FORCED INDUCTION OF DIFFERENTIATION IN OSTEOSARCOMA TUMOR INITIATING CELLS

By

MARGARET E. WHITE

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To my family
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NSG        NOD/SCID Gamma
OS         Osteosarcoma
p21Cip1   Cyclin-dependent Kinase Inhibitor 1
p27Kip1   Cyclin-dependent Kinase Inhibitor 1B
p38       p38 Mitogen Activated Protein Kinase
PERK       Protein Kinase R (PKR)-like Endoplasmic Reticulum Kinase
PFC        Preformed Complex
PI         Propidium Iodide
Runx2      Runt-Related Transcription Factor 2
SBE        SMAD Binding Element
TAK1       TGFβ Activated Kinase 1
TGFβ       Transforming Growth Factor beta
TIC        Tumor Initiating Cell
TME        Tumor Microenvironment
Abstract of Dissertation Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

FORCED INDUCTION OF DIFFERENTIATION IN OSTEOSARCOMA TUMOR INITIATING CELLS

By
Margaret E. White

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Osteosarcoma (OS) is a highly malignant bone cancer that is defined histologically by the secretion of immature osteoid. OS is believed to originate from osteogenic committed progenitor cells, involving disruption of extracellular matrix synthesis in favor of proliferation. Because Bone Morphogenetic Proteins (BMPs) play key roles in osteogenic maturation and the OS lineage of origin suggests an innate sensitivity to BMP proteins, we hypothesize that BMP stimulation will force the induction of differentiation in OS Tumor Initiating Cells (TICs) and will impair the ability of these cells to initiate and maintain tumor growth.

To begin, we determined the expression of BMP Receptors Type II and 1A in two primary OS-derived cell lines (OS521 and OS156), with similar expression in Mesenchymal Stem Cells. Furthermore, by treating OS TICs with BMP heterodimers, BMP2/7 or BMP4/7, we observed an activation of canonical BMP signaling, suggesting a functional signaling network. BMP4/7 stimulation of OS521 TICs in vitro showed an increase in p27 and p21 protein expression, leading to an expansion in the amount of cells halted in G1 Phase and a reduction over time in the cells’ ability to proliferate. Strikingly, time to tumor onset of BMP4/7 pretreated OS521 TICs in NSG mice was
significantly longer than in controls, in addition to a reduction in the total amount of mice that generate tumors.

To investigate the underlying mechanisms responsible for BMP4/7 inhibition of OS tumor formation, we attempted to simulate the OS tumor microenvironment (TME) using exogenous adenosine and calcium, in conjunction with exposure to hypoxia. OS TICs exposed to these stress inducers at physiological levels respond through replication of the biological characteristics of non-tumorigenic GFP- cells, showing reduced cell viability, a loss of proliferative capabilities, decreased activation of our tumorigenic reporter, and an increase in IL6 secretion. However, no additive effects were seen with BMP4/7 pretreatment and subsequent exposure to stress inducers.

In summary, our data suggests that BMP4/7 stimulation is initiating its inhibitory effect on tumor formation through its regulation of the cell cycle, however the induction of differentiation seen is due to the stresses of the TME in OS521 TICs.
CHAPTER 1
LITERATURE REVIEW

Osteosarcoma

Osteosarcoma (OS) is a highly aggressive bone cancer that is histologically defined by the production of disorganized and immature osteoid from malignant mesenchymal cells [1]. This disease remains fatal for many young people due to metastatic spread, most often to the lungs [1; 2; 3]. In addition to being a pediatric disease, OS is rare and classified by the United States Food and Drug Administration (FDA) as an orphan disease, with 4 to 5 cases per million people [2]. Characterized by local invasiveness, genomic instability, histological heterogeneity, and high metastatic potential, OS continues to be an extremely challenging form of cancer [1]. The high mortality and morbidity of OS highlights the need for new therapeutic approaches with heightened tumoricidal effects and reduced toxicity.

Following a bi-modal distribution, OS initially arises as the primary tumor during adolescence. OS can later occur during or after the 6\textsuperscript{th} decade of life, in which almost half are presented as secondary conditions following Paget’s Disease or as a result of previous treatments, suggesting that it may be distinct from that found in younger patients [1; 2; 3]. As a cancer of children, OS affects teens during the time of skeletal growth, arising in the growth plates of long bones, as these are the largest regions of active cell proliferation and differentiation [4]. Specifically, the most common primary sites of OS are the distal femur, the proximal tibia, and the proximal humerus, with more than half originating about the knee [2]. Because of the tumor’s typical metaphyseal location, its peak incidence during adolescence and early adulthood, and its male
predominance (60%), it has been postulated that there is an association between rapid bone growth and OS [2; 3].

Since OS has a high degree of genomic instability, it carries few recurrent, targetable mutations. Molecular studies to identify specific genetic loci involved in OS genesis are hindered by the lack of a known etiology and therefore an inability to identify normal counterparts for comparison, in addition to its characteristic genetic instability causing a general lack of reproducibility [5]. However, recognized risk factors include high cumulative radiation doses as well as high doses of some chemotherapies, as OS is most likely to develop after these treatments [6]. Additionally, certain genetic conditions predispose individuals to a number of malignancies, with OS being one of them: Hereditary Retinoblastoma, Li-Fraumeni Syndrome, Rothmund-Thomson Syndrome, and Bloom and Werner Syndrome, diseases that are associated with defects in cell cycle regulation and DNA repair [3].

**Clinical Presentation and Staging**

OS patients typically present with nonspecific clinical symptoms, including localized pain and swelling of the affected area, but mild blunt trauma is often reported as an antecedent event. A series of imaging tests are needed to describe and stage the disease. This includes radiographs to define the mass in the affected extremity, and magnetic resonance imaging, bone scans, and positron emission tomography to determine the extent of the primary tumor mass in detail but also to investigate the presence of metastases in other areas of the body [2; 3]. Lastly, a tumor biopsy is taken for histologic examination to confirm the diagnosis, which often is the final step of the staging process [2; 3].
At the time of OS diagnosis, 10-20% of patients present with macroscopic evidence of metastatic disease, most commonly in the lungs (90%), but sometimes in other bones (8-10%) and rarely in the lymph nodes [2]. However, 80-90% of patients are assumed to have micrometastatic disease, which is subclinical or undetectable using current diagnostic modalities [3]. Unfortunately, the most reliable prognostic indicator for OS survival is the detection of metastatic disease at the time of presentation, with long term survival reduced from 70% to 20% in such instances [3].

Surgical staging is performed using the Musculoskeletal Tumor Society staging scheme, originally developed and described by William Enneking: tumors are defined as being either low grade or high grade (I vs II), intracompartmental or extracompartmental (A vs B), and metastatic (III) [7]. Histological subtypes are based on the predominant features of the cells and their associated matrix: osteoblastic (60% of cases), chondroblastic, and fibroblastic, although without clear significant differences in clinical outcome [1; 2; 8]. In actuality, most OS demonstrate varying amounts of all three cell types and matrix, so generally it is meant to signify greater than 50% predominance of any histologic type [2; 4]. Based on anatomic location, predominant histologic subtype, and sometimes tumor grading, types of OS include: conventional, teleangiectatic, parosteal, periosteal, low grade central, and small cell, where conventional OS is the most common [4]. But despite its various phenotypic forms and appearances, the clinical behavior of high grade OS, which accounts for 80-90% of all cases [9], is remarkably homogenous.

**Treatment**

Prior to the current standard of care, clinically detectable pulmonary metastases usually evolved within the first year following amputation; this has been used to support
the concept that microscopic involvement of the lung was already present at the time of operation [10]. Overall survival remained stable at approximately 20% with amputation from the 1910s to the 1960s. Overall survival substantially improved to approximately 60% in the 1980s following the introduction of chemotherapy, where it currently remains [11]. Different dosing strategies and chemotherapy schedules have been studied since then, but have been shown not to affect survival rate [12; 13]. Due to the high malignancy of OS, current treatment now is aggressive and consists of cytotoxic chemotherapy both before and after wide resection surgery to remove the primary tumor and all operable metastases. The chemotherapy regimen for OS consists of a combination of doxorubicin, high dose methotrexate, and cisplatin [14].

The preoperative drug treatment offers several advantages: time for planning the limb salvage surgery and reconstructive procedures, an opportunity to study the histological effect of preoperative chemotherapy on the primary tumor (a better response to chemotherapy is strongly correlated with better outcome), and the ability to potentially modify postoperative chemotherapy accordingly. A good response to treatment is 90% necrosis within the tumor at the time of surgery: this still represents the most important prognostic factor to date, besides detectable metastatic disease. Studies have consistently demonstrated 5 year event free survival of 70-80% for good responders and 35-45% for poor responders [15]. However, it has been shown that there is no advantage specifically in event free survival for nonmetastatic OS patients given presurgical chemotherapy [16].

Complete surgical resections with wide margins, if feasible, remains essential for cure [1; 3]. Advances in imaging and surgical techniques and the positive effects of
preoperative chemotherapy have led to a major shift away from amputation and towards limb-salvage (conservative) surgery. Metastasectomy remains a critical and effective adjunct to multi-agent chemotherapy in the treatment of pulmonary metastases. However, surgical resection is only considered if all lung nodules can be removed and a sufficient amount of pulmonary tissue can be saved to maintain adequate pulmonary function.

Despite the aggressiveness of the current standard of care for OS, therapeutic strategies have thus far had limited efficacy in metastatic disease. The five-year survival rate has remained at ~60% for the last four decades, and patients most often succumb to their disease due to metastatic spread to the lungs. Of those that do respond initially to the current standard of care, a total of 30-40% patients with localized OS will develop a local or distant recurrence [17]. Furthermore, OS survivors, in addition to impaired limb function, often endure toxic side effects from chemotherapy, including long-term heart or lung damage, loss of hearing, slowed or decreased bone growth, and/or infertility [1].

**Etiology of OS**

Although the etiology of OS is unknown, there is accumulating evidence indicating OS to be of either mesenchymal stem cell (MSC) origin or originating from a more committed osteoblastic precursor. Some studies suggest that because OS has increased pluripotent potential, it may arise from a primitive precursor like the MSC. This implies that OS originating from an MSC might acquire its patterns of osteoblastic differentiation during transformation. Alternatively, some studies have described OS to arise from an osteoblastic population, and the pluripotent capacity is acquired through de-differentiation during the process of transformation [9].
Indeed, MSCs are characterized by high proliferative capacity (self-renewal) and multi-lineage potential. MSCs from the bone marrow can express gene programs of osteogenic, adipogenic, chondrogenic, neurogenic, or myogenic lineages. As an MSC progresses down the osteogenic differentiation lineage pathway, it becomes less proliferative and increases its extracellular matrix synthesis, resulting in a mature, specialized cell. Therefore, the genetic and epigenetic events that generate an OS tumor cell from a non-malignant MSC could cause epigenetic “deprogramming” of the cell, which is reflected in the tumor’s overall phenotype. Extensive epigenetic deprogramming causes tumor cells to become more resistant to signals that would induce differentiation-like characteristics, such as extracellular matrix (ECM) synthesis and silenced proliferation. Conversely, the greater the retention of the native differentiation programs, the less virulent the tumor cell and the better the response to treatment.

