrigel observed with the SaOS2-ABC and HOS-ABC cell lines could be attributed to simply an increase in cell number (proliferation) promoted by ABC. To determine if this was the case I measured cell proliferation in the various transfectants using the alamarBlue^{TM} Cell Viability Assay. This assay is able to quantify the cell proliferation by measuring Resorufin, a by-product of the alamarBlue^{TM} when cells proliferate.

i. SaOS2 and HOS transfectants

Over a 48-hour time period I measured the SaOS2 and HOS cell transfectants proliferative ability at 8-,18-,24-,48-hour intervals. My results demonstrate that there is no statistical difference in proliferative abilities between the transfectant groups within each cell line at all time periods (Figure 10A) (Figure 10B).

These results suggest that overexpression of both the ABC and β-catenin in the SaOS2 and HOS cell lines had no effect on proliferation.


A.
SaOS2-GFP
SaOS2-β-catenin
SaOS2-ABC
Cell Proliferation (Fold Change)
30
20
10
0
0
8
18
24
48
Hours

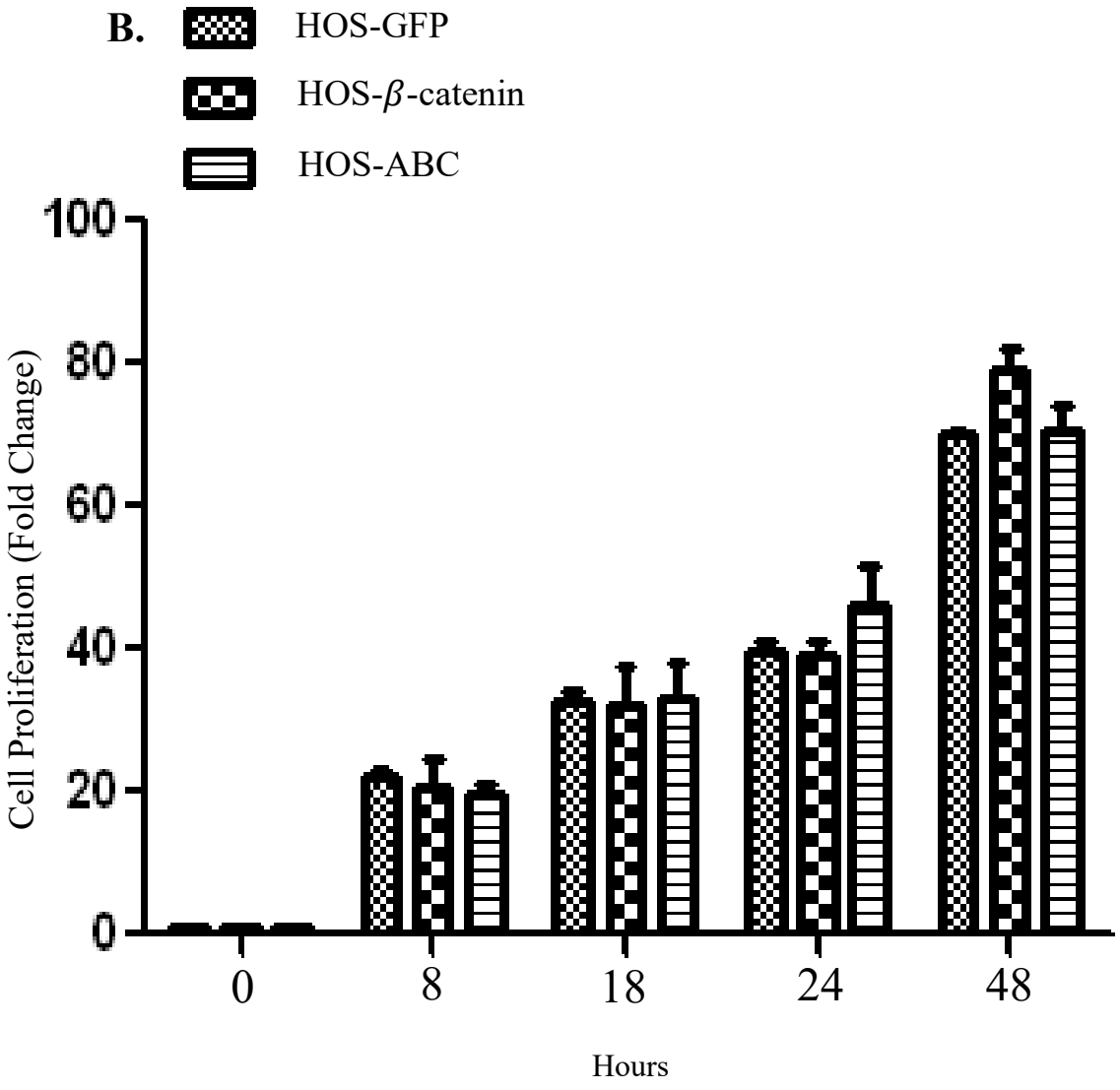

B.
HOS-GFP
HOS-β-catenin
HOS-ABC
Cell Proliferation (Fold Change)
100
80
60
40
20
0
0
8
18
24
48
Hours

Figure 10. Overexpression of ABC and β-catenin shows no impact on cell proliferation

A. SaOS2 transfectants. Using two-way ANOVA I compared the fold change in proliferation of the SaOS2 transfected cell lines at different time points. My results suggest there was no significant difference between cells overexpressing ABC and β-catenin; n=3, n=3, p=0.3689. **B. HOS transfectants.** When comparing the HOS transfected cell lines, there was also no statistically significant difference between cells overexpressing ABC and β-catenin; n=3, n=3, p=0.9352.

3.6. Overexpression of ABC causes an increase in MMP9 and MMP2 mRNA expression and a change in the RANKL:OPG ratio

MMP9 and MMP2 matrix metalloproteases are transcriptional targets of the canonical Wnt/β-catenin pathway. Furthermore, an important feature involved in the progression of OS to a more invasive phenotype is the activity of both MMP9 and MMP2. MMP9 and MMP2 transcriptional expression was quantified by evaluating the mRNA expression of both metalloproteases in SaOS2 and HOS cells overexpressing ABC or β-catenin. Another valuable piece of information is having an understanding of the characterization of OS as either osteoblastic or osteolytic which can be determined by the RANKL:OPG ratio. I quantified the mRNA expression of RANKL and OPG and then combined the two results to determine the RANKL:OPG ratio between SaOS2 and HOS cells overexpressing ABC and β-catenin. GAPDH was used as the housekeeping gene. This experiment was completed utilizing quantitative real-time Reverse Transcriptase polymerase chain reaction (RT-qPCR).

i. MMP9 and MMP2 mRNA expression from SaOS2 transfectants and LM-7

My results show that overexpression of ABC in the SaOS2 cell line led to a statistically significant increase in mRNA expression of MMP9 compared to the control (**$p<0.005$) (Figure 11A). These mRNA levels were similar to the mRNA levels seen in the LM7 cell line (**$p<0.005$) (Figure 11A). MMP9 mRNA expression was not altered in SaOS2 cells overexpressing β-catenin. My results on MMP2 mRNA expression show that there was no statistically significant difference between SaOS2 cells that overexpressed both ABC and β-catenin compared to control (Figure 11B). LM-7 cells exhibited a statistically significant increase in mRNA expression of MMP2 (*$p<0.05$) (Figure 11B).

ii. MMP9 and MMP2 RNA expression from HOS transfectants and 143B

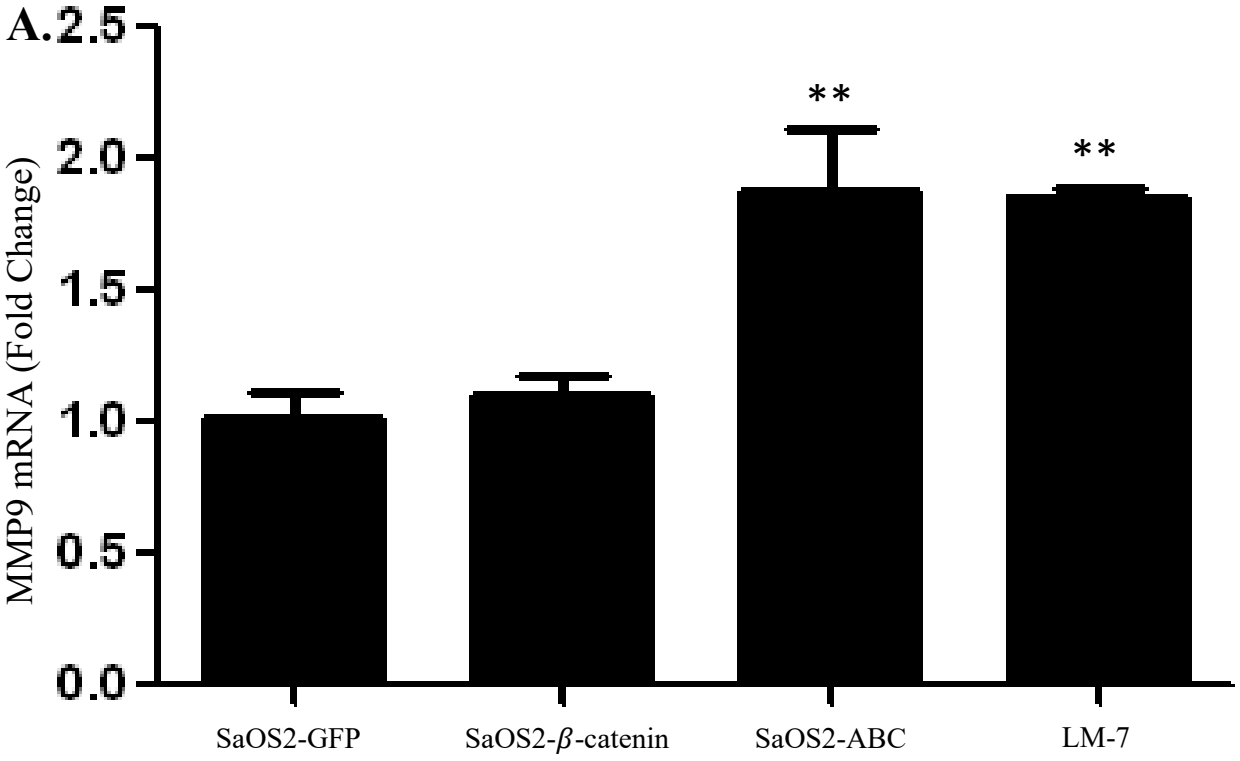
My results show that the overexpression of ABC in the HOS cells led to a statistically significant increase in MMP9 mRNA expression (*$p<0.05$) (Figure 11C). The 143B cell line also showed an increase in MMP9 mRNA expression but had a higher significance (***$p<0.001$) (Figure 11C). HOS cells overexpressing β-catenin did not show any alteration in MMP9 mRNA expression (Figure 11C). MMP2 mRNA expression in the HOS cells overexpressing β-catenin and in the 143B cell line showed that there was no statistically significant difference (Figure 11D).

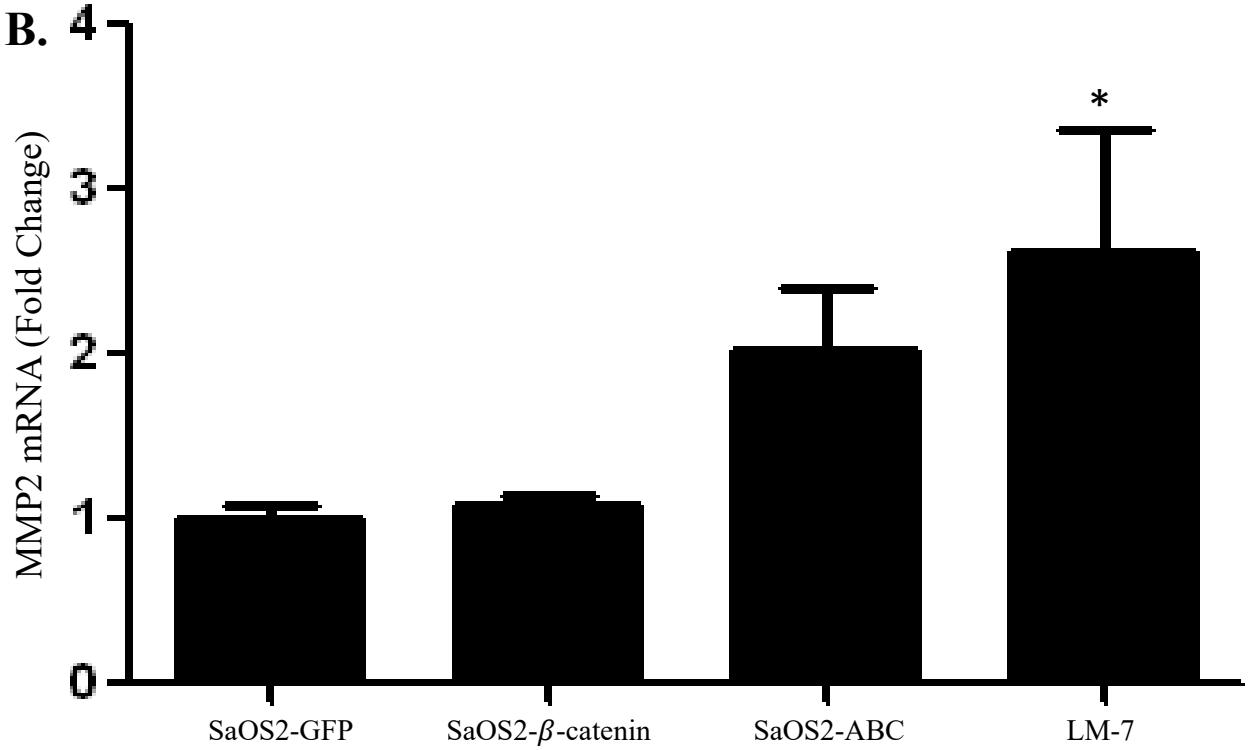
However, when HOS cells were overexpressing ABC, there was a statistically significant increase in MMP2 mRNA expression (*p<0.05) (Figure 11D).

