CHARACTERIZATION OF P27 IN OSTEOSARCOMA

by

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ABSTRACT

Osteosarcoma is an aggressive bone tumor that primarily affects children and young adults. Patients are treated with high-dose chemotherapy, and surgery. However, when metastasis or recurrence occurs it becomes incurable. Osteosarcoma tumors are heterogeneous and pose challenges for selecting targets for new treatments. Biomarkers are needed to screen tumors, and help in the treatment of the disease to better predict patient outcomes. Based on previous studies, our lab focuses on the p27/kip1 (p27) protein as a potential biomarker for osteosarcoma.

p27 is a tumor suppressor that regulates the cell cycle. In cancer cells, p27 localizes to the cytoplasm and interacts with proteins that promote invasion and migration. Therefore, it can also function as an oncoprotein. Osteosarcoma cells use this oncogenic activity to promote proliferation and resistance to therapies. However, a more comprehensive understanding of the role of p27 in osteosarcoma is needed. I hypothesize that nuclear p27 acts as a tumor suppressor, while cytoplasmic p27 acts as an oncoprotein that decreases the sensitivity of osteosarcoma cells to drug treatment.

In this study, I examined how human osteosarcoma cells rely on p27 to evade drug treatments, and allow cells to proliferate in the presence of drugs. I evaluated the activity of the cyclin-dependent kinase 4/6 inhibitor (CDK4/6) palbociclib combined with gemcitabine on the human osteosarcoma cell lines, HOS and 143B. I found that cell viability was reduced in response to gemcitabine and palbociclib given as single agents. When these two drugs were combined, the effect of administering gemcitabine before palbociclib, further reduced cell viability and the cells were sensitive to lower concentrations of palbociclib.

Further studies showed the effect of drug treatment on osteosarcoma cells was dependent on p27 expression. Using immunoblot analysis, I showed a correlation between p27 protein expression and cell viability. Gemcitabine decreased p27 protein levels that correlated with decreased cell viability, while palbociclib increased levels of nuclear p27, resulting in cell cycle arrest and higher cell viability. When the two drugs were combined, and gemcitabine was administered before palbociclib, the efficacy of the drugs was significantly increased and p27 expression was further downregulated.

I also found that the effect of drug treatment resulted in increases in cleaved p27 that correlated with an enhancement of cell death. Reverse Transcriptase-qPCR analysis revealed mRNA levels of CDKN1B (encodes p27) correlated with p27 protein expression. Further, by mass spectrometry analysis of p27 immunoprecipitated complexes, I identified novel p27 interacting proteins that are key components of signal transduction pathways for osteosarcoma. In summary, these data provide insight into the critical role p27 plays in osteosarcoma cell survival and identify novel p27 interactions that can be further studied as predictive biomarkers for drug response.

Chapter 1

INTRODUCTION

1.1 Osteosarcoma

Osteosarcoma is the primary type of bone cancer affecting children (Geller et al., 2010). The prognosis for osteosarcoma is a 70-80% survival rate at 5 years, but this drastically reduces to 20-30% for about 15 to 20% of patients who have metastases at presentation (Kager et al., 2006). The cause of osteosarcoma is unknown, but it is thought to arise from improper differentiation of mesenchymal stem cells. Tumors are heterogeneous and contain numerous genomic aberrations. However, these occur with low frequencies, that make these aberrations difficult to target (Bosse et al., 2016). Osteosarcoma is thought to be a developmental disease because it primarily affects children at the metaphyseal plate of the long bones (Taran, and Malapatil, 2017) during growth spurts. Genetic diseases like retinoblastoma, and Li-Fraumeni syndrome increase the risk of osteosarcoma, but these do not provide a known driving genetic factor for the disease (Kansara, 2007). Standard treatment includes chemotherapy consisting of [Methotrexate, Adriamycin (doxorubicin), and Cisplatin (MAP)] and surgery; however, survival has plateaued in the last 30 years and metastasis and recurrence are incurable (Allison, et al., 2012).

1.1.1 Etiology of osteosarcoma

The etiology of osteosarcoma is currently unknown. Inconsistent genetic differences lead to difficulty in identifying effective therapeutic targets. However, new

techniques continue to improve knowledge of the genome that are commonly associated with osteosarcoma. Copy number gains at chromosomes 1p, 1q, 6p, 8q, and 17p as well as copy number losses at chromosomes 3q, 6q, 9, 10, 13, 17p, and 18q have been discovered, however specific tumor suppressor genes and oncogenes remain undetermined (Martin et al., 2010). Many sarcomas can be characterized by specific chromosome translocations, and complex genomic rearrangements allowing for molecular targets of these tumors, but this is not the case for osteosarcoma. Many techniques and studies have been done to look at the cytogenetics, genomics, and karyotyping of osteosarcoma as well as fluorescence *in situ* hybridization, quantitative PCR, and single-strand conformation polymorphism analysis to name a few, but the etiology remains unclear (Savage, and Mirabello, 2011).