Histologically, OS has many characteristics of immature osteoblasts [18]. Osteoblasts develop from MSCs through a series of reasonably well defined, highly controlled, and tightly regulated intermediate precursor cell populations. The osteogenic differentiation program arises from both positive and negative regulation of gene expression and protein function, which are critical to proper osteogenic differentiation and bone formation. It is influenced by a variety of endogenous and environmental factors. However, coupled with its molecular and genetic complexity, the heterogeneous nature of OS tissue indicates the presence of cells arrested at various stages of differentiation, and its development coincides with periods when bone undergoes rapid growth or remodeling.
To study such a complicated topic, investigators have turned to experiments at the genetic level with transgenic mice using what little genetic information is known. The two most commonly inactivated genes and their associated pathways in OS are tumor suppressor genes Rb and p53, both of which are required for controlled osteoblast differentiation. More than 70% of all OS tumors demonstrate an overt mutation in the Rb gene [19] and up to 90% display a disruption in the p53 gene or pathway [20], suggesting that inactivation of these pathways is essential for the development of OS. One study proposed that a mutation in the p53 pathway could serve as an initiating event in OS, with a subsequent mutation in the Rb pathway strongly accelerating tumor development [21]. Additionally, it is conceivable that more elusive defects in their associated pathways exist, as well.

From transgenic mouse studies, it appears that a committed osteogenic precursor serves as a relevant and accurate “normal” control cell for future studies. Inactivation of p53 alone or in combination with Rb in committed osteogenic cell lineage populations led to nearly 100% OS development in mice [22; 23]. Alternatively, inactivation of Rb and/or p53 in early MSC of embryonic limb buds resulted in sarcoma development at a much lower incidence and an increased incidence of poorly differentiated soft tissue sarcomas [24]. Thus, at this level of study there is more evidence that osteoblastic commitment contributes to the OS phenotype.

**Differentiation Status**

In addition to the presence of osteoid, histopathologic tumor grading for OS takes into consideration cell differentiation characteristics, such as the volume and distribution of osteoid, coupled to malignant phenotypes, such as cellular atypia, mitotic figures, and the extent of necrosis [1]. The extent of differentiation seen in the
diagnostic biopsy is strongly associated with tumor behavior, where a lack of differentiation is associated with a worse clinical outcome [25]. Thus, differentiation status is intrinsically linked to malignant potential in OS and is an important prognostic factor.

Luo. and Wagner et al. have both argued that OS should be considered a disease of disrupted cell differentiation [26; 27; 28]. It is apparent that the process of differentiation confers a restraint on unrestricted proliferation and tumorigenesis [29], and must be overcome in the course of OS development. However, some aspects of osteogenic differentiation must be preserved, because the presence of osteoid is necessary to confirm an OS diagnosis.

Various similarities exist between stem cell biology and the nature of the OS disease [30]. It is theorized that the stage at which differentiation is interrupted likely correlates with the aggressiveness and metastatic potential of the various OS tumors [28; 30]. Classic osteogenic pathways are clearly disrupted in OS tumorigenesis, in addition to those seen in Rb and p53 pathways [30; 31]. Furthermore, studies have shown that OS can differentiate into various lineages with certain agents [30; 32]. However, not all osteogenic agents work, and it is argued that this may be due to specific defects in the differentiation cascade [30].

Differentiation-based therapies have been proposed as methods for treating advanced malignancies, with the goal of activating natural cellular maturation processes and, in turn, silencing malignant proliferation. Such treatments would ideally lead to tumor stasis and regression and have demonstrated success in acute myeloid
leukemia with all-trans-retinoic acid, and in glioma with Bone Morphogenetic Protein 4 (BMP4) [33].

**Inter- and Intratumoral Heterogeneity**

Inter- and intratumoral heterogeneity are common features for a variety of tumors and OS is no exception [34]. Even with its strict histological definition, OS is a form of cancer that exhibits considerable variability in its histologic features, grade, and anatomic location [4]. Furthermore, while cytotoxic chemotherapy has been effective for some patients, not all OS tumors respond equally and relapses are common. This is most likely due to the traditional assumption that a tumor, being clonally derived, is composed of a homogeneous population of cells, which are expected to respond uniformly to chemotherapy.

It is increasingly recognized that the cancerous cells within individual tumors are not biologically equivalent, but are heterogeneous. Variations in cell morphology, matrix production, proliferation rate, and tumorigenic and metastatic potential are frequently seen among cells within individual OS tumors. Thus, it is not surprising that the current chemotherapeutic cocktail, which only target rapidly dividing cells, often fails.

**OS Tumor Initiating Cell**

Despite reports of success identifying and isolating cancer stem cells through the use of cell surface markers: for example, EpCAM-1 for colon cancer and CD133 for brain tumor, this has not found broad acceptance [35]. OS cells that have been studied for cancer stem cell markers remain homogeneous in this aspect, and among tumors, all cells express the marker or all cells lack expression of the markers commonly used [36].
In previous work, our lab developed a method to selectively identify Tumor-Initiating Cells (TICs) in OS xenografts based on their ability to activate a transcriptional reporter comprised of a promoter for a human embryonic stem cell transcription factor (Oct4) linked to the coding sequence for Green Fluorescent Protein (GFP). Clonally derived, stably transfected OS Oct4/GFP+ cells are capable of initiating and maintaining the growth of new tumors and driving disease progression [36]. Furthermore, the GFP+ cells recapitulate the phenotypic heterogeneity of the parental tumor, demonstrating that benign GFP- cells arise from the tumorigenic GFP+ cells, a process we have termed “malignant reversion.” Additionally, differential analysis of global expression in the tumorigenic cells (GFP+) and their benign progeny (GFP-) from individual tumors has provided a detailed representation of their characteristic expression programs. The loss of GFP expression, and the subsequent loss of tumorigenic capacity, occurs during tumor formation in vivo, in response to prolonged stress either intrinsically from DNA damage or extrinsically to the growth environment. Growth under these conditions appears to reprogram the GFP+ cells, whereby they slow their proliferation and take on specialized secretory phenotype as an adaptive, survival mechanism no longer capable of activating the reporter. GFP- cells show increased endoplasmic reticulum (ER) stress, stress-induced differentiation pathways, and elaborate extracellular matrix components of specialized cells. Innate biochemically-induced survival responses to stress redirect transcriptional programs away from proliferation to survival and ECM synthesis, based on the residual native epigenetic program the cell has retained. This phenotypic change in response to stress is similar to that seen in normal bone physiological differentiation.
Bone Morphogenetic Proteins

Physiological Bone Development

The Bone Morphogenetic Protein (BMP) superfamily consists of more than 20 different phylogenetically conserved growth factors and is the largest subgroup of the Transforming Growth Factor β (TGFβ) superfamily. BMPs were initially discovered by their ability to induce ectopic bone formation [37], but are now known to be involved in a much broader array of developmental processes [38]. BMPs play an active role in embryogenesis, including dorsal-ventral patterning, limb patterning and formation, induction of the dermis, and early patterning of the central nervous system and neural crest, in addition to development of virtually every organ in the body [39; 40]. However, in post-natal life, BMPs are principally involved in bone induction. This includes both intramembranous and endochondral ossification, bone repair and regeneration, and the chondro-osteogenesis that is involved in ectopic bone formation [41; 42].

Although BMPs appear to be highly pleiotropic, developmental functions often overlap amongst BMP family members due to their amino acid sequence similarity. Furthermore, the non-viability of BMP knockout mice makes individual BMP isoforms difficult to study [43]. However, three general trends of BMP function are often featured: 1.) multipotent progenitor cell types respond to BMPs by increasing or decreasing their proliferation; 2.) BMPs depict a strong regulatory action to commit primitive mesenchymal progenitors into precursors of various phenotypes; and 3.) BMPs induce the differentiation of committed cells into specific tissues [41]. BMP activity is exceedingly regulated in these processes by the transient or highly localized expression of antagonists to permit controlled stem cell self-renewal for tissue maintenance and repair [44]. Importantly, differential gene induction by BMPs
depends on the specific nature of the “machinery” expressed in each responding cell type [40]. Therefore, BMP signaling is complex and requires concerted actions with other protein regulators to initiate certain cellular differentiation programs.

Pertinent to osteogenesis, BMPs are involved in normal bone formation by initiating and regulating the differentiation of MSCs into osteoblasts. At least seven factors control BMP signaling initiation: 1.) concentration of the ligand, 2.) type of ligand (isoform), 3.) ligand trapping antagonists, 4.) ligand trapping antagonist mediators, 5.) ligand receptor inhibitors, 6.) proteins that present ligands to the cells, and finally 7.) receptor combinations [45]. In the post-natal skeleton, BMPs are produced by periosteal cells and bone marrow MSCs. Bioavailability is imperative to BMP function, and is modulated by the extracellular environment in terms of production, secretion, and antagonism.

BMP ligands are typically divided into at least 4 different subgroups: I) BMP2 and 4 (with 92% amino acid sequence similarity in the mature part of these proteins); II) BMP5, 6, 7, 8a, and 8b; III) BMP9 and 10; and IV) BMP12, 13, and 14 [46]. BMPs have been observed as both homodimers and heterodimers in vivo and in vitro, and heterodimers appear to be more effective activators of the signaling pathways than their respective homodimers [38; 47; 48; 49; 50]. Furthermore, BMP isoforms 2, 4, and 7 play key roles in osteoblast differentiation and there is extensive data regarding their osteoinductive capabilities in the literature [41; 42; 43; 51; 52; 53]. Recombinant human BMP2 and BMP7 are FDA-approved for use in patients with non-healing bone fractures and spinal fusions [54; 55; 56; 57]. However, an implantation carrier is required for recombinant human BMP2 for efficient bone formation. Though it has
been shown that BMPs without a carrier can still induce bone formation; the carrier aids with localization, diffusion, and specified concentration [41].

As disulfide linked dimer ligands, BMPs bind to Serine/Threonine protein kinase BMP Receptors Types I and II (BMP Receptor Type II [BMPRII] and BMP Receptor Type 1A [BMPR1A] and 1B [BMPR1B]) [58]. BMP2 and 4 have higher affinities for BMPR1A and 1B, with lower affinity for BMPRII. Whereas BMP7 has higher affinity for BMPRII, its affinity for BMPRI is less pronounced, and so exemplifies a first level of regulation between BMP ligand and intracellular signaling [59].

Upon BMP dimer-receptor binding, the constitutively active BMPRII activates BMPRI via phosphorylation. BMPRI can then activate the Canonical Signaling Pathway by phosphorylating a BMP R-SMAD (1, 5 or 8), allowing interaction and complex formation with the Co-SMAD4. The specificity between the binding of BMPRI and the SMAD 1, 5, and 8 signaling components lies with the 9 amino acid L45 loop found in the Type I receptors, which recognizes specific L3 loops in the SMADs, thus conferring signal specificity and a second level of regulation between BMP ligands and intracellular signaling [58]. The R-SMAD and Co-SMAD4 protein complex will then translocate to the nucleus to affect gene expression in conjunction with other transcriptional regulators. Additional SMAD isoforms also exist, with SMADs 1, 5 and 8 specifically activated by BMP ligands, SMADs, SMAD 2 and 3 primarily activated by TGFβ isoforms, and finally the inhibitory SMADs 6 and 7, which can disrupt receptor-activated SMAD signaling [45].