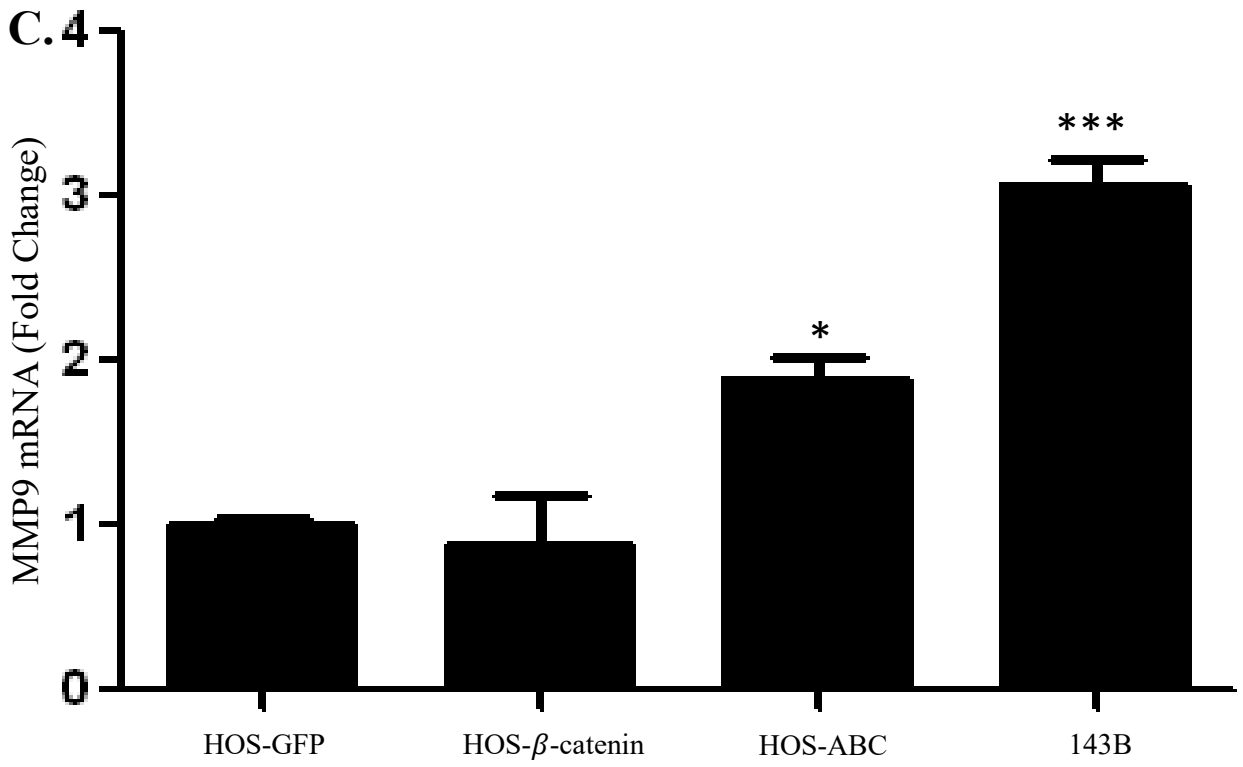
iii. RANKL:OPG ratio from SaOS2 and HOS transfectants and LM-7/143B

The RANKL:OPG ratio from all SaOS2 transfectants and LM-7 showed me there was no significant difference (Figure 12A). However, HOS cells overexpressing ABC showed a significant decrease, this shift in the RANKL:OPG ratio suggests a higher OPG mRNA expression compared to RANKL mRNA expression (**p<0.005) (Figure 12B). When analyzing the 143B cells RANKL:OPG ratio results suggested a similar trend that was seen in HOS cells overexpressing ABC however there was an even greater decrease in our RANKL:OPG ratio (***p<0.001) (Figure 12B).

These results suggest that when both SaOS2 and HOS cells overexpress ABC there is a significant increase in the mRNA expression of MMP9 (Figure 11A) (Figure 11C). However, this overexpression does not hold true for MMP2 mRNA expression as only HOS cells that overexpressed ABC had a statistically significant increase in MMP2 mRNA expression (Figure 11B) (Figure 11D). LM-7 cells showed a statistically significantly higher expressions of MMP9 and MMP2 mRNA while 143B cells only had a statistically significant higher expression of MMP9 mRNA (Figure 11A) (Figure 11B) (Figure 11C) (Figure 11D). The RANKL:OPG ratio is known to help distinguish the characteristic of OS, where an increased RANKL expression is seen in more typical osteolytic lesions and an increased OPG expression is seen in more osteoblastic lesions (Ando, Mori, Redini, & Heymann, 2008). The SaOS2 cells overexpressing ABC and β-catenin showed no significant difference in the RANKL:OPG ratio indicating a 1:1 ratio of RANKL to OPG mRNA expression (Figure 12A). However, HOS cells overexpressing ABC showed a statistically significant decrease in the RANKL:OPG ratio meaning that the RANKL:OPG ratio favours the mRNA expression of OPG over RANKL (Figure 12B). LM-7 cell line did not have any significant difference in its RANKL:OPG ratio while 143B showed a significantly lower RANKL:OPG ratio, heavily favouring the mRNA expression of OPG to RANKL (Figure 12A) (Figure 12B).


A.
2.5
2.0
1.5
1.0
0.5
0.0
MMP9 mRNA (Fold Change)
**
**
SaOS2-GFP
SaOS2-β-catenin
SaOS2-ABC
LM-7


B.
4
3
2
1
0
MMP2 mRNA (Fold Change)
*
SaOS2-GFP
SaOS2-β-catenin
SaOS2-ABC
LM-7


C.
MMP9 mRNA (Fold Change)
4
3
2
1
0
*
***
HOS-GFP
HOS-β-catenin
HOS-ABC
143B

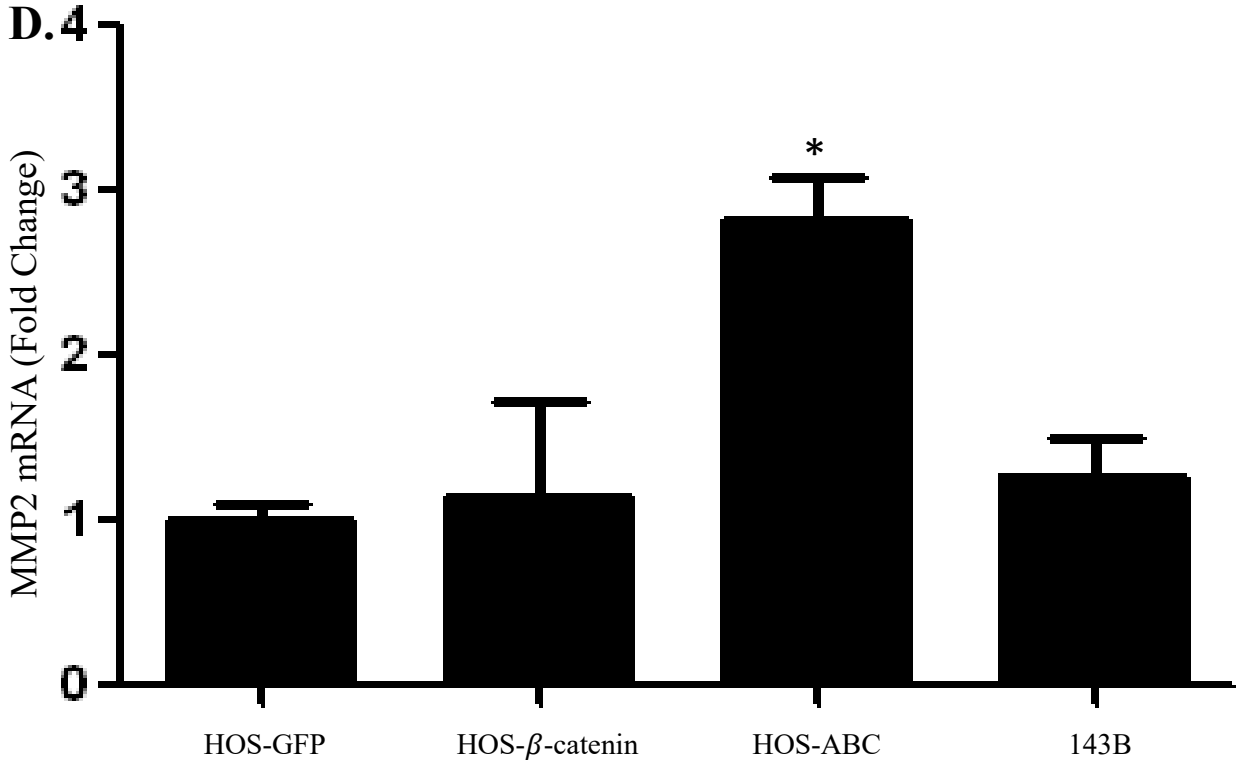
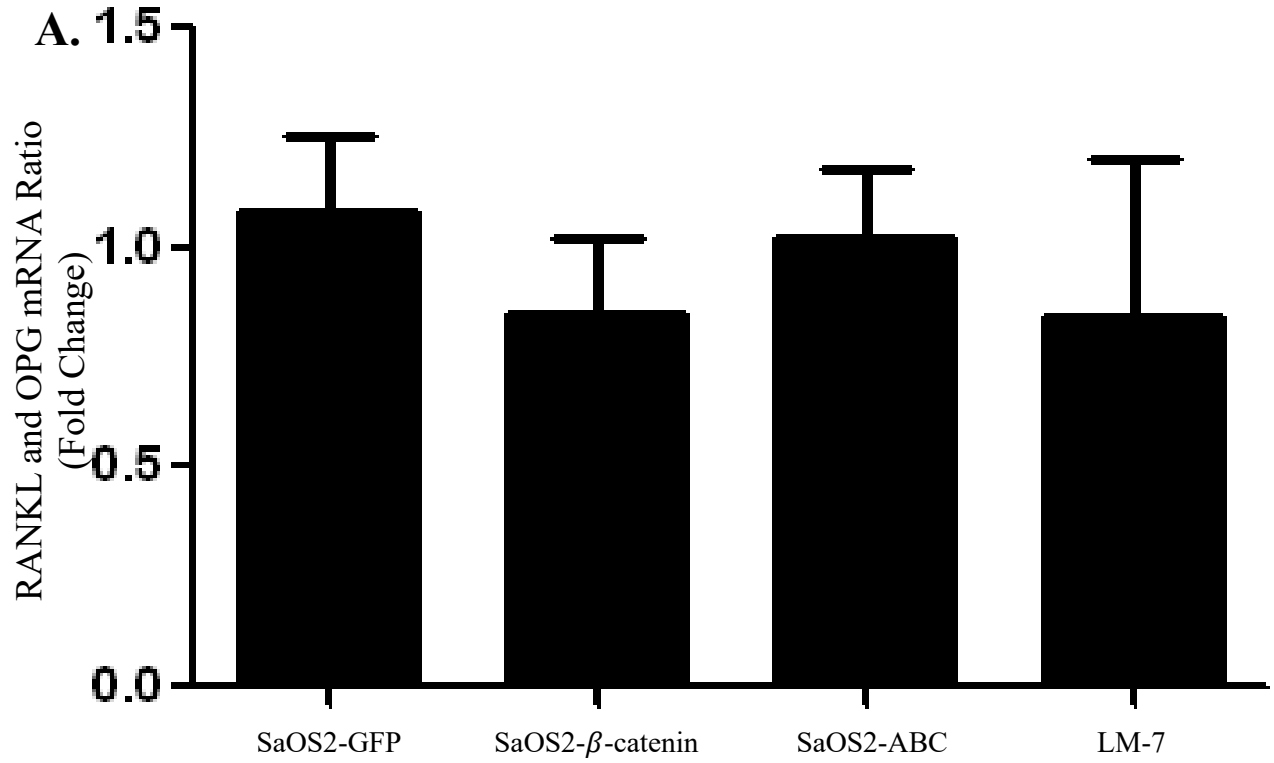

D.
MMP2 mRNA (Fold Change)
4
3
2
1
0
*
HOS-GFP
HOS-β-catenin
HOS-ABC
143B

Figure 11. Overexpression of ABC in SaOS2 and HOS cells utilizing RT-qPCR show an increase in both MMP9 and MMP2 mRNA expression

A. SaOS2 transfectants and LM-7 MMP9 mRNA. Overexpression of ABC in SaOS2 cells led to an increased mRNA expression of MMP9; n=3, **p<0.005. LM-7 cells also displayed a statistical increase in mRNA expression of MMP9; n=3, **p<0.005. Overexpression of β-catenin in SaOS2 cells did not show any statistically significant difference in mRNA expression of MMP9; n=3 **B. SaOS2 transfectants and LM-7 MMP2 mRNA.** MMP2 mRNA expression did not change when SaOS2 cells overexpressed ABC and β-catenin, however, there was a statistical increase in mRNA expression in the LM-7 cells; n=3, n=3, n=3, *p<0.05, respectively. **C. HOS transfectants and 143B MMP9 mRNA.** MMP9 mRNA expression was shown to increase in HOS cells overexpressing ABC and in 143B cells; n=3, *p<0.05, n=3, ***p<0.001, respectively. There was no difference seen in HOS cells that overexpressed β-catenin; n=3. **D. HOS transfectants and 143B MMP2 mRNA**. When HOS cells overexpressed ABC, there was a statistically significant increase in MMP2 mRNA expression; n=3, *p<0.05. There was no difference in cells overexpressing β-catenin and 143B cells; n=3, n=3, respectively.


A.
1.5
1.0
0.5
0.0
RANKL and OPG mRNA Ratio
(Fold Change)
SaOS2-GFP
SaOS2-β-catenin
SaOS2-ABC
LM-7

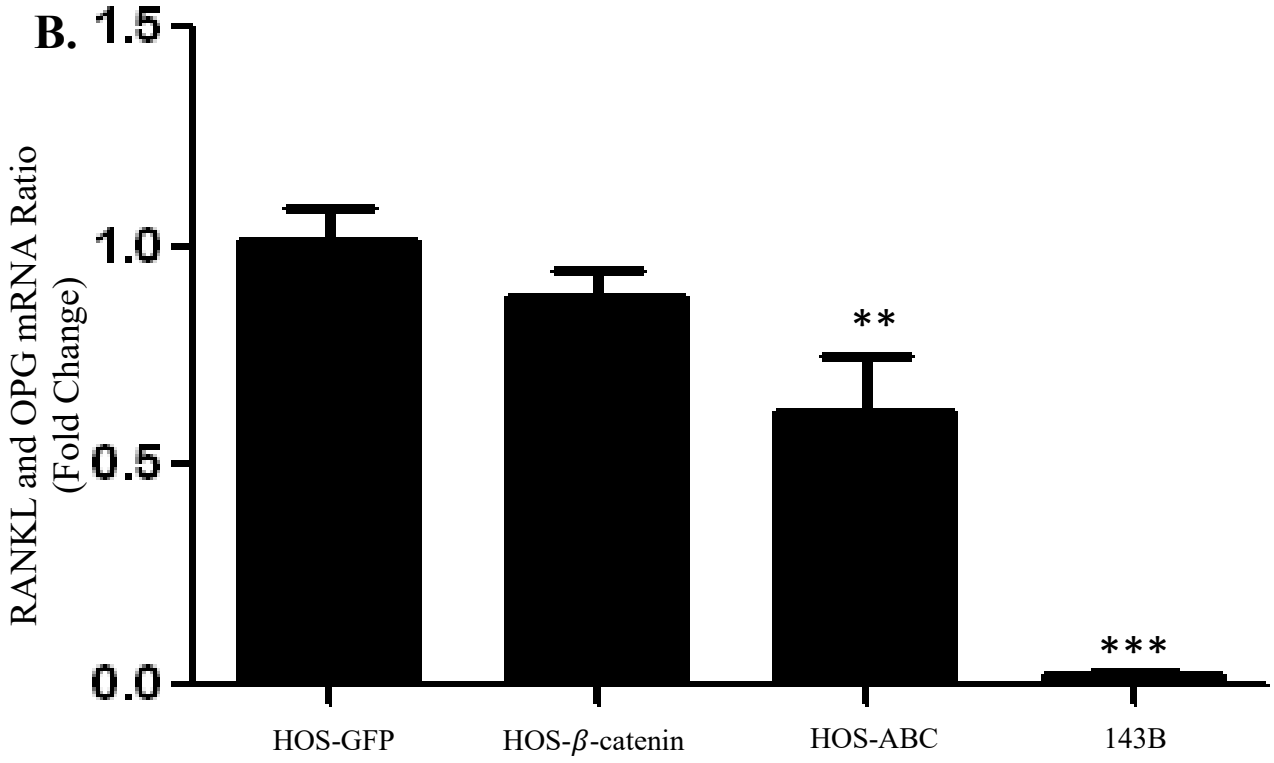

B.
1.5
1.0
0.5
0.0
RANKL and OPG mRNA Ratio
(Fold Change)
**
***
HOS-GFP
HOS-β-catenin
HOS-ABC
143B

Figure 12. RANKL:OPG ratio changes with overexpression of ABC in HOS cells

A. SaOS2 transfectants and LM-7 RANKL:OPG mRNA ratio. No statistically significant differences seen in the RANKL:OPG ratio in SaOS2 cells overexpressing ABC, β-catenin, and LM-7 cells; n=6, n=6, n=6, respectively. **B. HOS transfectants and LM-7 RANKL:OPG mRNA ratio** No statistically significant difference was seen in the RANKL:OPG ratio in HOS cells overexpression β-catenin; n=6. A significant decrease in the RANKL:OPG ratio was seen when HOS cells were overexpressing ABC; n=6, **p<0.005. An even greater decrease was seen in the RANKL:OPG ratio in the 143B cells; n=6; ***p<0.001.

3.7. Overexpression of ABC does not affect MMP9 and MMP2 activity

Because of the important roles MMP9 and MMP2 play in the invasive phenotype seen in OS I utilized gelatin zymography to detect the proteolytic activity of these two enzymes and to see if there was a difference when the SaOS2 and HOS cells overexpressed either ABC or β-catenin. I quantified the activity of both MMP9 and MMP2.

i. MMP9 activity: SaOS2 transfectants and LM-7

My results show that there is no statistically significant difference in MMP9 activity in SaOS2 cells overexpressing ABC or β-catenin, compared to control. (Figure 13A) (Figure 13C). However, The LM7 cells exhibited a statistically significant increase in MMP9 activity compared to control. (*$p<0.05$) (Figure 13A) (Figure 13C).

ii. MMP9 activity: HOS transfectants and 143B

My results show that there is no statistically significant difference in MMP9 activity in HOS cells overexpressing ABC or β-catenin, compared to control (Figure 13B) (Figure 13D) However, the 143B cells exhibited a statistically significant increase MMP9 activity compared to the control (***$p<0.001$) (Figure 13B) (Figure 13D).

iii. MMP2 activity: SaOS2 transfectants and LM-7

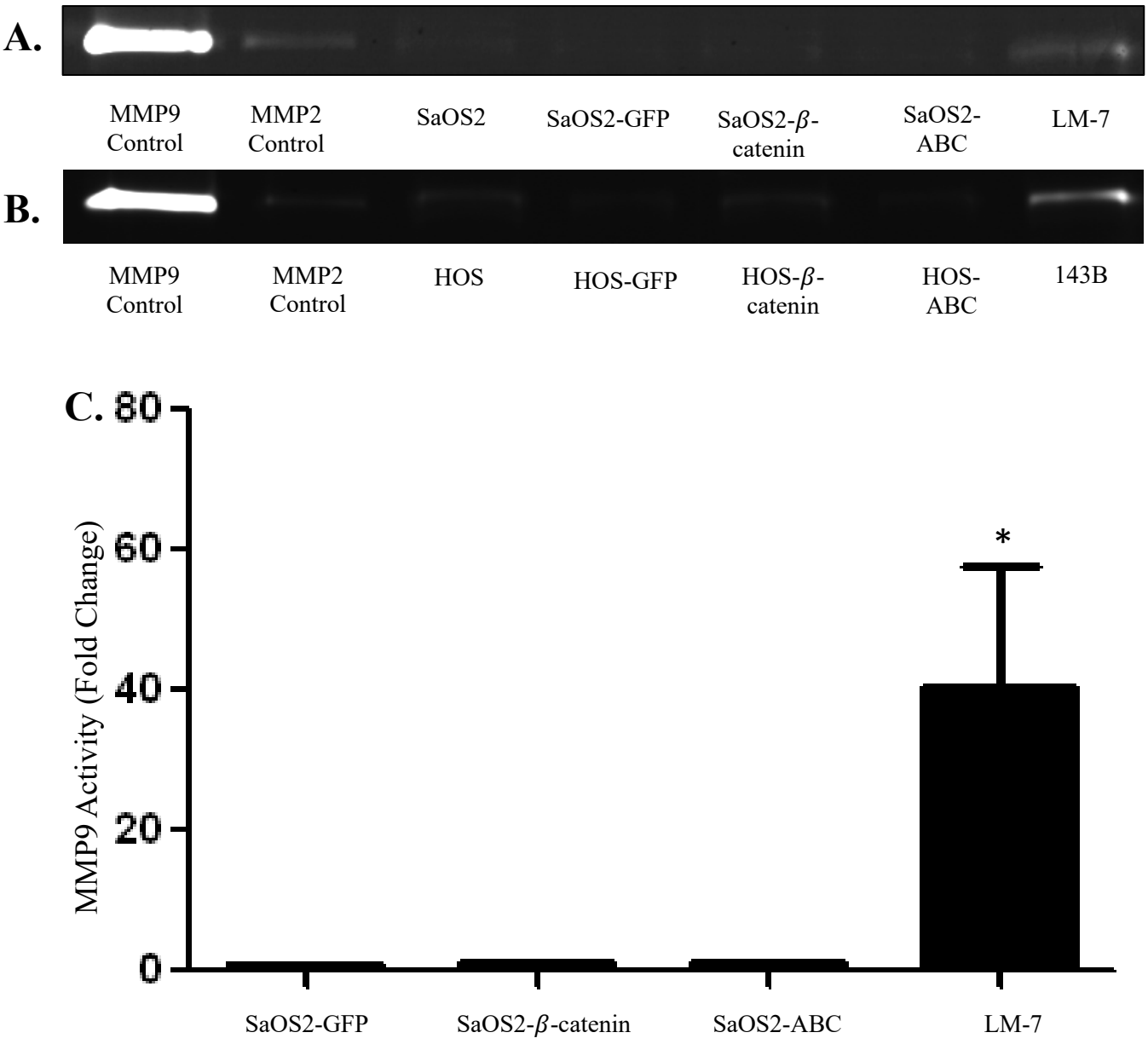
My results show that there is no statistically significant difference in MMP2 activity in SaOS2 cells overexpressing ABC or β-catenin (Figure 14A) (Figure 14C). LM-7 also showed no difference in MMP2 activity (Figure 14A) (Figure 14C).

iv. MMP2 activity: HOS transfectants and 143B

My results show that there is no statistically significant difference in MMP2 activity in HOS cells overexpressing ABC and β-catenin (Figure 14B) (Figure 14D) 143B cells also showed no statistically significant difference in MMP2 activity (Figure 14B) (Figure 14D).

These results suggest that the overexpression of ABC has no direct effect on the proteolytic ability of MMP9 and MMP2. However, it is important to note that the metastatic daughter lines of

LM-7 and 143B both showed a significant increase in MMP9 activity that was not seen in the transfectants of SaOS2 and HOS.


A.
MMP9 Control
MMP2 Control
SaOS2
SaOS2-GFP
SaOS2-β-catenin
SaOS2-ABC
LM-7
B.
MMP9 Control
MMP2 Control
HOS
HOS-GFP
HOS-β-catenin
HOS-ABC
143B
C.
MMP9 Activity (Fold Change)
80
60
40
20
0
*
SaOS2-GFP
SaOS2-β-catenin
SaOS2-ABC
LM-7

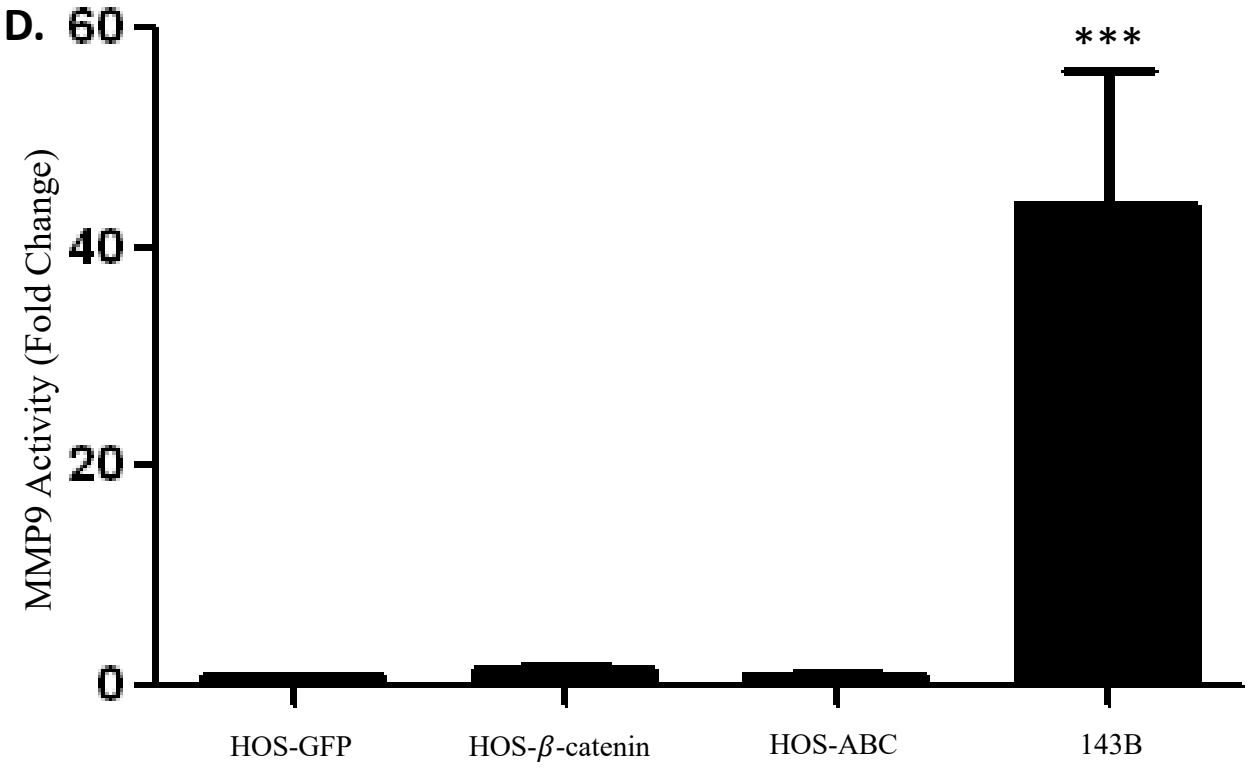
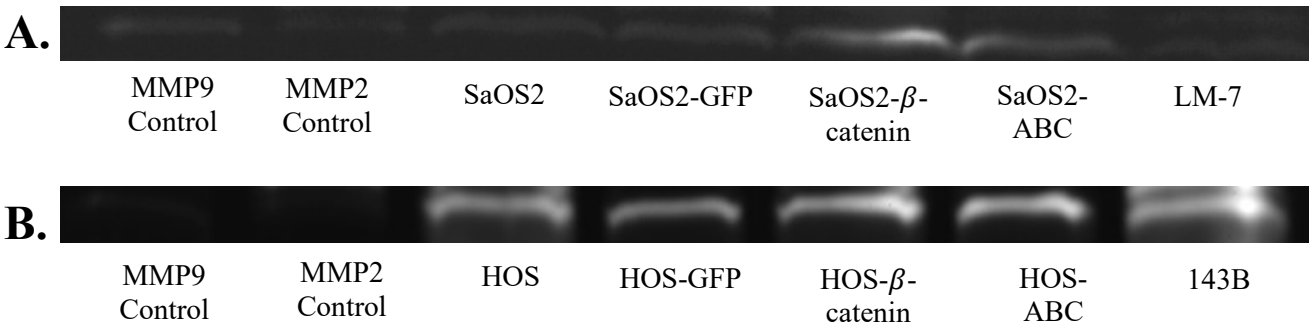
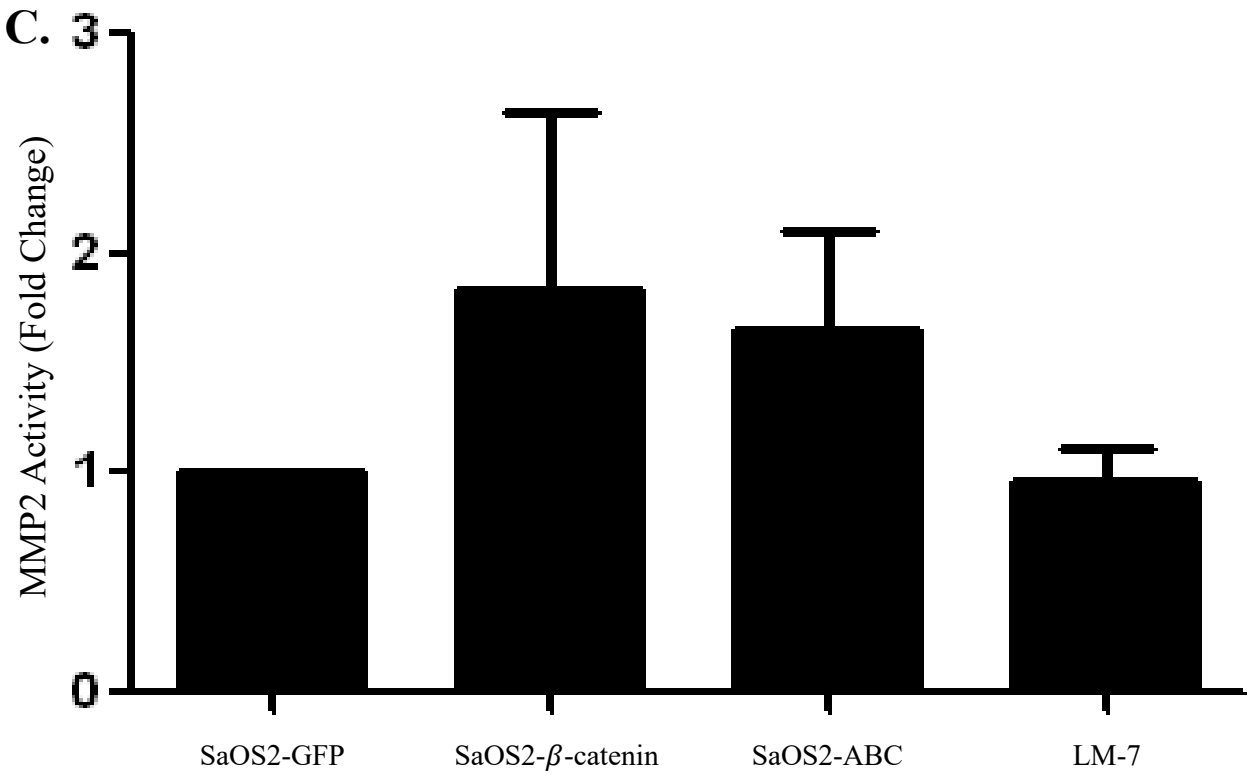