In all isoforms of the SMAD proteins, the DNA binding hairpins are identical in amino acid sequence and thus all recognize SMAD Binding Elements (SBEs).
However, the DNA binding hairpin positioning in SMAD1 is different from that of SMAD3, and thus might explain why BMP R-SMADs also bind GC rich sequences in addition to SBEs, whereas the TGFβ R-SMADs 2 and 3 primarily bind to canonical SBEs [45]. Additionally, all SMAD proteins bind DNA directly but with low affinity and low specificity, and therefore must have a transcriptional regulator to affect gene expression properly [45]. Thus, the SMAD 1, 5, and 8 specific DNA binding properties and the additional requirements for transcriptional regulators represent a third and fourth level of regulation between BMP ligands and intracellular signaling.

There is yet to be determined a ubiquitous DNA binding SMAD partner that regulates all BMP responsive genes, and thus the cells response to BMP signals is absolutely dependent on its molecular status at the time of receptor activation [40; 60]. BMP signaling culminates at the level of the transcriptional binding partners pertinent to each cell type. SMADs have been shown to have various co-factors once inside the nucleus, which include: 1.) Runt-Related Transcription Factor 2 (Runx2) and Osterix for induction of osteogenic lineage commitment and terminal differentiation [61], 2.) epigenetic modifiers such as p300 and CREB-Binding Protein (CBP), 3.) repressive transcription factors, including Msh Homeobox 1 and 2 (Msx1 and 2), and 4.) transcription factors Distal-Less Homeobox 5 and 6 (Dlx5 and 6) to express osteoid matrix genes [28].

SMADs are not the only molecules that can transduce signals from BMP ligands, but they are by far the best understood. Phosphorylated BMPRI can also activate the Non-Canonical Pathway through phosphorylating TGFβ Activated Kinase 1 (TAK1). Phospho-TAK1 will then activate Mitogen Activated Protein Kinase Kinase
3 and 6 (MKK3/6), which leads to phosphorylative activation of p38 Mitogen Activated Protein Kinase (p38). This allows for phospho-p38 to translocate to the nucleus [62; 63; 64]. These signaling modulators also bind with transcriptional regulators inside the nucleus to affect gene expression.

BMPRI kinase function is thought to define the nature and specificity of the BMP signals [59]. One study suggests that the way the receptor complex is formed allows for differential signaling to be induced [65]. The BMPRI and BMPRII receptor complex can be preformed on the surface of the cell prior to ligand binding, thus termed the Preformed Complex (PFC). Upon BMP binding to the PFC, the ligand-receptor complex becomes internalized via clathrin coated pits, and thus activates the Canonical Signaling through the R-SMADs [65]. Alternatively, when BMPs induce receptor complex formation on the outside of the cell, the ligand-receptor complex is termed the BMP Induced Signaling Complex (BISC). BISC formation results in internalization via caveolae, and thus activates the Non-Canonical Pathway [65]. However, the reproducibility of this data is unclear and more work is necessary to confirm this research.

BMP signaling activates several downstream cellular responses to induce osteogenic differentiation. In conjunction with gene expression changes, BMPs activate the ER Stress Response, specifically the Protein Kinase R (PKR)-like Endoplasmic Reticulum Kinase (PERK) and Inositol-Requiring Enzyme 1 (IRE1) pathways of the Unfolded Protein Response, which plays a central role in positive regulation and expression of extracellular matrix genes [66; 67; 68]. Furthermore, osteogenic differentiation is linked to reduced proliferation, and without exception, BMP
signaling in bone formation is associated with reduced proliferative potential [69]. As a
downstream effector of BMP signaling, Runx2 physically interacts with Rb to control
cell cycle progression [31]. Runx2 also induces cell cycle regulator p27 expression and
function, and the SMAD pathway has also been shown to be involved in transcriptional
regulation of the cell cycle regulator p21 [70; 71]. Overall, osteogenic differentiation is
confirmed through changes in various markers: Alkaline Phosphatase, Osterix, Runx2,
Osteopontin, Osteocalcin, and Collagen 1A Type 1 [28].

In conclusion, BMPs are involved in various processes, with a major role in
osteogenesis, and are regulated at four different levels: 1.) at the membrane level,
through certain receptors and receptor forming mechanisms; 3.) at the cytosolic level,
by the SMAD proteins; and 3 and 4.) at the nuclear level, by SMAD binding activity, in
conjunction with co-activators or co-repressors that modulate transcriptional activity
[42].

Previous Literature of BMP Treatment for OS

Almost all TGFβ superfamily members, including BMPs, Activins, Nodals, and
Growth and Differentiation Factors (GDFs), play active roles in embryogenesis and
adult homeostasis and thus make them a frequent target for deregulation in cancer
[44]. BMP ligands and their associated signaling pathways, which require balance and
regulation in their activity, can be disrupted in a number of ways [44]. Deregulation in
the interplay between BMPs and their antagonists are involved in the aggressiveness
of primary tumors and the ability of disseminated tumor cells to exit dormancy and
establish metastases in vivo [44; 72]. However, investigators have tried to correlate
BMP ligand expression and the presence of BMP signaling components with grade or
histopathologic subtype of OS disease, but no unifying differences in expression have
been found to date [72; 73]. In addition to different journal articles assigning differential tumorigenic functions for BMP isoforms (whether pro-tumorigenic or anti-tumorigenic), many review articles interpret the same published differences. The functions of BMPs and their role in tumorigenesis, the maintenance of tumor cells, metastasis and dormancy in not only OS but also other forms of cancer have been discussed in detail, but with no reproducible conclusions [26; 73].

Previous reports describing the effects of BMP stimulation on OS cell lines have been conflicting or inconclusive, with some reporting increased tumorigenicity and others the opposite [74; 75; 76], or additionally that there is no effect proven in a realistic model of local recurrence [77]. This controversy likely stems from the overuse of the limited number of commercially available OS cell lines (U2-OS, MG-63, and SaOS-2), their wide distribution, growth, and expansion under variable conditions, poor characterization for TICs, as well as the limited number of useful OS animal models. Furthermore, through the different methods of administration of BMPs, whether pretreatment, exogenously, systemically, or through Adenovirus [75], there are massive misconceptions regarding the role of BMP signaling in OS. Thus, there are clear deficits in several areas of the knowledge of BMP signaling in the cancer setting and specifically in the genesis of OS that need to be addressed.
CHAPTER 2
THE EFFECT OF BONE MORPHOGENETIC PROTEIN HETERODIMERS ON OSTEOSARCOMA TUMOR INITIATION

Introduction

Osteosarcoma (OS) is a highly malignant form of bone cancer defined histologically by the secretion of immature bone matrix. Primarily affecting children during skeletal growth, treatment is aggressive and consists of cytotoxic chemotherapy and wide surgical resection \([3; 32]\). Despite advances in surgical techniques, the chemotherapeutic regimen has not changed in the last 40 years. Similarly, the five-year survival rate has remained a dismal \(\sim60\%\), with the majority of fatalities arising from metastatic spread to the lungs \([11]\). The malignant cells within individual OS tumors are not biologically equivalent, but are in fact heterogeneous. Variations in cell morphology, matrix production, proliferation rate, and tumorigenic and metastatic potential are frequent \([1]\). Thus, it is not surprising that current chemotherapies, which target rapidly dividing cells, often fail. There is a need for chemotherapies with greater efficacy and a better understanding of the heterogeneous nature of OS biology.

In previous work, we developed a method to selectively identify Tumor-Initiating Cells (TICs) in OS xenografts based on their ability to activate a transcriptional reporter comprised of the human Oct4 promoter linked to the coding sequence for Green Fluorescent Protein (GFP). Following subcutaneous implantation in NOD/SCID Gamma (NSG) mice, clonally derived, stably transfected OS Oct4/GFP+ TICs form heterogeneous tumors comprised of tumorigenic GFP+ cells and nontumorigenic GFP- cells \([36]\). Differential gene expression profiling shows that the GFP- cells have transitioned away from malignancy to an extracellular matrix (ECM) secretory phenotype as an adaptive survival mechanism (Levings, et.al. in preparation). This was
confirmed by immunohistochemical staining in xenografts, where the loss of Oct4/GFP expression correlated with DNA damage, slowed proliferation, and avascular, hypoxic regions, suggesting a change in phenotype arising from intracellular stress. This stress-related phenotypic change is similar to processes seen in normal osteogenic differentiation.

During skeletal formation, Bone Morphogenetic Proteins (BMPs) (members of the Transforming Growth Factor β (TGFβ) Superfamily) play key roles in initiating osteogenic maturation. BMPs act as hetero- or homo-dimers and bind to BMP Receptors Type I and Type II (BMPR II and BMPR I) on mesenchymal stem cells (MSCs), activating BMP signaling pathways [45]. Upon BMP-receptor binding, BMPR II kinase activity phosphorylates BMPR I. BMPR I can then activate the Canonical Signaling Pathway by phosphorylating an R-SMAD (1, 5 or 8), allowing for complex formation with Co-SMAD4, which then translocates to the nucleus. Alternatively, phosphorylated BMPR I can activate the Non-Canonical Pathway by phosphorylating TGFβ Activated Kinase 1 (TAK1). Phospho-TAK1 then activates Dual Specificity Mitogen-Activated Protein Kinase Kinase 3 and 6 (MKK3/6), and in turn p38 Mitogen-Activated Protein Kinase (p38), allowing for phospho-p38 to translocate to the nucleus. These signaling modulators bind with transcriptional regulators inside the nucleus (such as Runt-Related Transcription Factor 2 (Runx2), a point of convergence for both the Canonical and Non-Canonical Pathways), and mediate transcription of genes associated with the osteogenic lineage and osteoid matrix production.

OS is believed to originate from MSCs or osteogenic committed precursors [18]; but, oncogenic transformation induces a hyper proliferative phenotype at the expense of
matrix synthesis. Our analyses of the comparatively benign, GFP- cells within OS tumors are consistent with an osteoid extracellular matrix (ECM) secretory phenotype, which suggests the cells retain remnants of an osteogenic program and an innate sensitivity to BMP proteins. We hypothesized that sustained BMP stimulation would force OS TICS to differentiate and thereby impair their ability to initiate and maintain tumor growth. Similar differentiation-based therapies have demonstrated success in acute myeloid leukemia using all-trans-retinoic acid [78] and in glioma using BMP4 [33].

In this study, to test the ability of BMPs to initiate osteogenic differentiation in OS, TICs derived from patient tumors of two different phenotypic subtypes were stimulated with either a BMP2/7 heterodimer or a BMP4/7 heterodimer. Despite both heterodimers exhibiting amino acid sequence similarity, coordinate analyses of a series of in vitro and in vivo assays show the ability of only BMP4/7 to induce an antitumor effect similar to differentiation.

**Methods**

**Cell Culture**

The OS521 and OS156 cell lines were previously established from patient biopsies using protocols approved by the Institutional Review Board of the University of Florida College of Medicine. TICs, as defined by Oct4/GFP+ fluorescence, were isolated from xenograft tumors using Fluorescence Activated Cell Sorting and maintained in complete culture medium (DMEM/F-12, 1% Penicillin/Streptomycin, and 10% Fetal Bovine Serum; Life Technologies) supplemented with 0.4 µg/mL G418 (Mediatech). Cell line OS521 was established from an aggressive, highly vascular, poorly differentiated, high grade OS of the distal femur. Cell line OS156 arose from an osteoblastic OS, also originating from the distal femur, with necrotic foci, poor vascularity and differentiation.
MSCs were also isolated from discarded surgical tissues using protocols approved by the Institutional Review Board of the University of Florida College of Medicine. MSCs were maintained in complete culture medium of DMEM, 1% Penicillin/Streptomycin, and 10% Fetal Bovine Serum (Life Technologies) supplemented with 10 ng/mL of Fibroblast Growth Factor (Peprotech).