Figure 13. Utilizing gelatin zymography to measure MMP9 activity results in only LM-7 and 143B being significantly increased

A. SaOS2 transfectants and LM-7 MMP9 image. Representation of MMP9 activity of SaOS2 transfectants and LM-7 cell line. **B.HOS transfectants and 143B MMP9 image.** Representation of MMP9 activity of HOS transfectants and 143B. **C. SaOS2 transfectants and LM-7 MMP9 activity.** LM-7 cells had a significant increase in MMP9 proteolytic activity while overexpression of ABC and β-catenin resulted in no change; n=3, *p<0.05, n=3, n=3, respectively. **D. HOS transfectants and 143B MMP9 activity.** 143B cells also showed a statistically significant increase in MMP9 activity while overexpression of ABC and β-catenin in HOS cells resulted in no change; n=3, ***p<0.001, n=3, n=3, respectively.


A.
MMP9 Control
MMP2 Control
SaOS2
SaOS2-GFP
SaOS2-β-catenin
SaOS2-ABC
LM-7
B.
MMP9 Control
MMP2 Control
HOS
HOS-GFP
HOS-β-catenin
HOS-ABC
143B


C.
MMP2 Activity (Fold Change)
0
1
2
3
SaOS2-GFP
SaOS2-β-catenin
SaOS2-ABC
LM-7

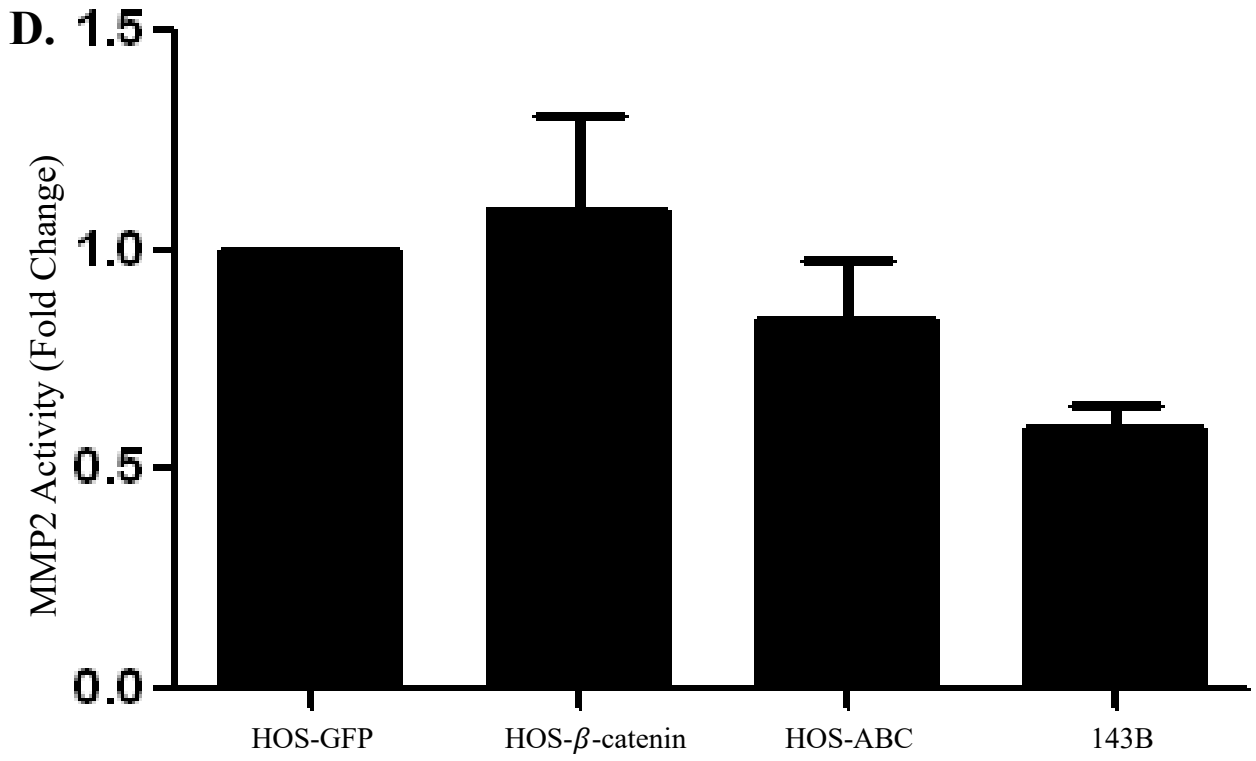


Figure 14. Utilizing gelatin zymography to measure MMP2 activity results in no significant change

A. SaOS2 transfectants and LM-7 MMP2 image. Representation of MMP2 activity of SaOS2 transfectants and LM-7 cell line. **B. HOS transfectants and 143B MMP2 image.** Representation of MMP2 activity of HOS transfectants and 143B. **C. SaOS2 transfectants and LM-7 MMP2 activity.** No statistically significant change in MMP2 activity between SaOS2 cells overexpressing ABC, β-catenin, and LM-7; n=3, n=3, n=3. **D. HOS transfectants and 143B MMP2 activity.** No statistically significant change in MMP2 activity between HOS cells overexpressing ABC, β-catenin, and 143B; n=3, n=3, n=3, respectively.

3.8. ABC has a potential role in anchorage independent growth however, fine tuning of protocol is required

An important characteristic of cancer progression is their ability to grow colonies that are anchorage independent. This is possible due to an ability of these cancerous cells to avoid undergoing anoikis (apoptosis caused by absence of attachment to ECM). I compared colony forming ability of SaOS2 and HOS transfectants and LM-7/143B. Unfortunately, this experiment over several trials did not produce very visible colonies. However, very small colonies under the microscope were visible but were difficult to image. Alterations to the experimental protocol will need to be made before attempting another series of experiments. Issues pertaining to the challenges faced will be further detailed in "Future Directions".

i. SaOS2 transfectants and LM-7

The results show that there is no significant difference in the number of colonies in SaOS2 cells that overexpress ABC or β-catenin compared to control (Figure 15A) (Figure 15B). The LM7 cells also did not form significantly greater number of colonies compared to control (Figure 15A) (Figure 15B)

ii. HOS transfectants and 143B

The results suggest that there is no significant difference between HOS cells that overexpressed ABC and β-catenin compared to control cells (Figure 16A) (Figure 16B) However, 143B cells were able to grow under these conditions and showed that if this project is done properly I could potentially see some more substantiated results (***$p<0.001$) (Figure 16A) (Figure 16B).

These results do present an opportunity to show that ABC could have an impact on anchorage independent growth which is crucial for OS progression. When looking at both graphs there is a noticeable trend of increasing colonies in both cell lines when overexpressing ABC, however they are not significant (Figure 15B) (Figure 16B). Fine tuning of the protocol will be required.

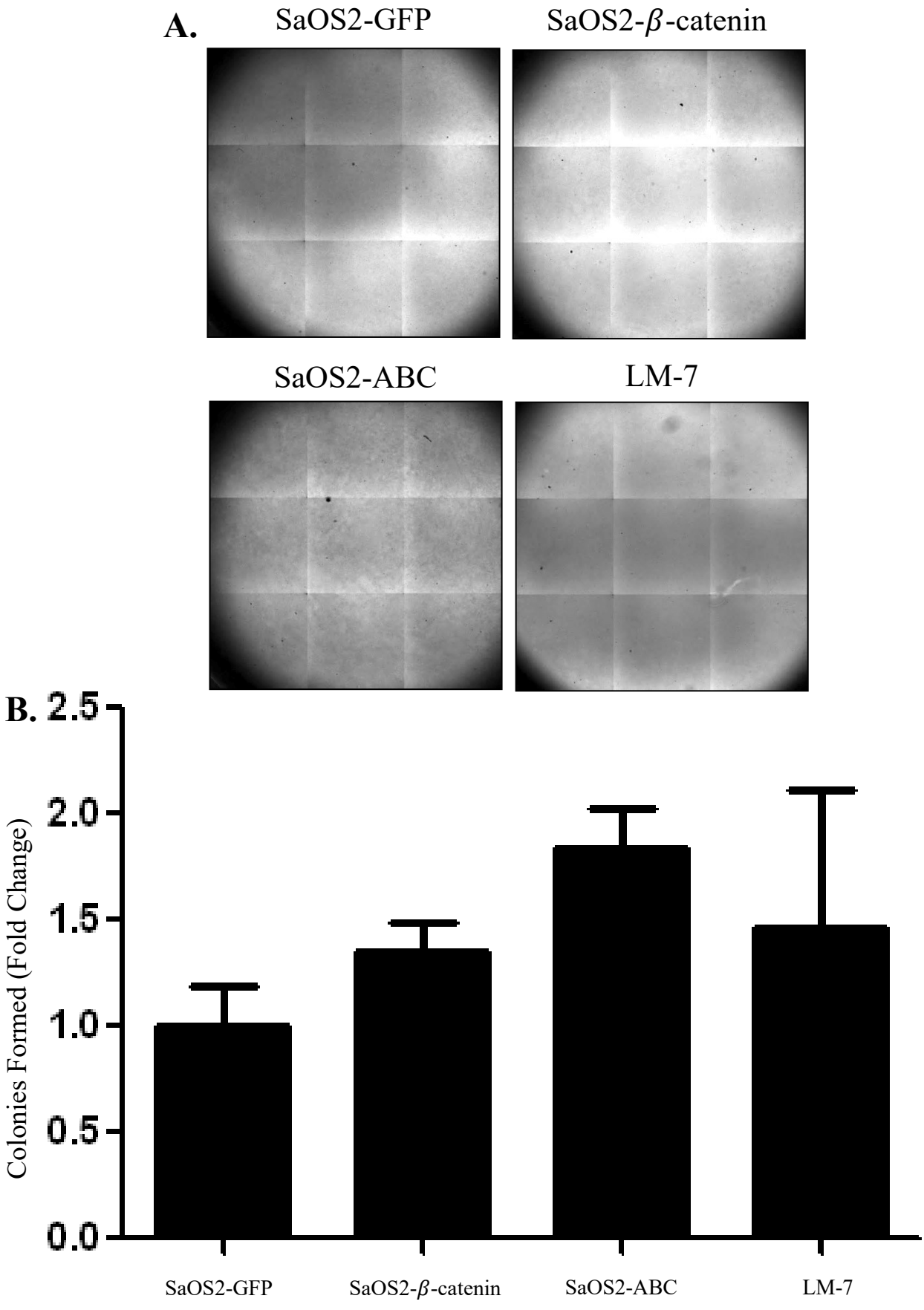

A.
SaOS2-GFP
SaOS2-β-catenin
SaOS2-ABC
LM-7
B.
Colonies Formed (Fold Change)
2.5
2.0
1.5
1.0
0.5
0.0
SaOS2-GFP
SaOS2-β-catenin
SaOS2-ABC
LM-7

Figure 15. Soft agar colony forming assays show possible role ABC has in anchorage independent growth

A. SaOS2 transfectants and LM-7 representative image. Images taken with High Content System showing soft agar colony forming assays depict lack of growth seen by transfected SaOS2 cell lines and LM-7. **B. SaOS2 transfectants and LM-7.** No statistically significant difference in SaOS2 cells overexpressing β-catenin and ABC, and LM-7 cells; n=3, n=3, n=3, respectively.