**BMP Receptor Expression**

The cells were removed from culture and resuspended in 0.05% Bovine Serum Albumin in PBS at $10^6$ cells/mL. The cells were blocked with human IgG (1 μg/10^5 cells) before incubation with antibodies targeting the following receptors: BMPR II (R&D Systems #BAF811), BMPR 1A (R&D Systems #BAF820), and BMPR 1B (R&D Systems #FAB5051P) (diluted 1:100). Flow cytometry was done using the LSRII flow cytometer (BD Biosciences).

**Bone Morphogenetic Protein Stimulation**

OS521 and OS156 cells were plated at a density of ~7,000 cells/cm², in complete culture medium containing 100 ng/mL of either BMP2/7 (#3229-BM-010/CF) or BMP4/7 (#3727-BP-010/CF) (R&D Systems). Media with fresh BMP protein was replaced at 24 hours, and the cells were trypsinized and collected for experiments at 48 hours.

**Western Blot Analyses**

Lysates were prepared by incubating $10^6$ cells in 100 μl RIPA buffer followed by 1 hour incubation on ice. Protein concentrations were measured using the Pierce BCA Protein Assay Kit (Thermo-Scientific). For each protein sample, 15 μg of protein was denatured by SDS-PAGE (BioRad) and then electrophoretically transferred to nitrocellulose membranes. Membranes were blocked with 5% milk for 1 hour and incubated with primary antibody at the indicated concentration at 4°C overnight followed
by 40 minutes of washing with Tris Buffered Saline with 0.1% Tween20 (Fisher Scientific). Secondary antibody was added for 1 hour at room temperature, and the membrane was washed for an additional 30 minutes. Proteins were visualized using ECL Prime Western Blotting Detection Reagent (GE Healthcare) and exposed on autoradiographic film (Denville Scientific Inc.). The following primary antibodies were used for analyses: Phosphorylated (Ser206) SMAD 1 and 5 (p-SMAD1/5) (Cell Signaling Technology #5753, diluted 1:2000), SMAD1 (Cell Signaling Technology #6944, 1:2000), Co-SMAD4 (Cell Signaling Technology #9515, 1:2000), Phosphorylated (Thr180/Tyr182) p38 MAPK (p-p38) (BD Transduction Laboratories #612288, 1:2000), p38 (BD Transduction Laboratories #512168, 1:2000), Phosphorylated (Thr202/Tyr204) p44/42 MAPK (p-ERK1/2) (Cell Signaling Technology #4377S, 1:2000), p44/42 MAPK (ERK1/2) (Cell Signaling Technology #4695S, 1:2000), P27 (KIP1) (BD Transduction Laboratories #554069, 1:4000), p21 (Santa Cruz sc-397, 1:2000), and Glyceraldehyde 3-Phosphate Dehydrogenase linked to Horseradish Peroxidase (GAPDH) (EnCor Biotechnology Inc. #MCA-1P4, 1:30000 dilution). Mouse secondary antibody was used at a dilution of 1:2000 except in combination with GAPDH when it was used at a 1:30000 dilution, and Rabbit secondary antibody was used at a dilution of 1:4000.

**Cell Viability and GFP Expression**

To determine cell viability and GFP expression, post treatment cells were trypsinized and resuspended in 0.05% Bovine Serum Albumin in PBS at 4 x 10^6 cells/mL. The cells were then incubated with Propidium Iodide (PI) for 5 minutes and PI and GFP fluorescence was measured using the LSRII flow cytometer (BD Biosciences).
Quantitative Reverse Transcriptase PCR

After BMP pretreatment for 48 hours, cells were collected and total RNA was isolated using phenol:chloroform extraction with Trizol (Life Technologies) according to the manufacturer’s protocol. The recovered RNA was quantified by spectrophotometry at 260/280 nm. cDNA was generated using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). qPCR reactions were run using Perfecta SYBR Green FastMix according to the manufacturer’s instructions (Quanta Biosciences) on an Eppendorf Mastercycler Realplex2 (Eppendorf). Target mRNA levels were normalized to GAPDH mRNA. Relative gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method.

Cell Cycle Analyses

BMP stimulated cells were re-suspended in DAPI Nucleic Acid Stain (Invitrogen) and incubated at room temperature for 10 minutes. UV fluorescence was determined using a LSRII flow cytometer (BD Biosciences) and cell cycle progression was analyzed using ModFit LT software.

Proliferation Assay

After BMP treatments, cells were plated in complete culture medium into 96 well plates (OS521 at 4000 cells/well and OS156 at 3000 cells/well). At the indicated time points, cells were fixed in 1% glutaraldehyde and stored in PBS at 4°C. At the end of the experiment, cells were stained with 0.5% crystal violet for 20 minutes and then thoroughly washed of excess. Crystals were dissolved with 15% acetic acid and optical density was read at 595 nm.
Invasion Assay

Following stimulation with BMPs, 2.5 x 10^4 cells in serum free medium were plated into the top chamber of transwell inserts (Falcon) coated with 10 μg/mL Fibronectin in PBS and 2.5% Bovine Serum Albumin in PBS. Complete culture medium was added to the lower chamber as a chemoattractant. After 24 hours, cells were fixed with 1% glutaraldehyde and stained with 0.5% crystal violet. Cells that did not invade through the pores were mechanically removed using a cotton swab. Images of cell invasion were taken using a Leica DMIL inverted fluorescence microscope (Leica Microsystems) and the Retiga 1300R camera (Q Imaging). The cells were counted in 5 randomly selected fields and the mean values were calculated. All experiments were performed in triplicate and repeated.

Tumorigenicity Studies

All animal experiments were approved by the University of Florida Institutional Animal Care and Use Committee. Tumors were formed in six-week-old NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ (NSG) mice (Jackson Laboratory) by subcutaneous injection of cells in 100 μL Opti-MEM (Gibco) in the scapular region at indicated concentrations. Tumor onset was recorded when a physical mass could first be manually palpated. Tumors were harvested at a diameter of 1.3 to 1.5 cm or 90 days post injection. The lungs and liver from the mice were also harvested for histological analyses.

Immunohistochemistry

Xenograft tumor specimens, lungs, and liver were fixed in 4% paraformaldehyde at 4°C for 24 hours and paraffin embedded. The embedded tissues were sectioned at 4 μM and stained using Hematoxylin and Eosin. Histologic images were captured using a 3.3 MPX Camera (Imaging Planet) mounted on a Zeiss Axioskop 40 microscope (Carl
Zeiss Microimaging) and analyzed using Image Planet Capture software (Imaging Planet)

**Statistical Analyses**

The Log Rank Mantel-Cox Test was used to determine statistical significance between the different treatment groups in the tumorigenicity studies. A two-tailed student t-test was used to determine statistical significance unless otherwise specified. Asterisks indicate level of significance found: *p≤0.05, **p≤0.01, and ***p≤0.001.

**Results**

To gauge their susceptibility to exogenous BMP stimulation, flow cytometric analyses were performed to determine the levels of BMPR II and two isoforms of BMPR I (BMPR 1A and 1B) on unstimulated OS521 and OS156. MSCs were used as a positive control to assess normal receptor levels. Whole population analysis displayed uniformly high expression of BMPR II and BMPR 1A on OS cells, but minimal expression of BMPR 1B, showing both OS cell lines appearing potentially sensitive to BMP stimulation (Figure 2-1).

To determine whether the receptor-signaling pathways remain functional, cell cultures were stimulated with BMPs. Following normalization to GAPDH protein, BMP4/7 stimulation increased levels of phosphorylated R-SMAD 1 and 5 (p-SMAD1/5) (2.0 densitometric units for OS521, 3.4 for OS156, and 2.1 for MSCs) (Figure 2-2). Increased levels of p-SMAD1/5 after BMP2/7 stimulation were also seen in OS521 and partially in OS156. R-SMAD1 and Co-SMAD4 were present under all conditions. Only minimal changes were seen in levels of phosphorylated p38 (p-p38) of the Non-Canonical Pathway in OS cells. Together these observations indicate exogenous BMP
stimulation induced activation of the Canonical Pathway in all cell lines tested; however, the amplitude was both BMP heterodimer-specific and cell line-specific.

Since differentiation is associated with reduced proliferation [69], we looked at levels of phosphorylated Extracellular Signal-Regulated Kinase 1 and 2 (p-ERK1/2), a proliferative MAPK. In both OS521 and OS156, no changes were observed in p-ERK1/2 after BMP stimulation (Figure 2-2). Alternatively, we determined protein levels of cyclin-dependent kinase inhibitor 1 (p21^{Cip1}) and cyclin-dependent kinase inhibitor 1B (p27^Kip1), two G1-checkpoint cyclin-dependent kinase inhibitors. OS156 showed a decrease in both p27 and p21. Interestingly, OS521 stimulated with BMP4/7 displayed a slight increase in p27 and p21 (0.8 and 0.9 densitometric units, respectively), suggesting the involvement of cell cycle regulators with BMP-SMAD signaling.

We saw no changes in Oct4 promoter-regulated GFP expression with either BMP protein or in either OS cell line (Figure 2-3A). There were no changes observed in cell viability after 2 days of BMP stimulation in any cell line (Figure 2-3B). Finally, BMP stimulation had no universal effect on osteogenic gene marker transcription (Figure 2-4). Therefore, although BMP stimulation was measurable, its overall impact on cellular differentiation was not readily discernable.

To directly determine the effect on cellular division, we performed cell cycle analysis on BMP treated cultures. Two days of BMP stimulation was not enough to induce any cell cycle changes in MSCs (Figure 2-5). OS156 showed a 39% decrease in the fraction of cells in G1 Phase after both BMP2/7 and 4/7 stimulation. In BMP2/7 treated OS521, the amount of cells in G1 Phase remained relatively unchanged. However, in BMP4/7 treated OS521, the percentage of cells in G1 increased 20%, with
a 38% reduction in the amount of cells in G2, showing reduced mitotic entry as a consequence of BMP4/7 stimulation.

To investigate further the role of cell division regulation in BMP stimulated OS cells, we next looked at proliferative ability. The OS521 cell cycle changes induced by BMP4/7 stimulation do not materialize into proliferative changes until Day 8, where there was a significant decrease in relative cell density (Figure 2-6). BMP2/7 stimulated OS521 proliferation was affected but to a lesser extent. Alternatively, BMP treated OS156 show an increase in relative cell density at Day 8, consistent with their increase in cells in G1 Phase from Figure 2-5.

To study the effect of BMP stimulation on one aspect of metastases, invasion capacity through a complex of Fibronectin and Bovine Serum Albumin was analyzed. OS521 stimulated with either BMP2/7 or 4/7 showed an increase in invasion (Figure 2-7). However, BMP4/7 treated OS156 showed a 33% reduction in invasive capability, compared to untreated controls, suggesting an alternative effect of BMP4/7 stimulation in a more differentiated cell line.