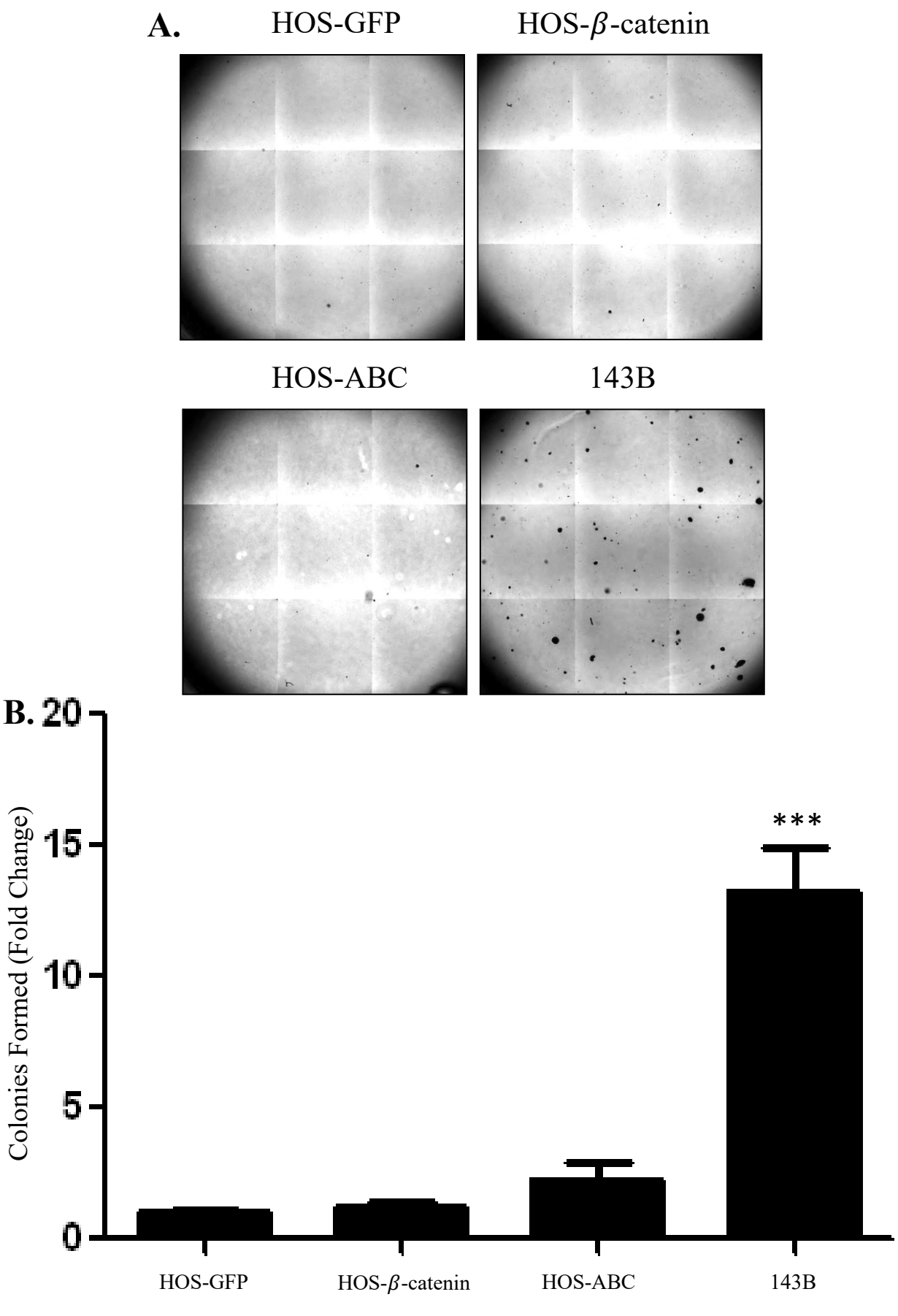

A.
HOS-GFP
HOS-β-catenin
HOS-ABC
143B
B.
Colonies Formed (Fold Change)
20
15
10
5
0
***
HOS-GFP
HOS-β-catenin
HOS-ABC
143B

Figure 16. Soft agar colony forming assays show possible insight into whether ABC has a role in anchorage independent growth

A. HOS transfectants and 143B representative image. Images taken with High Content System showing soft agar colony forming assays depict lack of growth seen by transfected HOS cell lines. 143B cells were able to grow and colonies can be seen. **B. HOS transfectants and 143B.** No statistically significant difference in HOS cells overexpressing ABC and β-catenin; n=3, n=3, respectively. 143B cells were very efficient at growing in these conditions; n=3, ***$p<0.001$.

3.9. Preparation of Stable SaOS2 cell lines expressing pEGFP-C2-β-catenin and pEGFP-C2-ABC for future research

An important aspect of our labs research is the continuation of the project and seeing how ABC promotes OS progression and metastasis. One of the future directions for our research is utilizing stably transfected cells to inject *in vivo*. One of the many experiments I worked on was the development of these stable cell lines.

i. SaOS2 stables

To see whether or not the stable cell lines were expressing the GFP-tagged proteins, utilization of immunofluorescent and microscopy was required. The images taken suggest that the SaOS2 cell lines transfected with pEGFP-C2-ABC and pEGFP-C2-β-catenin were able to integrate the plasmid and consistently produce the GFP-tagged proteins to form our now stable SaOS2-ABC and SaOS2-β-catenin cell lines (Figure 17) (Figure 18).

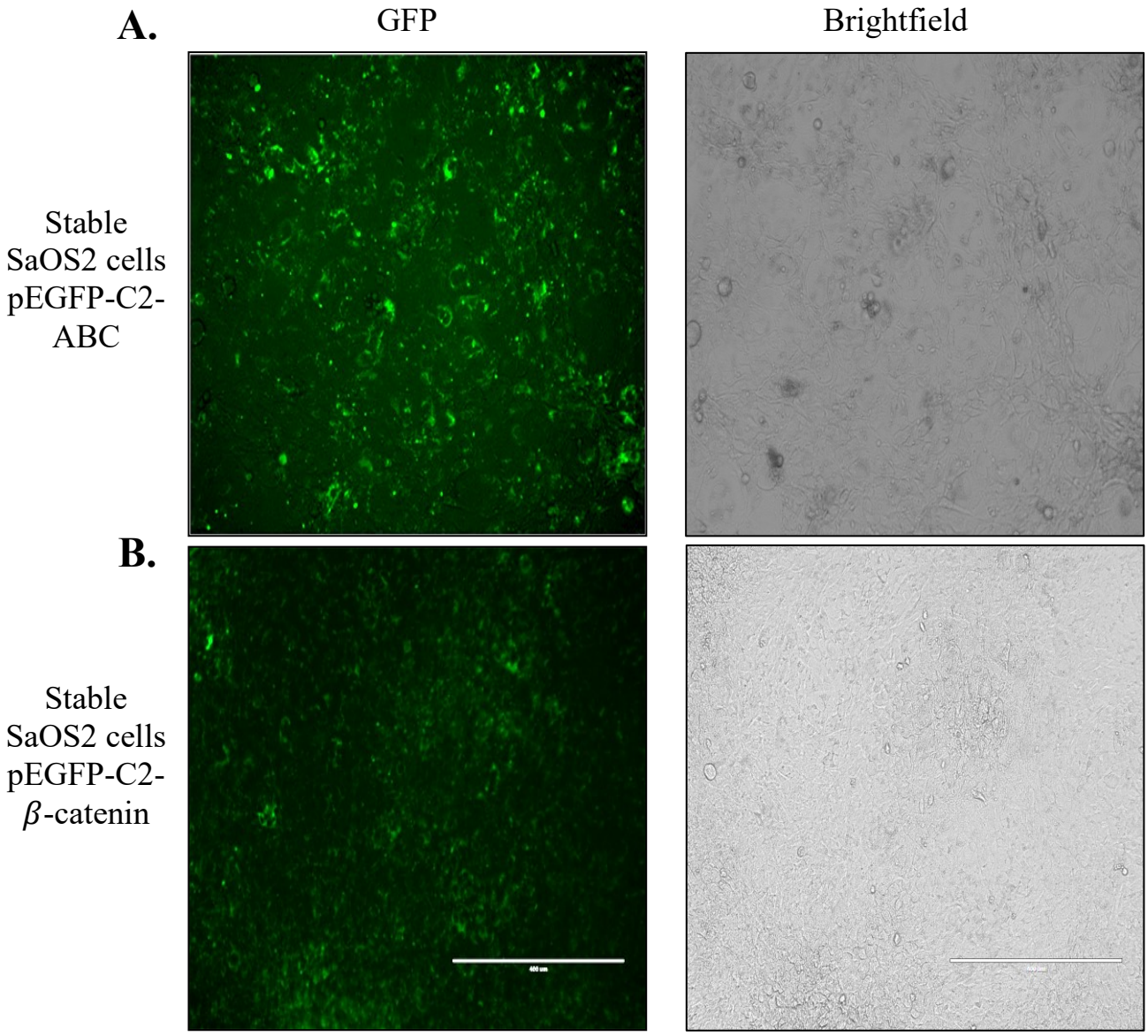


Figure 17. GFP expression is substantial and widespread in SaOS2 stable cell lines

A. SaOS2-ABC stable image. SaOS2 stable cell lines show constant expression of GFP-ABC on the left. SaOS2 cells under Brightfield on the right **B. SaOS2-*β*-catenin stable image.** SaOS2 stable cell line showing constant expression GFP-β-catenin on the left. SaOS2 cells under Brightfield on the right.

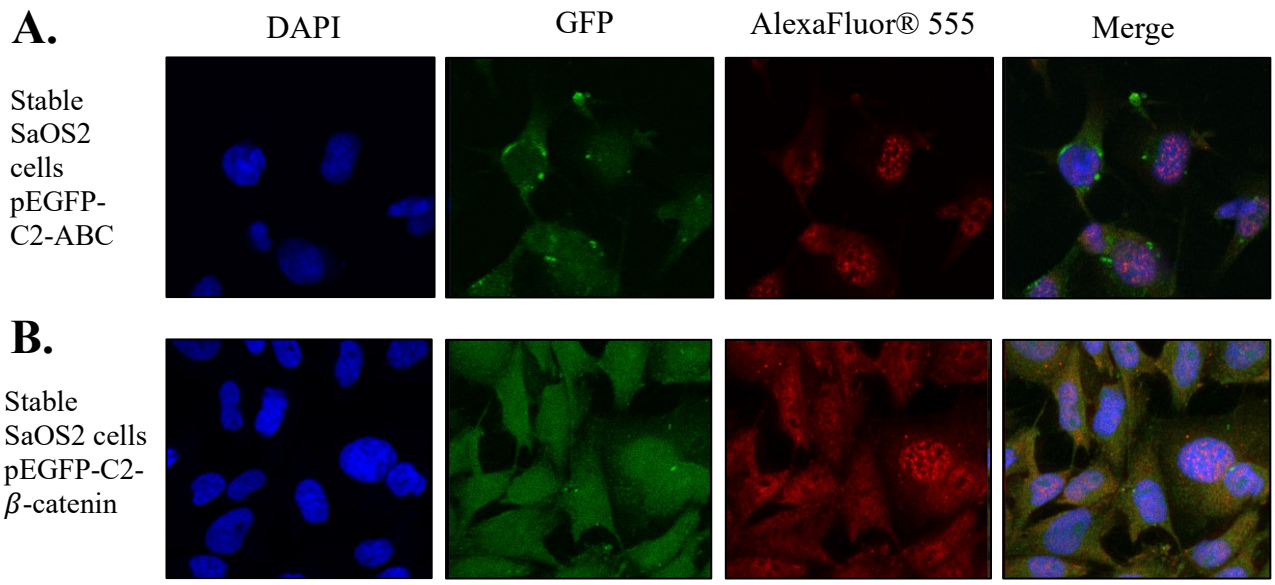


Figure 18. Stable cell lines show similar colocalization seen in transient transfectants

A. SaOS2-ABC stable immunofluorescence. SaOS2 cell line with stable transfection of pEGFP-C2-ABC shows that colocalization at the nucleus is consistent with endogenous ABC. **B. SaOS2-*β*-catenin stable immunofluorescence.** SaOS2 cell line with stable transfection of pEGFP-C2-*β*-catenin shows that colocalization throughout the cytoplasm is still consistent with endogenous *β*-catenin.

3.10. Pediatric OS patient tissue samples shows positive association with high levels of ABC and metastasis

My *in vitro* results have suggested that there is a relationship between ABC levels and how it affects OS progression. To further investigate these results and how they can be applied clinically I carried out IHC of ABC on OS tissue samples. The OS tissue samples came from thirty patients with pre-treatment biopsies with matched resection specimens. There was an equal sex distribution (15 males & 15 females). The average age was 13.4 years for females and 11.6 years for males. Our colleagues at the University of British Columbia evaluated the ABC levels in these tissue samples and the possible relationship between necrosis and metastasis at both the time of diagnosis and resection. ABC expression was calculated by quantifying the number of ABC positive nuclei over a given section. Scores were performed twice and averaged. ABC score was assigned as low (<25% nuclear positivity) and high (≥25% nuclear positivity). A total of eight cases (27%) showed high ABC nuclear staining, while 22 (73%) showed low ABC staining. The results suggest that analysis of "aggressive" disease, which is determined by metastasis at diagnosis or resection showed an association with high ABC levels and metastases at both time of diagnosis ($p=0.029$) and resection ($p=0.007$) (Figure19B) (Figure 19C). The results also looked to see if there was an association between high ABC and necrosis, but results showed no association between the two ($p=1.0$) (Figure 19A).

These results support that ABC may have the potential to be a prognostic biomarker of "aggressiveness" as defined by metastatic potential both at the time of diagnosis and resection.

A.

ABC and Necrosis		
	ABC High	ABC Low
≥ 90% Necrosis	4	11
< 90% Necrosis	4	11
Fisher (Two-Tailed): 1.0		

B.

ABC and Metastasis (at diagnosis)		
	ABC High	ABC Low
Metastasis at resection	4	2
No Metastasis at resection	4	20
Fisher (Two-Tailed): 0.029		

C.