To investigate the effect of BMP stimulation in vivo, OS521 and OS156 were treated with BMP2/7 or 4/7 for two days in cell culture, then trypsinized and inoculated into NSG mice at varying cell doses. BMP2/7 had no measurable effect on OS521 tumor formation or histology, compared to unstimulated controls (Figure 2-8A and B). However, pre-incubation with BMP4/7 significantly decreased OS521 tumor penetrance and increased time to tumor onset (Figure 2-8A). Tumors that were able to form from BMP4/7 treated cells appeared to have more necrotic areas and a lack of cohesion.
amongst cells (Figure 2-6B). No metastases were observed following any treatment of OS521 tumors.

BMP2/7 stimulation of OS156 showed a slightly reduced time to onset for the lowest cell dose, relative to controls, suggesting a somewhat enhanced tumor forming ability. Additionally, there was an increase in mice with metastases when inoculated with BMP2/7 treated OS156 cells (Figure 2-6C). However, similar to OS521, pretreatment of OS156 with BMP4/7 trended towards decreased tumor penetrance and increased time to tumor onset, although no significance was found. No obvious differences were found in the histology of any of the tumors or metastases (Figure 2-6B).

**Discussion**

The current standard of care for OS patients includes cytotoxic chemotherapy both before and after aggressive surgery to remove the primary tumor and any visible metastases. This chemotherapy regimen has remained unchanged in the last four decades and still includes a combination of high dose methotrexate, cisplatin, and doxorubicin [3]. When the disease presents as nonmetastatic, these drugs are effective, but produce very toxic side effects. Alternatives to these therapies are greatly needed.

Previous reports describing the effects of BMP stimulation on OS cell lines have been conflicting or inconclusive, with some reporting increased tumorigenicity and others the opposite [75; 79]. This likely stems from the overuse of the limited number of commercially available OS cell lines (U2-OS, MG-63, and SaOS-2), their wide distribution, growth, and expansion under variable conditions, poor characterization for TICs, and a limited number of useful OS animal models [80]. Furthermore, although OS is diagnosed histologically by the presence of osteoid, intratumoral heterogeneity is evident in the many cell types that are present (e.g. osteoblastic,
chondroblastic, fibroblastic) and their corresponding production of matrix [3]. However, the extent of differentiation, i.e. how much matrix is produced, within tumors often holds prognostic value.

OS521 and OS156 were chosen for study because they represent two different sides of the spectrum of differentiation status in OS: OS521 as a poorly differentiated cancer and OS156 as a well differentiated cancer. The intertumoral heterogeneity depicted here not only describes the variabilities seen in diagnostic pathology but evolves into differences in outcome for the patients. Specific to this study, the intertumoral heterogeneity is highlighted in each cell lines’ response to BMP stimulation, molecularly and biologically.

OS521 and OS156 are receptive to BMP stimulation through BMPR II and 1A, thus activating the Canonical SMAD Signaling Pathway (Figure 2-1 and 2-2). However, there is a differential response to BMP stimulation between the two OS cell lines and we believe this can be attributed to the inter-tumor heterogeneous nature of the disease. Activation of the Canonical Signaling Pathway is a necessary precursor to the start of BMP induced osteogenic differentiation [45]. Yet, there is no evidence in this study to confirm that BMP stimulation induces osteogenic differentiation in our OS cell lines (Figure 2-4). Previous literature has shown that it takes a full 2-3 weeks for MSCs to produce substantial osteoid matrix in cell culture [81]. Therefore, two days of BMP stimulation in vitro is not enough to determine if our OS cell lines can osteogenically differentiate. Interestingly, when OS521 cultures are stimulated with BMP4/7, there is an increase in p27 and p21 expression. This leads to an expansion in the amount of cells halted in G1 Phase and a reduction over time in the cells’ ability to proliferate (Figure 2-
5 and 2-6). Because of this, we believe that BMP4/7 stimulation is primarily working through a cell cycle regulated mechanism in OS521, a feature similar to what takes place during the process of cellular differentiation. Alternatively, there is evidence to support an anti-invasive mechanism in BMP4/7 stimulated OS156, as shown by reduced invasion through a complex of Fibronectin and Bovine Serum Albumin (Figure 2-7).

These two different BMP heterodimer combinations were chosen based on their potent osteoinductive potential [41]. However, it was surprising to note that not only do BMP2/7 and 4/7 act differently in vitro, but also in vivo. Tumor-free survival data of OS521 reflected experiments done in vitro. Confirming the cell cycle regulated mechanism imposed by BMP4/7 in cell cultures, BMP4/7 stimulated OS521 cells showed a lapse in tumor development and a significant overall inhibition of tumor generation (Figure 2-8A). Interestingly, the enhanced invasiveness in BMP2/7 and 4/7 treated OS521 does not appear to manifest itself in vivo; considering OS521 does not metastasize at all in our mouse model, it suggests that this assay does not accurately represent clinical OS biology in this cell line.

In OS156, BMP stimulation resulted in only slight effects seen in vivo, with a nonsignificant trend of BMP2/7-enhanced and BMP4/7-inhibited tumor formation (Figure 2-8A). Interestingly, whereas cell culture experiments showed a BMP4/7 inhibition of invasion, the same amount of metastatic animals injected with BMP4/7 stimulated cells was seen as metastatic animals injected with untreated cells, whereas there was actually an increase in the amount of animals with metastases that were inoculated with BMP2/7 stimulated cells (Figure 2-8C). Again, as an example of the disconnect between
data gained *in vitro* and *in vivo*, this confirms that the 3D microenvironment plays a role in the cells’ behaviors. One potential explanation for the lack of BMP4/7 induced effect in OS156 is that not enough of the cells were affected by stimulation *in vitro*, as shown by the lack of signaling transduction shown in western blotting experiments (Figure 2-2).

Taken together, BMP4/7 could be a potential treatment for poorly differentiated OS, as shown by its significant anti-tumorigenic activity in OS521. It would be interesting to study the combinatory effect of BMP4/7 stimulation with traditional chemotherapies. However, more studies are needed to determine the efficacy of BMP4/7 treatment in the setting as an actual therapy.
Figure 2-1. Unstimulated OS cells express BMPR II and 1A but not BMPR 1B. (A) BMP receptor expression was examined on OS521, OS156, and MSCs. Graphs presented are representatives from one experiment (n=3). Profiles of cells incubated with isotype control are presented in grey lines, and profiles of cells incubated with BMPR antibodies are presented in black lines. (B) BMPR expression was quantified and is shown as mean percent of cells that are BMPR-positive and the standard error of the mean of independent measurements.
Figure 2-2. OS cells are responsive to exogenous BMP stimulation. OS521, OS156, and MSCs were stimulated with BMP2/7 or 4/7 for 2 days in cell culture. Western blots of whole cell lysates were performed to determine the relative levels of proteins indicated. p-SMAD1/5, SMAD1, and Co-SMAD4 represent the BMP Canonical Pathway; p-p38 and p38 represent the BMP Non-Canonical Pathway; p-ERK1/2 and ERK1/2 represent indications of cellular growth; and p27 and p21 are cell cycle regulators. Bands were analyzed using ImageJ software, where each band is normalized to GAPDH, and densitometric values are presented below each blot.
Figure 2-3. BMP stimulation does not affect GFP expression or cell viability. Cells were stimulated in the same manner as in Figure 2-2. Cells were then collected and GFP expression (A) and cell viability following incubation with PI (B) was investigated. Data is shown as mean percent of cells that are GFP+ and GFP- in (A) and viable cells in (B) with the standard deviation of the mean of independent experiments (n=2).
Figure 2-4. BMP stimulation does not induce universal osteogenic gene expression. Relative fold change, determined by RT-qPCR of osteogenic marker genes normalized to GAPDH, following BMP stimulation for 48 hours (n=2).
Figure 2-5. BMP stimulation alters OS cell cycle progression. Post BMP treatment, cells were collected and cell division was investigated following incubation with DAPI. ModFit LT cell cycle graphs are presented in (A) and quantified data as the percent of cells in each phase in (B) (n=2).
Figure 2-6. BMP stimulation has differential effects on OS proliferation. BMP treated cells were collected and plated for a proliferation assay. Data is presented as percentage of relative cell density, with each group normalized to its own group on Day 0. Asterisks indicate significance found only at Day 8.
Figure 2-7. BMP4/7 stimulation increases OS521 invasion and decreases OS156 invasion. BMP treated cells were plated into transwells coated with a complex of Fibronectin and Bovine Serum Albumin, allowed 24 hours to invade, and then stained with crystal violet and counted (A). (B) Quantified data is presented as mean percent of cells that invaded and the standard deviation of the mean of independent measurements (n=2).
Figure 2-8. BMP stimulation in vitro decreased tumor formation in vivo. (A) Kaplan-Meier plots for animals that remained tumor-free after injection with BMP stimulated cells or untreated cells. OS521 cell doses for each injection are: 3 x 10^4 (Highest, n=8 mice/group), 3 x 10^3 (Medium, n=10), and 3 x 10^2 (Lowest, n=8). OS156 cell doses are: 1 x 10^5 (Highest), 1 x 10^4 (Medium), and 1 x 10^3 (Lowest); n=6 mice/group. Only OS521 pretreated with BMP4/7 was found to be statistically significant: Highest at p=0.0002; Medium at p=0.0001; and the Lowest at p=0.0202. No significance was found with OS156. (B) Primary tumors, lungs, and liver from animals in (A) were taken for Hematoxylin and Eosin staining. (C) Upon tumor harvest for mice inoculated with OS156, metastasis was investigated. Data is presented as number of mice that develop lung or liver metastases, per cell dose as indicated.
CHAPTER 3
SIMULATING THE OSTEOSARCOMA TUMOR MICROENVIRONMENT WITH ADENOSINE, CALCIUM, AND HYPOXIA

Introduction

In Darwinian dynamics, crucial selection forces provided by the microenvironment control proliferative capability and inevitably survival. Thus, the phenotypic changes that emerge during OS development must represent successful adaptations to these microenvironmental selection forces [82]. Indeed, the differential gene expression analyses between the tumorigenic cells (GFP+) and their benign progeny (GFP-) from individual tumors has provided a detailed representation of their characteristic transcriptional programs as an adaptive response to DNA damage and to their microenvironment (Levings, et al., in preparation). The loss of GFP expression, and the subsequent loss of tumorigenic capacity, occurs during tumor formation in vivo, in response to prolonged intracellular stress. Growth under these conditions appears to reprogram the GFP- cells, whereby they slow their proliferation and take on a “differentiated” secretory phenotype as an adaptive, survival mechanism. Furthermore, these phenotypic changes seen in response to stress within the OS tumor microenvironment (TME) are similar to that seen in normal bone physiology. Microenvironmental cues are critical to developmental processes, whereby for example hypoxia and physical stress play a role in osteoblast differentiation from mesenchymal stem cells and subsequently the resulting formation of bone [83; 84].

Though we observed an anti-tumorigenic effect associated with stimulation from Bone Morphogenetic Protein 4/7 Heterodimer (BMP4/7), our in vitro assays did not uncover a specific mechanism other than activation of the BMP Canonical Signaling Cascade. BMP stimulation in cell cultures for two days had no effect on the activation of
the Oct4/GFP reporter, cell viability, or osteogenic differentiation. Therefore, we hypothesized that the anti-tumorigenic properties of BMP4/7 on OS Tumor Initiating Cells (TICs) in vivo require the presence of microenvironmental stresses which do not occur in standard cell culture. To test this, we started by investigating whether exposure to hypoxic conditions would alter the response to BMP stimulation, as previous data in our laboratory and others have shown that this is a major factor present in the TME (Levings, et.al. in preparation). However, we soon speculated that stress from multiple sources would be needed.

The differential gene expression programs in GFP+ and GFP- cells showed that in addition to responding to a lack of oxygen, cells that have lost their malignant potential display amplified signaling as a result of adenosine accumulation and increased intracellular calcium. Adenosine is present at low levels in unstressed tissues, but accumulates rapidly due to pathophysiological conditions, such as hypoxia, inflammation, and regional necrosis [85]. After being released from stressed and dying cells, Adenosine Triphosphate (ATP) is converted to adenosine and internalized via purinergic receptors where it serves as a danger signal. Following uptake, adenosine activates Mitogen-Activated Protein Kinase (MAPK), Extracellular Signal-Regulated Kinase (ERK), and c-Jun N-Terminal Kinase (JNK) signaling as well as 5’ AMP-Activated Protein Kinase (AMPK) pathways, which drives the cell to increase ATP production [85]. Adenosine dampens the immune system while increasing angiogenesis; depending on the context, it can regulate numerous cellular processes, such as proliferation, differentiation, and apoptosis, and therefore directs the course of cancer development [85; 86; 87].
Calcium functions as a second level signaling molecule that has primary roles in both proliferation and apoptosis [88; 89]. It is stored within the cell in the mitochondria and endoplasmic reticulum, and is released following activation of inositol trisphosphate receptors, ryanodine receptors, and the sodium-calcium exchangers [88]. Upon calcium release, several signaling pathways are activated, including the ERK pathway, the IP3-DAG-PLC pathway, and at sustained high levels, intracellularly intrinsic apoptotic pathways. Within tumor cells, calcium signaling is exploited, driving both tumor progression via increased cell proliferation or activation of differentiation and anti-apoptotic cell survival pathways [88; 90].

Our differential gene expression analyses reveal that adenosine and calcium related pathways as well as hypoxia are major contributors to the stress signaling within the OS TME, and cause the differentiation of the GFP+ cells and emergence of the less tumorigenic GFP- cells. It is important to note that not only are these components important individually in their role in the TME, but there is a large degree of inter-relatedness between the activities of adenosine, calcium, and hypoxia [85; 88; 91; 92; 93; 94; 95; 96; 97; 98; 99; 100; 101; 102]. The goal of this research was to exogenously simulate the stresses of the TME, in order to study the mechanism by which BMP4/7 inhibits OS tumor formation.

Methods

Cell Culture and Hypoxia

The OS521 and OS156 cell lines were established from patient biopsies using protocols approved by the Institutional Review Board of the University of Florida College of Medicine. TICs, as defined by Oct4/GFP+ fluorescence, were isolated from xenograft tumors using Fluorescence Activated Cell Sorting and maintained in complete culture
medium: DMEM/F-12, 1% Penicillin/Streptomycin, and 10% Fetal Bovine Serum (Life Technologies) supplemented with 0.4 µg/mL G418 (Mediatech). Adenosine suitable for cell culture was purchased from Sigma-Aldrich (#A4036) and resuspended in DIH₂O at a stock concentration of 100 mM. Calcium was provided to cell cultures through calcium chloride (Fisher Scientific) resuspended in DIH₂O at a stock concentration of 1 M. Working concentrations are indicated in text.

Hypoxic conditions were achieved using a Modular Incubator Chamber (Billups-Rothenburg, Inc.) at 1% oxygen for 72 hours. Control cultures were incubated under normoxic conditions at ~21% oxygen for the same duration.

**Cell Viability and GFP Expression**

To determine cell viability and GFP expression, stress treated cells were trypsinized and resuspended in 0.05% Bovine Serum Albumin in PBS at 4 x 10⁶ cells/mL. The cells were then incubated with Propidium Iodide (PI) from 5 minutes and PI and GFP fluorescence was measured using the LSRII flow cytometer (BD Biosciences).

**Cell Cycle Analyses**

Post stress treatments, OS cells were re-suspended in DAPI Nucleic Acid Stain (Invitrogen) and incubated for 10 minutes at room temperature. UV fluorescence was determined using a LSRII flow cytometer (BD Biosciences) and the data were analyzed using FCS Express 5 software.

**Survival and Proliferation Assay**

OS cells were plated in 96 well plates (OS521 at 4000 cells/well and OS156 at 3000 cells/well). For survival assays, cells were incubated with various concentrations of adenosine or calcium as indicated in the figures, in complete culture media for three
days in a tissue culture incubator. Cells were then fixed in 1% glutaraldehyde, stained with 0.5% crystal violet, and optical density was read at 595 nm.

For proliferation assays, cells were incubated with 100 nM adenosine and 10 mM calcium in hypoxia for 10 days. Throughout the experiment, media was replenished every 4 days. At the indicated time points, cells were fixed in 1% glutaraldehyde and stored in PBS at 4°C. At the end of the experiment, cells were stained with 0.5% crystal violet and optical density was read at 595 nm.

**Luciferase Expression**

Transfections were carried out in 12 well plates. Plating densities were optimized for each cell line to generate ~70% confluence at the time of transfection. At 8 hours after plating, cells were transfected with 0.05 μg of pGL-Renilla and 1 μg of a pGL plasmid (where a 4 kb Oct4 promoter drives the expression of luciferase) using 4 μL Lipofectamine per well (Invitrogen). Transfection media was removed after 4 hours, and replaced with media supplemented with 300 nM adenosine, 10 mM calcium, or a combination thereof. The transfected cells were incubated under normoxic or hypoxic conditions for 72 hours and were subsequently trypsinized and pelleted. Cells were lysed at -80 for 24 hours and then a luciferase assay was performed using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer’s recommendations, with a few modifications. In short, the pellets were thawed and re-suspended in 50 μL PBS; 50 μL reagent Dual-Glo was added and luciferase read using a Berthold Detection System. 50 μL Stop&Glo was added to stop the reaction and Renilla was subsequently measured using the same aperture. Background reads (from untransfected cells) were subtracted from the readings and the relative luciferase activity was calculated.
**Interleukin 6 Secretion**

Cells were plated into 12 well plates and media was changed to stress conditions containing either 300 nM adenosine, 10 mM calcium, or a combination thereof, and incubated under normoxic or hypoxic conditions. After 72 hours, conditioned media was collected and assayed for concentration of Interleukin 6 (IL6) using the Human IL6 Quantikine ELISA Kit (R&D Systems). Cells were trypsinized and total cell lysates were prepared by incubating $10^6$ cells in 100 µl RIPA buffer followed by 1 hour of incubation on ice. Protein concentrations were then measured using the Pierce BCA Protein Assay Kit (Thermo-Scientific). The viable cell count was calculated through a standard curve of protein concentration and counted cells (Trypan Blue Exclusion). IL6 secretion was then normalized to viable cell count.

**Bone Morphogenetic Protein Stimulation**

Recombinant Human BMP2/7 Heterodimer (#3229-BM-010/CF) and BMP4/7 (#3727-BP-010/CF) were purchased from R&D Systems. The lyophilized proteins were resuspended and stored according to the manufacturer’s instructions. OS521 and OS156 cells were plated at a density of ~7,000 cells/cm², in complete culture medium containing 100 ng/mL of either BMP2/7 or 4/7. Media was replaced with fresh BMP-supplemented media at 24 hours, and at 48 hours cells were trypsinized and collected for experiments.

**Quantitative Reverse Transcriptase PCR**

After BMP4/7 pretreatment for 48 hours and exposure to stress conditions for 72 hours, cells were collected and total RNA was isolated using phenol:chloroform extraction with Trizol (Life Technologies) according to the manufacturer’s protocol. The recovered RNA was quantified by spectrophotometry at 260/280 nm. cDNA was
generated using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). RT-qPCR reactions were run using Perfecta SYBR Green FastMix according to the manufacturer’s instructions (Quanta Biosciences) on an Eppendorf Mastercycler Realplex2 (Eppendorf). Target mRNA levels were normalized to GAPDH mRNA. Relative gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method.

**Statistical Analyses**

A two-tailed student t-test was used to determine statistical significance unless otherwise specified. Asterisks indicate level of significance found: *p≤0.05, **p≤0.01, and ***p≤0.001.

**Results**

To determine whether a lack of oxygen would enhance the response of OS TICs to exogenous BMP stimulation, cultures of OS521 and OS156 were incubated with BMPs under conditions used previously and split into two groups. The first group was incubated in hypoxic conditions (~1% O$_2$) for three days, while the second group was maintained in normoxia as a control. This strategy was designed to emulate the delivery of BMP pretreated cells into NSG mice. Afterwards, cells were analyzed for changes in cell death using PI staining, GFP expression, and cell cycle progression.

No meaningful changes in cell death were observed in OS521 TICs responding to BMP pretreatment and exposure to hypoxia (Figure 3-1A). Conversely, prestimulation of OS156 TICs with BMP2/7 and subsequent hypoxia increased cell death to 23%. This suggests that the added “stress” from BMP2/7 stimulation before hypoxia causes toxicity. Additionally, as activation of the Oct4/GFP reporter has been associated with tumorigenic potential, we measured GFP expression by flow cytometry in the surviving
fraction of parallel cultures. No differences were observed in the percentage of GFP+ cells in either OS cell line (cells remained at ~90% GFP+) (Figure 3-1B).

Since differentiation is associated with reduced proliferation [69], we looked for BMP induced changes in cell cycle progression after hypoxia. BMP4/7 stimulated OS521 TICs under hypoxia showed an increase in the amount of cells in G1 phase (12%), with a corresponding reduction in the amount of cells in S phase (7%). A similar increase was noted after only 48 hours of BMP4/7 stimulation, where there was a 20% increase in the amount of OS521 cells in G1 (Figure 2-5). This suggests that hypoxia maintains some of the impact that BMP4/7 has on cell cycle in OS521, which could lead to the reduction in tumors formed in our mouse model. Alternatively, exposure of OS156 TICs to hypoxia, regardless of BMP pretreatment or not, resulted in an overall increase in the amount of cells in G1 phase, suggesting that hypoxia regulates cell division in this cell line.

In conclusion, this data demonstrates that both cell lines showed differential sensitivities to hypoxia. However, our in vitro findings did not demonstrate significant effects to explain our results in vivo. We speculate that exposure to a hypoxic environment alone is not sufficient to recapitulate the complex microenvironment of tumors in vivo, and that additional stress-induced signaling pathways are likely required to elicit modulation of BMP effects of OS TICs in vitro.

Modifying our previous hypothesis, that hypoxic stress is required to reveal the anti-tumorigenic effects of BMP4/7 in vivo, we now propose that the addition of adenosine and calcium in combination with hypoxia is required to replicate the effects in vivo of pretreatment with BMPs in cell culture. To begin, we determined the
concentrations at which OS TICs are sensitive to adenosine and calcium individually. Adenosine imparted an IC50 of 1.73 mM and 1.22 mM for OS521 and OS156, respectively (Figure 3-2). In contrast, calcium had different effects on each cell line: OS521 were more sensitive to calcium with a lower IC50, as compared to OS156 (32.11 mM and 53.63 mM, respectively). The differential sensitivities to calcium may in part explain the different inherent biological behaviors of both cell lines, which include their capacity for proliferation, clonability, migration and invasion, ability to form tumors in vivo, and also their expression profiles.