ABC and Metastasis (at resection)		
	ABC High	ABC Low
Metastasis at resection	5	2
No Metastasis at resection	3	20
Fisher (Two-Tailed): 0.007		

Figure 19. IHC Analysis on 30 Pediatric Patient Tissue Samples shows association between high levels of ABC and metastasis at both diagnosis and resection

A. ABC and necrosis. Analysis using Fisher's exact test shows that on a cohort of 30 pediatric patient tissue samples there is no association between ABC levels and necrosis (p=1.0). **B-C. ABC and metastasis at diagnosis and resection.** The same analysis demonstrated an association with ABC levels and metastases at time of diagnosis (p-0.029) and at time of resection (p=0.007), respectively.

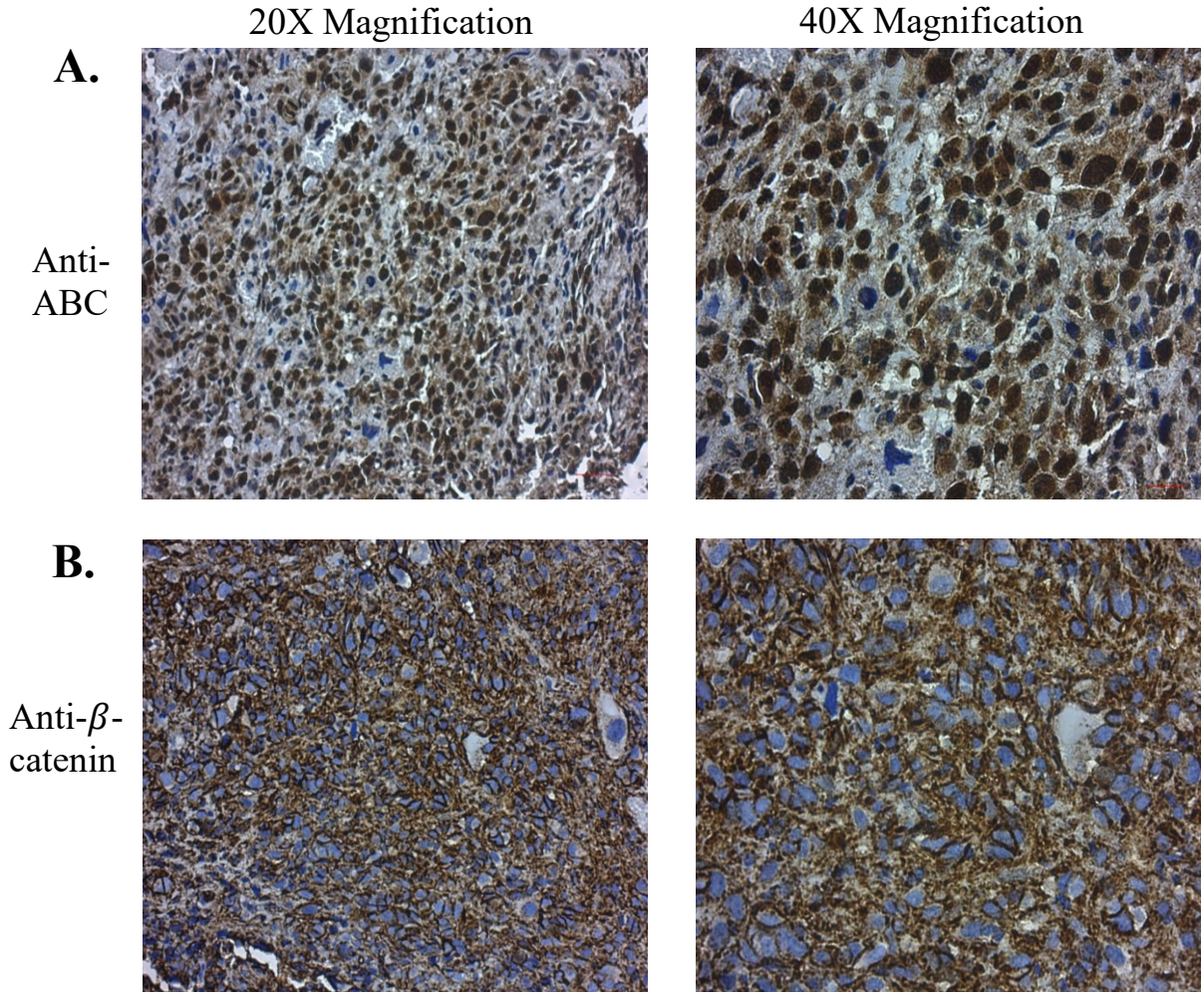


Figure 20. 20X and 40X magnification of ABC and β-catenin staining on a pediatric OS tissue sample

The images above show the 20X and 40X magnification of the staining results of the IHC with the brown indicating the staining of ABC or β-catenin and the blue indicating the nuclei of the cells. **A. Anti-ABC staining.** ABC staining of the tissue sample shows the localization of the ABC mainly in the nucleus, indicated by the brown circles. **B. Anti-β-catenin staining.** β-catenin staining shows that β-catenin is located throughout the cytoplasm of the cell and not the nucleus.

3.11. Results previously done in our lab show that OS cell lines with increased ABC levels associate with increased levels of invasion

Previously completed results in our lab that were unpublished show that within multiple OS cell lines the number of invaded cells was significantly higher in OS cell lines that had higher nuclear levels of ABC. These results were compared to two normal bone cell lines hFOB and NHOST which elucidated almost no invaded cells and undetectable ABC. The cytoplasmic ABC, and β-catenin are consistent throughout the OS cells but the difference between the different OS cell lines is with the nuclear ABC.

These results show that OS cell lines that have higher levels of invasion are also associated with having higher levels of ABC compared to other cell lines who showed lower comparative levels of invasion and lower ABC. These results also show that the process of osteosarcomagenesis increases ABC levels, depicted by undetectable levels of ABC, which can be assumed to associate with the lack of invasion seen in the normal bone cells compared to the OS cell lines.

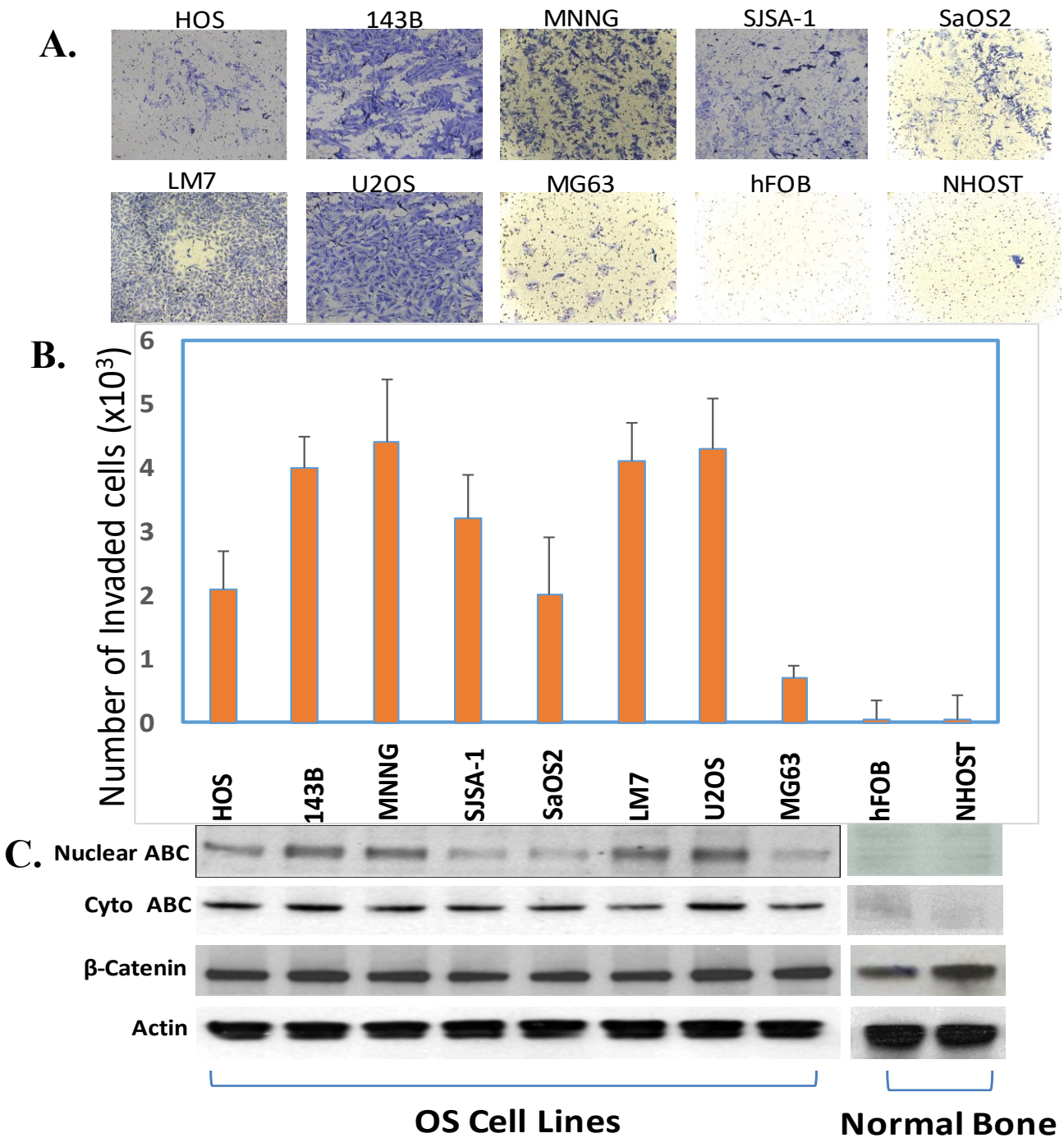

A.
HOS
143B
MNNG
SJSA-1
SaOS2
LM7
U2OS
MG63
hFOB
NHOST
B.
Number of Invaded cells (x10³)
0
1
2
3
4
5
6
HOS
143B
MNNG
SJSA-1
SaOS2
LM7
U2OS
MG63
hFOB
NHOST
C.
Nuclear ABC
Cyto ABC
β-Catenin
Actin
OS Cell Lines
Normal Bone

Figure 21. Cellular levels of ABC increase with OS metastatic potential

A. Multiple OS cell lines and normal bone cell lines representative images. The upper panel of images show representation of the invasion assay results. **B. Multiple OS cell lines and normal bone cell lines invasion assay.** The graph depicts the number of invaded cells across multiple OS cell lines and two normal bone cell lines, n=4. **C. Multiple OS cell lines and normal bone cell lines Western blot analysis.** Western blot analysis shows nuclear ABC levels were higher in OS cell lines that showed higher level of invasion. Cytoplasmic ABC and β-catenin amongst all the OS cell lines shows very little difference. Actin was used as a control. The Western blot analysis also shows that the normal bone cells had undetectable levels of ABC both nuclear and cytoplasmic. β-catenin levels were consistent with the OS cell lines and Actin was used as a control, n=4.

Chapter 4

Discussion

4.0 Discussion

OS is one of the most common primary sarcoma malignancies and with its alarmingly high mortality rate it is crucial that more research into understanding OS is done (Taran et al., 2017). The Wnt/β-catenin pathway is dysregulated in OS and has been implicated in cell proliferation, differentiation, and invasiveness (Danieau et al., 2019) (Ali et al., 2019). I believe that the dysregulation of the Wnt/β-catenin pathway seen in OS and the expression of ABC has an important role in promoting an invasive phenotype commonly seen in the progression of OS. To show that there is an association between ABC and the promotion of the invasive phenotype, my research projected used two pairs of cell lines, SaOS2/LM-7 and HOS/143B, which involves the use of both a parental cell line and a metastatic daughter cell line. LM-7 and 143B were used as a demonstration of what the peak invasive phenotype observed in OS progression looks like, since previous studies in our lab have shown that LM-7 and 143B have a higher level of ABC compared to SaOS2 and HOS (Ali et al., 2019). In order to understand how ABC specifically plays a role in the invasive phenotype seen in OS I transfected the parental cell lines SaOS2 and HOS with plasmids that would allow these cells to overexpress either ABC or β-catenin. Our studies, using Western blotting and immunofluorescence, showed that the presence of the GFP-tagged proteins of ABC and β-catenin upon transfection of the respective plasmids (pEGFP-C2-ABC or pEGFP-C2-β-catenin) and that the proteins localized similarly to their respective endogenous proteins (Figure 4) (Figure 5). This confirmation of GFP-ABC and GFP-β-catenin expression allowed me to continue with the assumption that any changes I saw between the GFP transfected SaOS2 and HOS cells and the SaOS2 and HOS cells transfected with GFP-ABC and GFP-β-catenin would be attributed to the overexpression of ABC or β-catenin. I next determined if ABC has a potential role in the promotion of an invasive/aggressive phenotype. For this, I used invasion assays, migration assays, and anchorage independent growth assays (Figure 6) (Figure 7) (Figure 8) (Figure 9) (Figure 15) (Figure 16). Furthermore, I analyzed the expressions and activities of two matrix metalloproteases MMP2 and MMP9, that are known to be involved OS invasion, this was done using RT-qPCR and gelatin zymography (Figure 11) (Figure 13) (Figure 14). To determine whether ABC plays a role in promoting OS tumor characteristic (osteoblastic or osteolytic) I investigated the mRNA expression ratio of the bone specific markers RANKL:OPG (Figure 12) I also utilized the technique of IHC on pediatric OS tissue samples and measured ABC and β-catenin

levels to see if there was a correlation between pediatric patients with “aggressive” OS and ABC (Figure 19) (Figure 20)

To determine if ABC played a role in the progression of the invasive phenotype in OS I used OS cells that were transiently transfected with GFP-tagged ABC and β-catenin to determine how overexpression of these proteins affected the cells invasive abilities. An important idea to consider when doing *in vitro* research in cancer is the inability to mimic the conditions within the human body, like that of the tumor microenvironment. However, that being said *in vitro* studies on cancer cell lines continues to be done due to the ease of growing cancer cells and the ability to study specific molecular mechanisms that are involved in tumor progression (Katt, Placone, Wong, Xu, & Searson, 2016). To ensure that the changes I saw in our experiments were due to overexpression of ABC and β-catenin I had to determine that the GFP-tagged ABC and β-catenin encoded by our plasmids were:

i. present when SaOS2 and HOS cells were transfected with pEGFP-C2-ABC and PEGFP-C2-β-catenin using Western blot analysis
ii. both acting similarly to the endogenous forms of ABC and β-catenin using immunofluorescence.