For further experiments, the selected concentrations of adenosine and calcium in combination with hypoxia allowed for cells to remain viable for study. Moreover, we wanted a concentration for both stress inducers that was still physiologically relevant to the TME [85; 88; 90; 91; 103; 104]. Therefore, 300 nM of adenosine and 10 mM of calcium was chosen as our working concentrations for the remaining experiments.

To analyze our ability to simulate the OS TME by inducing stressful conditions, we characterized the effects of adenosine and calcium in combination with hypoxia on OS TICs, starting with proliferation. OS521 exposed to adenosine alone were able to overcome this stress and increase in cell number to the same extent as untreated cells (Figure 3-3A). When OS521 was exposed to either calcium or hypoxia or combinations including these components, there is a much more reduced ability to proliferate (Figure 3-3B). However, OS521 under these conditions were still able to increase or maintain cell numbers under these conditions, ranging between 2-30% from Day 0.

OS156 TICs exposed to adenosine or calcium were able to maintain their proliferative phenotype but at a slightly reduced level, reaching a 136% and 86% cell
number increase at Day 10, respectively, compared to untreated cells at 312% (Figure 3-3C). However, as soon as hypoxia was added to the combination, OS156 cell density decreased (Figure 3-3D). This data highlights the level of intertumoral heterogeneity seen in this type of cancer, where there is variability amongst OS tumors even in the proliferative responses to different stress conditions.

To study the effects of these stress conditions on survival, we analyzed OS TIC viability after three days of incubation with adenosine and calcium under hypoxia (Figure 3-4A). Exposure of OS521 to calcium alone or any combination involving calcium results in a significant reduction in their cell viability by 43-48%, consistent with the data seen in Figure 3-2. Interestingly, OS521 in hypoxia maintain their cell viability, until calcium is added into the combination. Alternatively, OS156 TICs exposed to adenosine, calcium, or hypoxia results in a decrease in cell viability in an additive manner, reduced to only 3% of cells surviving when exposed to all three stress components together. This trend in OS156 correlates with the effects seen in their proliferative ability in Figure 3-3C and D.

To determine how these stress inducers affect our tumorigenic reporter, we transfected the OS TICs with a plasmid containing the Oct4 promoter regulating expression for luciferase, in order to get a more immediate and sensitive readout. OS521 exposed to calcium, and calcium with adenosine, and all three stresses together, reduce expression of luciferase (Figure 3-4B). Conversely, OS156 luciferase expression is reduced with any stress combinations involving hypoxia, consistent with the level of toxicity seen Figure 3-4A.
Using Interleukin 6 (IL6) as a marker for reprogramming (Levings, et al., in preparation) [105], we analyzed the ability of exogenous adenosine and calcium in combination with hypoxia in vitro to induce OS TICs to secrete IL6. OS521 increased IL6 secretion in response to calcium exposure, both calcium and adenosine, and incubation with calcium, adenosine and hypoxia altogether (Figure 3-4C), in agreement with the pattern of cell viability loss seen in Figure 3-4A. Alternatively, adenosine and hypoxia, and adenosine, calcium, and hypoxia altogether increased IL6 secretion in OS156, where this also matches the amount of toxicity seen in Figure 3-4A.

To determine the influence these stress conditions have on cellular division, we investigated the effects of adenosine, calcium, and hypoxia on OS TIC cell cycle progression. OS521 exposed to calcium and hypoxia, or adenosine, calcium, and hypoxia altogether result in an increase in the amount of cells in G1 Phase (to 66% and 67%, respectively, compared to 49% of untreated cells), with a subsequent reduction in the amount of cells allowed into S Phase (Figure 3-4D). Interestingly, the G1:S:G2 phase ratio of OS521 TICs under adenosine, calcium, and hypoxia (67%:17%:16%) in Figure 3-4D is very consistent with the G1:S:G2 phase ratio of non-tumorigenic OS521 GFP- cells analyzed right after tumor harvest (66%:17%:17%) (Levings, et al., in preparation). Alternatively, OS156 TICs exposed to any combination involving hypoxia respond with an increase in the amount of cells in G1 phase (to 51-55% of cells), correlating with the hypoxia regulation of cell division seen initially in Figure 3-1C.

Using exogenous adenosine and calcium in combination with hypoxia to simulate the stresses of the TME in cell culture, we next wanted to study the mechanism of BMP4/7 pretreatment, starting with cell cycle analysis. OS521 TICs were stimulated
with BMP4/7 for two days and subsequently exposed to stress components for three days, again designed to emulate the delivery of BMP treated cells into NSG mice. OS521 TICs pretreated with BMP4/7 and exposed to all three stresses showed the most cells in G1 Phase (71% of cells) with the least amount of cells in S (16%), compared to untreated cells (59% in G1 and 23% in S). However, no additive effects with BMP4/7 pretreatment were seen when compared unstimulated cells exposed to all the stresses combined (Figure 3-5).

To analyze how BMP4/7 pretreatment affects adaptation to our stressful environment, we looked for changes in IL6 secretion. OS521 TICs stimulated with BMP4/7 and exposed to adenosine and calcium under hypoxia maintain an increased production of IL6, compared to unstressed cells (Figure 3-6A). This confirms the level of stress induced when exposed to these components, regardless of BMP pretreatment.

To determine if stress induced by BMP4/7 pretreatment and exposure to adenosine, calcium, and hypoxia results in any indication of osteogenic differentiation of OS521 TICs, we analyzed changes in gene expression of osteogenic master regulator Runt-Related Transcription Factor 2 (Runx2) and osteoid matrix protein Collagen Type 1. BMP4/7 stimulated OS521 incubated in hypoxia has the highest fold change in Runx2 expression, but increases were also seen with cells exposed to adenosine, calcium, and hypoxia altogether, and with BMP4/7 pretreatment (Figure 3-6B). However, no changes were observed in Collagen Type 1 (Figure 3-6C), indicating that the process of osteogenic differentiation is initiated with Runx2 expression but not far enough along to produce osteoid matrix with Collagen Type 1.
Discussion

To begin, we used hypoxia to simulate inoculation of OS TICs into animals and elicit a better understanding of the workings of BMP4/7 pretreatment, showing that BMP4/7 stimulated OS521 in hypoxia maintain the increase in the amount of cells held in G1 Phase (Figure 2-5 and 3-1). Alternatively, OS156 TICs show a cell cycle regulated response to hypoxia, regardless of BMP stimulation. Because more changes were anticipated, we believe that more stress is needed, in addition to hypoxia, to replicate the stress of the OS TME and implicate the effects of BMP4/7 stimulation on tumor formation in vivo.

We next attempted to replicate the OS TME by incubating TICs with exogenous adenosine and calcium in hypoxic conditions. Similar characteristics between cells exposed to these stressful conditions in vitro and the transcriptional profile of GFP- cells directly from a tumor would indicate successful replication of the TME and a reasonable method of study for future experiments [36] (Levings, et al., in preparation). The response of cultured OS TICs to adenosine, calcium, and hypoxia is consistent with the loss of tumorigenicity in vivo with respect to inhibition of proliferation, reduced activation of Oct4 promoter (as shown by a reduction in luciferase expression), increased IL6 secretion, and altered cell cycle progression (increased cells in G1 Phase). Differences were observed between the response of OS521 and OS156 TICs to these stress conditions, and this is consistent with the level of intertumoral heterogeneity seen in this form of cancer.

The data shows that indeed adenosine, calcium, and hypoxia can simulate the TME and result in stress-induced differentiation of OS521 TICs with an increase in Runx2 expression, consistent with what is seen in the GFP- transcriptional profile.
However, BMP4/7 pretreatment is not enough to additively increase the amount of cells held in a G1 arrest (Figure 3-5) and furthermore, is not enough to increase osteogenic differentiation with more Runx2 expression (Figure 3-6). BMP4/7 stimulation might be initiating its effect on inhibiting tumor formation through its regulation of the cell cycle, however the induction of differentiation is due to the stresses of the TME in OS521 TICs.
A

OS521

Percent of Dead Cells

Untreated | BMP2/7 | BMP4/7

Normoxia | Hypoxia

OS156

Percent of GFP+ Cells

Untreated | BMP2/7 | BMP4/7

Normoxia | Hypoxia

B

Percent of Cells in Each Phase

G1 | S | G2

Untreated | BMP2/7 | BMP4/7

Normoxia | Hypoxia

C
Figure 3-1. Hypoxia after BMP stimulation elicits differential responses in OS TICs in vitro. (A) Cell death was measured by flow cytometry following incubation with PI. Data is presented as percent of dead cells. (B) The Oct4p/GFP plasmid was used as an indicator for tumorigenicity; flow cytometry was used to quantitate how many cells were GFP+. (C) Cell cycle progression was determined following incubation with DAPI; data was analyzed and is presented as the percent of cells in each phase.
Figure 3-2. OS TICs are sensitive to increases of exogenous adenosine and calcium \textit{in vitro}. OS TICs were exposed to increasing concentrations of either adenosine or calcium for three days. Data is presented as average percent survival with standard error, with cells exposed to each concentration normalized to untreated cell survival (n=3). Dotted line indicates half maximal inhibitory concentration (IC50). IC50s for OS521 TICs and OS156 TICs for each stress inducer presented above the graphs.
Figure 3-3. Adenosine, calcium, and hypoxia inhibit OS TIC proliferation \textit{in vitro}. Cells were exposed to 300 nM adenosine, 10 mM calcium, hypoxia at 1% oxygen, or combinations thereof over the course of 10 days. Time points were collected every 2 days. Data is presented as average cell number with standard error, with each group normalized to cell number at Day 0 ($n=3$). Complete data is presented for OS521 TICs in (A) and for OS156 TICs in (C). Data is presented with broken axis and altered scaling for OS521 in (B) and for OS156 in (D).
Figure 3-4. Exposure of OS TICs to adenosine, calcium, and hypoxia *in vitro* results in a survival phenotype. After exposure of OS TICs to stress treatments for three days, cell viability was measured by flow cytometry using incubation with PI; data is presented as average percent of viable cells with standard deviation (n=2) (A). (B) Cells were transfected with a plasmid containing the Oct4 promoter regulating expression for luciferase, and then exposed to stress conditions for three days. Relative luciferase was measured and quantified as the ratio between luciferase and constitutively expressed Renilla. Data is presented from one representative experiment as the average relative luciferase with standard deviation (n=2). Experiment was independently replicated. (C) Post stress exposure, conditioned media was collected and IL6 secretion was determined via ELISA. IL6 secretion was then normalized to protein concentration as a measurement of viable cells. Data is presented from a representative experiment of IL6 secretion normalized to viable cell count (n=3); experiment was independently replicated. (D) Cell cycle was investigated following incubation with DAPI; data is presented as the average percent of cells progressing through each phase with standard deviation (n=3).
Figure 3-5. BMP4/7 pretreatment and adenosine, calcium, and hypoxia incubation increased OS521 TIC G1 Phase halt in vitro. After BMP4/7 pretreatment and then exposure to stress conditions for three days, cell cycle was investigated; data is presented from one representative experiment and shown as the percent of cells progressing through each phase; experiment was independently replicated (n=2).
Figure 3-6. OS521 TICS treated with BMP4/7 and exposed to adenosine, calcium, and hypoxia maintain IL6 production while initiating osteogenic differentiation in vitro. (A) After BMP4/7 pretreatment and then exposure to different stresses for three days, conditioned media was collected and IL6 secretion was determined via ELISA. IL6 secretion was then normalized to protein concentration as a measurement of viable cells. Data is presented from a representative experiment of IL6 secretion normalized to viable cell count; experiment was independently replicated (n=2). (B) and (C) Relative fold change, determined by RT-qPCR of osteogenic marker genes Runx2 and Collagen Type 1 normalized to GAPDH, following stimulation with BMP4/7 for 48 hours and exposure to stress conditions for three days.
CHAPTER 4
CONCLUSIONS

Osteosarcoma as a Disease

Osteosarcoma (OS) presents itself as a challenging form of cancer, both in the clinic and in the laboratory. Important questions still require answers if the field of OS research is to progress and aid in the development of new and targeted therapies, and most importantly improve its dismal survival rate. These include OS etiology, the molecular and cellular biology of the disease, prognostic factors describing cancer progression, improved in vitro assays and in vivo translational models. Wider recognition of the level of inter- and intratumoral heterogeneity found in OS has helped to advance research and increased emphasis is placed on patient derived biopsies providing novel and improved findings. With investigations of primary OS tumors studied, rather than the handful of commercially available lines, a new level of understanding can be appreciated and exploited for more effective prognoses and treatments.