My Western blotting results were able to show me that the transfection was successful because the SaOS2 and HOS cells were expressing the GFP-tagged proteins (Figure 4). However, when looking at the SaOS2 gels that were incubated with the anti-β-catenin antibody there are faint bands that show up around the 119 kDa location in the SaOS2-GFP and SaOS2-ABC lanes. I believe this could have something to do with our protocol, in the lab protocol we use non-fat dried milk because it is inexpensive and easy to use, however after doing some research I found that milk is not good for phosphorylated proteins and since both ABC and β-catenin are our proteins of interest and differ due to a post translational modification that affects phosphorylation I think that potentially we are seeing non-specific binding and background noise. I suggest that the use of 5% BSA with 0.1% TBS-Tween be tried in the future. But with that said, when viewing the other gels this issue is not as apparent so knowing that the protein was present allowed me to conclude that changes I would see between our transfectant cell lines (SaOS2 and HOS cells transfected with pEGFP-C2-ABC and pEGFP-C2-β-catenin) compared to the control cell lines transfected with the empty vector (SaOS2 and HOS cells transfected with pEGFP-C2), could be

attributed to ABC and β-catenin overexpression. I initially used SaOS2 and HOS cells as my control which were subject to the same conditions as the pEGFP-C2, pEGFP-C2-ABC, and pEGFP-C2-β-catenin transfected cells but I realized that using these cells was not a true indication of the total changes I would see. This was because SaOS2 and HOS cells that were not transfected with anything did not undergo the same stresses as those that actually had a plasmid inserted into them thus reducing the difference between the control and pEGFP-C2-ABC and pEGFP-β-catenin transfected cells. I utilized immunofluorescence as a way to visualize the co-localization of the GFP-tagged ABC and β-catenin with the endogenous ABC and β-catenin. It could be challenged that in the SaOS2 cells that we do not see the endogenous proteins within cells that are not expressing the plasmid, however in my HOS cells one can see that when the image is correctly calibrated it is possible to show that the endogenous proteins of ABC and β-catenin are fluorescing red. In the future, before publication, SaOS2 cell images will need to be edited. However, in the end I believe that we have adequate evidence that co-localization was completed and that this matched with previous research done in our laboratory by Ali et al., (2019) when looking at localization patterns of ABC and β-catenin.

One potential issue that I questioned me initially was how specific the Anti-ABC antibody and the anti-β-catenin antibody were. According to the catalogues the anti-ABC antibody binds to the non-phosphorylated serine 37 and threonine 41 while the anti-β-catenin antibody binds to corresponding residues around aspartic amino acid residue 56. So, I asked myself, how would I know that I was not staining both ABC and β-catenin? This question was answered after the completion of my Western blot and immunofluorescence results. The results show that the anti-ABC antibody when tagged with the secondary antibody was fluorescing red in the nucleus, where ABC is found. The Western blot results also show that only the SaOS2 and HOS cells that were transfected with the pEGFP-C2-ABC plasmid showed a band at 119kDa, which is the size of the ABC construct plasmid, this meant that the anti-ABC antibody was specific for ABC. I also know that the anti-β-catenin antibody was specific to β-catenin and not ABC because in my immunofluorescence results, β-catenin which is known to be cytosolic was seen in the cytoplasm of the cell, fluorescing red throughout the cell. The Western blot results show that only the cells that were transfected with the pEGFP-C2-β-catenin had a band show up at 119kDa, if I saw a band at 119kDa in the cells that were transfected with pEGFP-C2-ABC when incubating them with the

anti-β-catenin antibody then I would know that the antibody was not specific to β-catenin but also bound to ABC, but I did not see that meaning that these two antibodies were specific to ABC and β-catenin.

To measure the impact that overexpressing ABC and β-catenin may have on promoting invasive phenotype in the OS cell lines I first looked at two important proteins, MMP9 and MMP2. MMPs are zinc-dependent endopeptidases that degrade ECM and are crucial for both pathological and physiological processes (Zhou, J., Liu, & Wang, 2018). MMPs have a role in tumor progression by increasing cell growth, migration, invasion, and metastases (Zhou et al., 2018). MMP9 degrades type IV collagen, a major component of basement membrane in ECM (Zhou et al., 2018). MMP9 was studied because a meta-analysis showed that patients with OS that expressed MMP9 were significantly associated with neoplasm metastasis and poor survival compared to those who were negative with MMP9 (Zhou et al., 2018). My findings show that when SaOS2 and HOS cells overexpressed ABC the mRNA expression of MMP9 significantly increased, to the point that MMP9 mRNA expression in SaOS2 cells expressing ABC were on a similar level of the more metastatic cell line LM-7 (Figure 11A) (Figure 11C). These results are consistent with that of a study that measured the effect of a Connexin-43 (Cx43) knockdown, a gap junction protein, which ultimately led to the activation of the Wnt/β-catenin pathway and an increase in mRNA and protein expression of MMP9 (Xie, D. et al., 2017). This suggests that the Wnt/β-catenin pathway has a role involved in mRNA expression of MMP9. However, my gelatin zymography results which were used to measure the proteolytic activity showed no association between MM9 activity and overexpression of ABC in SaOS2 and HOS compared to our control (Figure 13C) (Figure 13D). But the LM-7 and 143B cell lines both had much higher MMP9 activity compared to all of our transfectants, this contradiction between mRNA expression and MMP9 activity between the metastatic line and parental cell line brings me to believe that another mechanism or pathway involved in OS progression, other than the Wnt/β-catenin pathway is most likely associated with the activity of MMP9 (Figure 13C) (Figure 13D).

As previously discussed, MMPs play an important role in cell growth and tumor metastasis, so along with examining MMP9 I also examined MMP2. MMP2 has a confirmed role in the invasion and metastasis of many tumors (Zhang, M. & Zhang, 2015). MMP2 assists in the cellular

invasion seen in OS by contributing to the destruction of the collagen in the ECM (Zhang & Zhang, 2015). My results showed that only an overexpression of ABC, and not β-catenin, resulted in a significant increase in mRNA expression of MMP2 in HOS cells (Figure 11D). When looking at the fold change, there was a visual increase in MMP2 mRNA expression in SaOS2 cells that overexpressed ABC, but it was not statistically significant (Figure 11B). Just like our MMP9 results, gelatin zymography showed no change in MMP2 activity when overexpressing ABC and β-catenin in SaOS2 and HOS, however this was also seen to be true with LM-7 and 143B cells (Figure 14C) (Figure 14D). Zhang & Zhang, (2015) discovered that OS tissues had higher MMP2 expression compared to other tissues. In the Zhang & Zhang, (2015) study, they compared 45 OS patients tissues with an average age range of 14.3 years old to 45 non-cancerous tissues and found that MMP2 mRNA expression was significantly upregulated in the OS groups. These results indicate that OS is associated with an increased MMP2 mRNA expression, and I believe that our results show that the Wnt/β-catenin pathway, and specifically ABC plays a role (Figure 11B) (Figure 11D). One thing that is important to note is the difference in my study to theirs, they did their RNA extraction on tumor tissue samples and normal tissue samples. My results then suggest that even though my results are looking at the cells that are involved in the primary tumor and how the expression of ABC can induce metastatic tumor formation both our results show that there is increased MMP2 mRNA expression in OS tumors. On another note, an interesting result observed was the 143B cell line did not show any significantly higher expression of MMP2 mRNA expression compared to the control cells (Figure 11D). While in the other paired cell line LM-7 cells exhibited higher MMP2 mRNA levels compared to the control, which was similar to what was seen with the MMP9 (Figure 11A) (Figure 11B). These results are confounding and to better understand why this may be, more research into understanding MMP2 expression in 143B cells must be explored. To summarize, our findings suggest that overexpression of ABC may have a role in MMP9 and MMP2 mRNA expression is not the sole contributor to the proteolytic activity of the MMP proteins.

Along with looking at the specific activity of MMP9 and MMP2 which both have crucial roles in invasion and metastases of OS, I also used *in vitro* invasion and migration assays to directly measure if overexpressing ABC would have an effect on invasion and migration. My results suggest that overexpression of ABC in both SaOS2 and HOS caused an increase in their invasive

capacity (Figure 6) (Figure 7). These results seem to contradict what was shown earlier in regard to the proteolytic activity of MMP9 and MMP2 in our gelatin zymography assay since overexpression of ABC resulted in no change in proteolytic activity of both MMP9 and MMP2 (Figure 13C) (Figure 13D) (Figure 14C) (Figure 14D). This observation though seems to strengthen the notion that ABC has an important role in the transcription of MMP9 and MMP2 but may not be directly involved in the activation of these proteins. Further investigations are needed to understand the potential mechanisms involved in increasing invasive potential. Regardless of the contradicting results our invasion assay results seem to be consistent with many other studies that showed that any decrease in the Wnt/β-catenin pathway led to a decrease in invasion. (Kang et al., 2007) showed that overexpression of RECK reduced tumor invasiveness while (Zhou, L. et al., 2020) confirmed that RECK is an inhibitor of the Wnt/β-catenin pathway. Another study ((Qi, Yang, Si, & Nie, 2020) measured the effects of Piperine, a compound that was shown to downregulate β-catenin and its downstream oncogenic proteins, and the results of the study showed a decrease in invasion in the U2OS and 143B cells. These studies along with my results suggest that overexpression of ABC, hence activation of the Wnt/β-catenin pathway, is involved in promoting the increased ability of OS cells to invade. However, to understand why the overexpression of ABC promotes invasion and mRNA expression of MMP9 and MMP2 but not the proteolytic activity will require future research to determine on what other pathways or molecules may be involved in order to understand how the invasion of OS cells fully works. Along with the invasion assays I also completed migration assays and my results showed that overexpression of both ABC and β-catenin did not have a substantial effect other than a small but significant increase in migration of SaOS2 cells overexpressing ABC (Figure 8). This suggest that the Wnt/β-catenin pathway may play a relatively small role in the OS cells migratory ability. This can be further supported when looking at the more metastatic cell lines, LM-7 and 143B, and seeing how much more strongly significant the results were (Figure 8) (Figure 9).

An important consideration that came up in discussing the invasion and migration assay results was that the increased invasive activity observed in the SaOS2 and HOS cells overexpressing ABC may simply be a reflection of increased numbers of cells due to increased rate of proliferation. And since the Wnt/β-catenin signaling pathway is known to be essential for proliferation, putting this idea to test was important (Danieau et al., 2019) (Fang et al., 2018).

Using alamarBlue™ to measure cell proliferation I saw that over a 48-hour period there was no difference in proliferation seen in SaOS2 cells overexpressing ABC or β-catenin, this was the same for the HOS cells. Although my research does contradict the notion on how the Wnt/β-catenin is essential for proliferation, Danieau et al., 2019 states that the bone tumor microenvironment plays a large role in proliferation and that this involves the complex interactions between many cell types, osteoblasts, osteoclasts, stromal cells, mesenchymal stem cells, immune and non-cellular components, which are not present in my *in vitro* study suggesting that the overexpression of ABC alone may not be enough to enhance proliferation. An interesting finding in my results in the SaOS2 cells show that after 18 hours the proliferation measured by the cells does not change. As to why this may be future research into measuring SaOS2 cell's ability to grow over a long period of time should be done.

RANKL and its decoy receptor OPG are regulators of bone metabolism (Navet et al., 2018). RANKL is involved in the mediation of osteoclastogenesis and the activation of mature osteoclasts whereas OPG, which is secreted by osteoblasts, inhibits osteoclast differentiation and activity along with bone resorption (Navet et al., 2018). The relationship between RANKL and OPG has been shown to be important when determining the function and differentiation of osteoclasts (Raggatt & Patridge, 2010). Studies have shown that in OS there is a deregulation of the RANKL:OPG ratio (Navet et al., 2018). My results suggest that an overexpression of ABC and β-catenin in SaOS2 cells results in no change in the RANKL:OPG ratio, this was also seen LM-7 cells (Figure 12A). However, overexpression of ABC did cause a significant decrease in the RANKL:OPG ratio in HOS cells while 143B cells showed a larger and more significant decrease (Figure 12B). One issue that I would like to comment on in regard to our LM-7 results was the observation of a large error bar due to heterogeneity in the results, I believe that reviewing these results may elicit a more substantiative conclusion. Studies have shown that in OS that there is an increased level of OPG mRNA and a lack of RANKL mRNA which would be seen as a decreased RANKL:OPG ratio, which matches our results seen in our HOS and 143B results (Mori, Redini, Gouin, Cherrier, & Heymann, 2006). The role that the Wnt/β-catenin plays in this RANKL:OPG ratio change was shown when the activation of the pathway in osteoblast lineage cells caused the expression of OPG to be enhanced while simultaneously suppressing osteoclast differentiation

(Maeda et al., 2019). In conclusion my results suggest that the Wnt/β-catenin pathway and specifically ABC has a role in promoting an osteoblastic characteristic in OS.

Anoikis is the programmed death of cells that is caused by non-attachment to the ECM.. (Gao et al., 2019). Cancer cells in OS must avoid anoikis because metastases requires the cancer cells to continue to survive while not attached to the ECM during their journey through the vascular system (Gao et al., 2019). Studies have shown that the PI3K/Akt pathway may be involved in the inhibition of anoikis, more specifically the interleukin-8/C-X-C-motif chemokine receptor 1IL-8/CXCR1 axis has been shown to activate Akt signaling which results in the resistance to anoikis in OS (Yang, C. et al., 2020). Another study showed that the downregulation of PI3K/Akt induced by Celecoxib, a drug that enhanced the effects of the anti-cancer reagent Cisplatin caused an induction of anoikis in OS by the downregulation of β-catenin (Liu, B., Yan, Qu, & Zhu, 2017). The study concluded that the inhibition of anoikis was dependent on the PI3K/Akt pathway, but that β-catenin also played a role (Liu et al., 2017). Since cross talk between Wnt/β-catenin pathway and PI3K/Akt pathway promotes tumorigenesis and resistance to cancer therapy there is good reason to believe that increased ABC transcriptional activity is involved in the inhibition of anoikis (Zhang, Y. & Wang, 2020). My evaluation of the role of ABC in promoting anchorage independent growth did not go as expected. I ran into some issues with my protocol and with the difficulties brought on by the COVID-19 pandemic it made it that much harder, however, the results I obtained, although not ideal were sufficient to carry out a preliminary analysis (n=3). My findings suggested that there is a trend of increasing colony formation in SaOS2 and HOS cells overexpressing ABC, however, these results were not significant (Figure 15) (Figure 16). In the 143B cell line, these cells were able to grow effectively and showed that the more metastatic line was able to form colonies successfully in the experimental conditions. I believe that our results when done properly will elicit colony formation, this can be seen in a study done by Lauvrak et al., (2013) where they showed the colony forming ability in SaOS2, HOS, and 143B cells. However, this study did not show the colony forming ability in LM-7 cells, and after a thorough search I did not find an article that explicitly stated LM-7 cell lines ability to grow in anchorage independent conditions like that of the Lauvrak et al., (2013) study. However, a study done by (Salah et al., 2015) did show that LM-7 can develop colonies and that it was more than the SaOS2 cells. In conclusion I believe with future research and a proper protocol for the soft agar colony

forming assay we will get a definitive result and conclusion into how ABC affects anoikis resistance.

The use of immortalized cell lines in *in vitro* research has many advantages: they are cost effective, easy to use, provide countless ways to do research, and can bypass the ethics requirements associated with research using animal or human tissue (Kaur & Dufour, 2012). However, there is also importance in imaging and analyzing tissue which can add a clinical aspect to the research (Maiques, Georgouli, & Sanz-Moreno, 2019). The eventual goal of our research is to be able to understand how ABC affects target genes and what phenotypic changes it causes in OS cells, how ABC is formed, and whether ABC can be used as a prognostic biomarker in OS. Therefore, the use of tissue samples and microscopy is crucial for that type of translational research (Maiques et al., 2019). IHC is a method that can used to derive histopathological information with reference to tissue expression of the biomarker of interest (Maiques et al., 2019). One of the experiments we (University of British Columbia and University of Alberta) completed was the examination of the levels of ABC in thirty pediatric OS tissue samples, at diagnosis (pre-treatment biopsies) and resection (post-neoadjuvant chemotherapy biopsies). Our findings suggest that patients with high ABC levels were associated with both metastasis at diagnosis and resection, these results are independent of the post-neoadjuvant chemotherapy response (Figure 19). Our results are supported by other studies on OS patient samples, where it was also observed that there was significantly higher levels of β-catenin in OS tissue compared to adjacent healthy tissue and that this association correlated to a poor prognosis and lung metastases (Danieau et al., 2019). Using IHC, (Lu et al., 2015) discovered that the majority of the OS cases showed significantly higher expression of β-catenin compared with osteochondroma tissue samples. Lu et al., (2015) also showed that there was an increase in cytoplasmic β-catenin and a decrease in membrane associated β-catenin in the most advanced tumors, these results would represent the population of ABC. OS metastasis is associated with a high mortality rate and Lu et al., (2015) reported that upregulation of β-catenin was correlated with decreased patient survival, showing that the use of ABC as a prognostic biomarker for metastatic disease is supported. A meta-analysis that studied the relationship between overexpression of β-catenin and the prognosis of OS by analysing a large sample cohort concluded that the overexpression of β-catenin was associated with metastases and poor prognosis (Xie, X. et al., 2020). Even though these studies provide support to our claim of

ABC being used as a potential prognostic biomarker, an issue that I have noticed throughout my investigation of literature is that there is still a disparity in calling the β-catenin that is in the cytoplasm/translocating to the nucleus as just β-catenin when there are studies such as from our lab that recognizes that this β-catenin is ABC (Ali et al., 2019) (Persad et al., 2016). This disparity leads to their being a disconnect in results saying that β-catenin does what ABC does when they both have different roles and if one were to study β-catenin and its effects they would find that there was no difference, as I did in my study when overexpression of β-catenin resulted in non-significant results. I believe that one reason that this could be happening, which is based off my personal experience doing research on ABC is that there are antibodies for β-catenin that bind to both the N- and C-terminal, where the N-terminal antibody binds to β-catenin and the C-terminal antibody binds to both ABC and β-catenin. If you were to use the C-terminal antibody not knowing that both β-catenin and ABC require two different antibodies you would not be able to differentiate between both. Now credit is due to both of these studies because they do mention that β-catenin via the Wnt pathway enters the nucleus and binds to the LEF/TCF to activate Wnt target genes, however, they did not explore it any further (Lu et al., 2015) (Xie et al., 2020). In another study (Liu, W. et al., 2018) measured the effect Dioscin had on OS and found that it decreased the OS like properties and tumor growth by suppressing the Akt/GSK3/β-catenin pathway. However, the important aspect of the study was that they understood the importance of β-catenin translocating the nucleus, so they measured the effects Dioscin had on both nuclear and cytosolic β-catenin (Liu et al., 2018). Their results showed that Dioscin reduced both cytosolic and nuclear β-catenin but there was an even greater significant decrease in nuclear β-catenin (Liu et al., 2018). In summary the IHC results and the results from the other studies suggest the potential usage of ABC as a prognostic marker, however a larger, well powered study will need to be completed to determine its utility and the possible use of becoming a routine pathology practice.

In summary, after analysis and discussion of my results I conclude that ABC *in vitro* plays a vital role in the promotion of the invasive phenotype that is seen in OS progression by upregulating the transcription of MMP2 and MMP9 mRNA, increasing the ability to degrade ECM and become motile, and the promotion of an osteoblastic characteristic. I also conclude that ABC also has the potential to be used a prognostic biomarker for metastatic OS due to high levels of ABC being associated with metastasis at diagnosis and resection.

Chapter 5

Future Directions

5.0 Future Directions

My current findings suggest that ABC has a role in the promotion of the invasive phenotype and the preliminary data on 30 pediatric OS samples shows the potential of ABC being used as a prognostic biomarker for metastatic OS. In order to strengthen our results, there are some experiments and protocols that we need to redo and revise, respectively.

5.1 Improvements for current study

i. Western blot analysis of plasmid expression in SaOS2 and HOS cells.

   Although my Western Blot data does show the presence of GFP-ABC and GFP-β-catenin my blots currently are not clean enough to be published. In order to have results that are publishable we will need to review my Western blot protocol. This issue is apparent in my anti-β-catenin blots since β-catenin we can see that there are supposed bands that appear in the SaOS2-GFP and SaOS2-ABC lanes. I believe this could have something to do with our protocol, in the lab protocol we use non-fat dried milk because it is inexpensive and easy to use, however after doing some research I found that milk is not good for phosphorylated proteins and since both ABC and β-catenin are our proteins of interest and differ because of phosphorylation I think that potentially we are seeing non-specific binding and background noise. I suggest that the use of 5% BSA with 0.1% TBS-Tween be tried in the future. Once we are able to reach more visually appealing blots we will produce more results, at least n=3 to properly do an analysis.

ii. mRNA expression of MMP2 in SaOS2 and HOS cells and RANKL:OPG ratio in SaOS2 cells.

   One issue that I would like to address is the MMP2 mRNA expression in SaOS2/LM-7 cells. In the SaOS2/LM-7 cells results I observed large error bars due to heterogeneity and I believe that another set of experiments will help alleviate those issues along with help determine if the increasing trend of MMP2 mRNA expression seen in ABC overexpressing SaOS2 cells is statistically significant. Along with measuring the MMP2 mRNA one more set of experiments on the SaOS2/LM-7 cells measuring mRNA expression in RANKL and OPG is also critical because again there is a large amount of heterogeneity.

iii. Measuring MMP9 and MMP2 activity with gelatin zymography

The MMP9 activity is increased in the more metastatic cell lines and there is no difference between cells that overexpress β-catenin and ABC, however doing another set of experiments on the LM-7 and 143B cell lines will help reduce the error bars observed. My results on the MMP2 activity showed no change throughout all of my results but in the SaOS2 results I did observe a decent amount of heterogeneity so redoing these results for both SaOS2 and HOS should be done.

iv. Soft agar colony forming assay protocol review

Some of the issues that I ran into when doing the soft agar colony forming assays was that this assay in my opinion requires at least two people due to the speed at which the gel solidifies and during the COVID-19 pandemic the rules of lab interactions changed drastically and reduced the amount of teamwork that was required during this experiment. Another issue was the detachment of the gels from the wells which would then cause the medium that was added to the top of the gels to help the cells grow would flow around the sides and sink in the bottom causing the cells to not have any nutrients. Lastly I believe that I added too little cells when I seeded these plates and that increasing this the number to 3000 cells vs the 1250 I initially seeded will allow for better results.

v. Completion of the TOPFLASH assay

The TOP/FOPflash assay should be done to quantitively show that the overexpression of ABC in the transfected cell lines is greater than that of the control. This assay is a luciferase reporter assay and is able to quantify the amount of ABC activity because ABC interacts with TCF/LEF which in this assay would result in the Luciferase activity. The FOPflash assay is a negative control to the TOPflash assay. These results if conclusive would help corroborate with the results we saw *in vitro*.

vi. Invasion assay images

The HOS-GFP image is in a different magnification than the rest of the images and before publication it will be required to retake an image in the proper field.

5.2 Prospective Studies

My study's aim was to evaluate the role ABC has in the promotion of the invasive phenotype in OS. I did this by overexpressing ABC and β-catenin in SaOS2 and HOS cells by transfecting them with a plasmid that would produce a GFP-tagged version of the protein. My

Western blots and immunofluorescence results were done to show that the plasmids entered the cells, expressed the GFP-tagged ABC and β-catenin, and colocalized with the endogenous ABC and β-catenin. Once I knew that the cells were overexpressing ABC and β-catenin I measured markers that are important for measuring the invasive phenotype in OS. My study included measuring the mRNA expression of MMP9, MMP2, RANKL, and OPG with RT-qPCR, MMP9 and MMP2 activity with gelatin zymography, invasion and migration assays, and using soft agar colony forming assays to measure anchorage independent growth. My results suggest that the overexpression of ABC does have an impact on invasive properties and MMP9 and MMP2 mRNA expression. Along with the additional work that will have to be done to clean up some of the results and revisiting some of the protocols I believe that once these corrections are made it will help conclude the effect ABC has on the invasive phenotype in OS. For future research I believe that there should be four major studies completed: (i) the use of in vivo studies to investigate the role of ABC in OS development and metastasis, (ii) how ABC regulates metastasis in OS using next gen sequencing, (iii) understanding how ABC is formed, (iv) determining whether ABC can be a biomarker of aggressive metastatic disease using a larger cohort of pediatric OS tissue samples, and (v) exploring the relationships involved in the microenvironment of the primary tumor and the metastasized cells within the lungs.

i. Studying the effect of SaOS2 and HOS cells overexpressing ABC *in vivo* is a critical step in order to understand how ABC promotes OS development and metastasis. This study will be completed using SaOS2 and HOS cells stably expressing the pEGFP-C2, pEGFP-C2-ABC, and pEGFP-C2-β-catenin as an orthotopic xenograft in NSG SCID mice. Tumour growth rate and metastasis will be compared in the following three models: stable pEGFP-expressing SaOS2 and HOS cells, stable SaOS2 and HOS expressing pEGF-C2-ABC or stable SaOS2 and HOS expressing pEGFP-C2-β-catenin. Primary tumour burden and lung metastasis will be tracked *ex vivo* by fluorescent imaging of GFP.
ii. Using next-generation sequencing, specifically the technique of RNA-seq we can look at transcriptional changes of many genes, both known and unknown that ABC may be regulating in OS. This will be important when we compare the effect the overexpressing

ABC OS cells to the control group which is important for solidifying what we know already about MMP9, MMP2, RANKL, and OPG and the potential discovery of other genes.

iii. Currently we know that β-catenin undergoes post translational modification that forms ABC so an understanding of this process in OS is important. In our laboratory we have shown that in melanoma, breast and prostate cancer cell lines the Wnt pathway regulates β-catenin levels while the PI3K pathway and the protein PP2A regulate the cellular ABC levels (Persad et al., 2016). This ultimately supported an interaction between the PI3K and Wnt pathway in the regulation and formation of ABC and future studies should confirm this happens in OS as well.

iv. Can ABC be used as a biomarker of aggressive metastatic disease? We believe from our IHC results which showed that increased ABC levels were associated with more "aggressive" OS in pediatric patients that ABC could be used as a potential prognostic biomarker. Calculations were done to detect a difference in ABC levels in OS tumor samples at 95% power using one-way ANOVA. In order to detect a difference, we require 84 patients samples, and the good news is that we are currently in the process of obtaining 90 pediatric OS patient samples from the Children's Oncology Group (COG) to carry out this analysis.

v. OS tumors grow in the microenvironment of the bone, and as I have mentioned earlier there are many cells that are active in this environment such as osteoclasts, osteoblasts, osteocytes, MSC's, bone-lining cells, megakaryocytes, and immune cells. However, there are other cells that are involved such as fibroblasts, endothelial cells, pericytes, and not only that but the components of the ECM, the many secreted cytokines, chemokines and soluble growth factors, and lastly extracellular vesicles which are considered to be an important factor for communication between cells (Corre, Verrecchia, Crenn, Redini, & Trichet, 2020). With all of these factors playing a role in the growth of the tumor it creates an environment that is suitable for cells to survive and grow (Corre et al., 2020). Understanding and exploring these relationships in OS to further learn about the biological mechanism that progress towards aggressive OS is vital. In the future, there are many techniques that can be done to study this unique microenvironment. Ultracentrifugation is a common technique used to isolate the extracellular vesicles and to study them within the natural bone biology techniques labeling these extracellular vesicles with fluorescent dyes

can done (Shupp, Kolb, & Bussard, 2020). The use of dyes and small fluorescent molecules to measure cell-cell communication via the gap junctions (Shupp et al., 2020). Using multiplex arrays and proteomics to study cytokines, chemokines, and soluble growth factors is a viable option (Shupp et al., 2020). There is also the use of three-dimensional models which includes the use of scaffolds, hydrogels, and bioreactors to study the bone microenvironment (Shupp et al., 2020). As one can see the future of OS research is filled with many techniques and strategies to study the complex microenvironment and this will help further push our understanding of this disease.

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