Determining the etiology of OS and furthermore studying the molecular and cellular nature of this disease has been difficult because of the high degree of intertumoral heterogeneity. OS can arise in different anatomical positions, with a variety of histological grades, subtypes, and differentiation statuses [1; 2; 8]. Furthermore, as a cancer primarily affecting children, OS arises at an age during rapid bone growth when many hormonal and developmental processes are taking place [1; 2; 3]. However, it is only postulated that there is an association between OS onset and the processes involved at the epiphyseal regions of long bones [3]. The biology during skeletal growth leading up to OS genesis need to be determined. In defining the etiology of OS, normal
counterparts could be used to study the disease at the genetic, transcriptional, and cellular level.

The presence of metastases remains the primary index of prognostic outcome [3], not age of onset, primary tumor location or size, histological subtype, presence of a certain receptor or protein, etc. that could otherwise indicate disease progression. This highlights the fundamental lack of knowledge behind OS. Moreover, despite the various phenotypic forms and appearances of OS, the clinical behavior of high grade OS is remarkably homogenous. With a better understanding of OS at the molecular, cellular, and tumor level, there could be better biomarkers for cancer progression and its response to treatment.

Like many cancers, in OS a lack of histologic evidence of differentiation is associated with a worse clinical outcome [25]. Differentiation status is evaluated as the volume and distribution of osteoid production amongst malignant mesenchymal cells, together with amount of cellular atypia. However, although differentiation status is implicated in patient outcome, there is little discussion of the rationale behind this and the mechanistic determinants of differentiation status. Furthermore, there is speculation that OS reflects a disconnect between malignancy and differentiation [26; 27; 28; 29]. Yet, there is no consensus on this in the field of OS research, and how to take this theory into account to improve patient survival. More research is needed in this area in order to get a better understanding of how to address differentiation status and thus formulate effective differentiation-based therapies.

The overall survival rate for OS has remained unchanged in the last 40 years [1]. There have been no significant upticks in survival rate with alternative chemotherapy
dosing schedules or drug combinations [12; 13]. In addition to the loss of limb and/or function attributed to surgery to remove the primary tumor, the chemotherapeutic side effects can be severe and long lasting [1]. Over the last four decades, there have been no new FDA approved drugs that have been successfully implemented into the standard of care for OS patients. This can largely be attributed to the high level of genetic instability in OS tumors [20], but ultimately responsibility lies with the lack of research progress.

Within the laboratory, studying the nature of OS disease presents many obstacles. Unfortunately, there are not many authentic pediatric OS cell lines to represent the nature of the bone tumor. Of the cell lines used most frequently, challenges include: plasticity of cell lines adapting to the 2D cell culture system, the lack of intratumoral heterogeneity in vitro, the poor growth of OS cell lines in mouse models in vivo, the lack of reproducibility of results in general and the failure of “successful” laboratory results to translate into human treatment. What is well-known now is that patient derived OS biopsies provide models that are most reflective of original disease, and most advantageous for study. The use of biopsy material needs to be adopted as the experimental standard.

**The Effect of BMP4/7 Stimulation of Osteosarcoma Tumor Initiating Cells**

The data presented here investigates the effect of Bone Morphogenetic Protein (BMP) heterodimers on the induction of differentiation of OS Tumor Initiating Cells (TICs) from primary biopsies. Through activation of the BMP Canonical Signaling Cascade, OS521 treated with BMP4/7 showed reduced tumorigenic activity in vivo, presumably through cell cycle inhibition, and could be a potential treatment for poorly
differentiated OS. However, the specific mechanism by which BMP4/7 activity inhibits OS TIC tumor formation had not emerged from our original assays *in vitro*.

For the last few decades, research has been aimed at teasing apart the functions of individual BMP isoforms on specific cell types. However, although certain themes of signaling are featured, BMPs require further study to get a complete understanding their signaling power and specificity, particularly with regard to cancer [45]. The literature frequently highlights the need for cellular context in understanding BMPs. The data gathered here emphasize context, with the variable consequences of BMP heterodimer stimulation in two different cell lines of dissimilar differentiation status. This encourages continued study for therapeutic potential of BMP4/7, and the benefits that could be gained from it. BMP4/7 stimulation in the more differentiated OS156 cell line appears to block invasiveness but nonetheless has minimal impact on tumorigenesis *in vivo*. As the metastatic process is critical in OS disease progression, BMP4/7 anti-invasive potential could limit metastatic spread.

Preclinical data suggests that treatment with BMPs or strategies to boost the activity of endogenous BMPs may be effective alone or with chemotherapy [33; 44; 106]. Future studies to determine how BMP4/7 stimulation might augment or synergize with the current chemotherapy regiment in OS521 and OS156 would be interesting. However, characterizing the level of osteogenic deregulation in OS tumors is critical for the development of future treatments, especially in the context of differentiation based therapies. Ideally, with the advent of genomic DNA and RNA sequencing, OS tumors will soon become better classified genetically with an improved understanding of tumor cell ecology and predictive indices of treatment response. In the interest of using
BMP4/7 as a potential treatment, the literature shows that cells cannot respond to BMP signals and induce osteoblastic differentiation without the transcriptional regulator Runt-Related Transcription Factor 2 (Runx2) [61]. The presence of Runx2 could be determined at the time of diagnosis in order to shape the subsequent chemotherapy/differentiation regiment.

BMP4/7 pretreatment shows anti-tumorigenic potential in vivo, but more is needed to confirm a differentiation-based mechanism in our assays in vitro as well as its efficacy in vivo when administering to growing tumors. Along these lines, our data demonstrate the disconnect between experiments in vitro and that which occurs in vivo. In vitro assays are used to characterize certain biochemical signals that are one aspect of the whole tumor phenotype. The challenges of translating to in vivo preclinical studies beg to question reliability of in vitro methods and what is lacking in the experimental setup. Various models, including patient derived xenograft transplants and 3-dimensional printing, have gained popularity in order to address these shortcomings.

**Simulating the Osteosarcoma Tumor Microenvironment with Adenosine, Calcium, and Hypoxia**

Hanahan and Weinberg explained that seven biological capabilities are acquired during the multistep development of human tumors, and these underlie the complexities of disease: 1.) sustaining proliferative signaling, 2.) evading growth suppressors, 3.) resisting cell death, 4.) enabling replicative immortality, 5.) inducing angiogenesis, 6.) activating invasion and metastasis, and 7.) evading the immune system [34]. However, there is no explanation of how these phenotypical changes specifically arise other than random mutation. In Darwinian dynamics, microenvironmental selection forces provide barriers that must be overcome for tumor proliferation and ultimately survival.
Understanding intrinsic mechanisms driving cellular response to the microenvironment can provide insight into a better understanding of OS biology.

Our differential gene expression analyses between the malignant, GFP+ cells and the benign, GFP- cells illustrate some of the workings of the tumor microenvironment (TME) found in individual OS tumors. The malignant and non-malignant cells together establish an ecology that promotes tumor growth and survival. Moderate stress drives the malignancy seen in the GFP+ cells. Alternatively, high stress inhibits malignancy driving the GFP+ cells to inactivate the reporter for the cells to survive and reprogram their genome into an adaptive differentiation response. Adenosine and calcium related pathways as well as hypoxia are major contributors to the stress signaling within the OS TME, and potentially to the differentiation and development of the less tumorigenic GFP- cells.

By incubating OS TICS in 300 nM adenosine, 10 mM calcium, and 1% oxygen, we attempted to simulate the stresses of the TME and compare how this matched with gene expression in the GFP- cells in vivo. The response of cultured OS TICs to adenosine, calcium, and hypoxia is closest in agreement with the emergence of the GFP- cells in vivo with respect to inhibition of proliferation, altered cell cycle progression, reduced activation of Oct4 promoter, and increased IL6 secretion. Furthermore, the differing response to the stress inducers correlates with the level of intertumoral heterogeneity seen in this type of cancer, where differential phenotypes, genotypes, and responses to drugs are expected.

Through our simulation of TME, we attempted to replicate the delivery of BMP4/7 pretreated cells into NSG mice. However, adenosine, calcium, and hypoxia does not
appear to be enough to mimic the stresses of the TME in OS521 TICs, through its minimal effect on cell cycle progression and induction of osteogenic gene markers. Thus, we provide more reasoning for the use of a 3D model to better replicate the TME and determine the effect of BMP4/7 pretreatment on OS TICS. Given the population of patients selectively targeted and the lack of progress made improving the efficacy of treatment in the last four decades, OS is a disease that continues to need attention. Treatment with BMP4/7 could provide scientific insight for future differentiation based therapies. [94]
LIST OF REFERENCES


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BIOGRAPHICAL SKETCH

Margaret White was born in Mesa, Arizona in 1990. She grew up in New Smyrna Beach, Florida and graduated from Spruce Creek High School summa cum laude in 2008. Margaret then attended the University of Miami studying general biology. From 2010 to 2012, she worked in the lab of Dr. Ian Hentall at The Miami Project to Cure Paralysis researching spinal cord injury. She earned her Bachelor of Science in 2012, with a major in biology and minors in chemistry and sociology.

Margaret then started her PhD in the Interdisciplinary Program in Biomedical Sciences (IDP) at the University of Florida (UF) College of Medicine in 2012. Working under the mentorship of Dr. Steven Ghivizzani and Dr. C. Parker Gibbs, Margaret investigated the effect of Bone Morphogenetic Protein stimulation in Osteosarcoma Tumor Initiating Cells. Margaret had the opportunity to present her research at several international conferences, such as: the 2014 Cold Spring Harbor Mechanisms and Models of Cancer, the 2015 World Pre-Clinical Congress, and the 2016 and 2017 Annual Meetings of the American Association for Cancer Research (AACR).

Outside of her dissertation research, Margaret has been an active member of the student community at UF. Her activities have included teaching high school, undergraduate, and graduate students, through the Student Science Training Program with the UF Center for Precollegiate Education and Training, the Science for Life Research Undergraduate Seminar with the Howard Hughes Medical Institute, and Core Course Discussion Groups with the first year IDP graduate students. In 2016, she served as Treasurer in the Organization for Graduate Student Advancement and Professional Development, which also included managing the UF Graduate Student
Research Day – a major annual event held on the UF campus. Upon completion of her Ph.D. program in August 2017, Margaret aspires to remain in the cancer research field.