Although no mutations occur in high enough frequency to drive the disease, some mutations are at a consistently higher incidence. For example some germline mutations caused by the inactivation of genes that are linked with an increased risk of osteosarcoma include Li-Fraumeni syndrome (*TP53)*, hereditary retinoblastoma (*RB1*), Rothmun-Thomson syndrome (*RECQL4)*, Bloom syndrome (*BLM*), and Werner syndrome (*WRN*) (Savage, and Mirabello, 2011). Li-Fraumeni is normally caused by a mutation that silences the *TP53* tumor suppressor gene. Humans have only one copy of this gene therefore when this gene is mutated it leads to a high risk of developing cancer (Wang, 2005). Approximately 70% of families meeting the criteria of Li-Fraumeni syndrome have germline *TP53* mutations. In a study *TP53* exons were sequenced in 765 osteosarcoma cases and found a high frequency of osteosarcoma cases (age <30 years) carrying a known LFS- or likely LFS-associated mutation (3.8%) or rare exonic variant (5.7%) with an overall frequency of 9.5%, compared to

none in case patients over the age of 30 (Mirabello, et al., 2015). Savage and Mirabello in 2011 found that *TP53* is associated with loss of heterozygosity (LOH) or deletion of the region 17p13.1 which is present in 29-42% of cases. The mutation of the region 181 (MCR 18q21-q23) is present with 10-39% of cases while the deletion of this region is associated with 31-64% of cases, however the presence of germline *TP53* mutations in sporadic osteosarcoma has been reported to be 3% to 7% (McIntyre et al., 1994). Another mutation involves the retinoblastoma (*RB1)* tumor suppressor gene. The mutation or loss of *RB1* causes an increased risk of retinoblastoma, an eye cancer that affects children. Complex and largely inconsistent genetic alterations are typical of conventional osteosarcoma. Savage, and Mirabello, 2011 also analyzed multiple studies to find that LOH or deletion of *RB1* has been detected in about 19-67% of cases, *RB1* mutations are present in about 25-35% of cases and about 50% of cases have either type of alteration and are associated with inactivation of *RB1* expression. Children with this gene mutation have an increased risk of developing osteosarcoma especially if they are treated with radiation (Calvert, et al., 2012).

Specific treatments may target gene mutations and amplifications for example cyclin dependent kinase 4/6 (CDK4/6) is amplified in 11% of osteosarcoma tumors which can be targeted by cell cycle checkpoint inhibitors such as CDK4/6 inhibitors (Sayles, et al., 2018). Osteosarcoma cells overexpress disialoganglioside (GD2) protein on the surface of the cells. Drugs that target GD2 are used to treat neuroblastoma, so for GD2 expressing osteosarcoma tumors, clinical testing of these treatments for osteosarcoma is warranted (Yang, and Sondel, 2010). Gene changes and differences in cellular components may be used to predict the migration ability,

response of drug effects, and even patient outcomes. Other components of the pRb pathway are also common genetic alterations in osteosarcoma. Loss of cyclin-dependent kinase inhibitors (*CDKN*) genes are also present. *CDKN* is a gene that encodes tumor suppressor proteins that inactivate CDKs. Genes *CDKN2A/p16/INK4A*, *p14/ARF*, and *CDKN2B/p15/INK4B* are located at chromosome 9p21 and have been associated with development of osteosarcoma (Mohseny, et al., 2009). This chromosome is deleted in 5-21% of osteosarcomas (López-Guerrero, et al., 2004).

Bones are formed by osteoblasts that build bones and osteoclasts that reabsorb the bone and (Chen et al., 2018). Osteosarcoma is thought to arise from mesenchymal bone forming cells that produce malignant osteoid that is normally present around the metaphyseal plate of long bones (Mohseny et al., 2009). Osteosarcoma tumor cells resemble mature osteoblasts, yet they are most likely held in the immature osteoblast stage rather than fully differentiating into osteocytes. The tumors are heterogeneous because they contain mixed populations of undifferentiated cancer cells that affect the formation and organization of normal bone tissue. This implies disruptions along the developmental process of osteoblastogenesis.

One of the main features of osteosarcoma is the ability to deregulate bone remodeling. Evidence supports a coupled relationship between the decline in proliferative activity and the subsequent induction of genes associated with extracellular matrix maturation and mineralization. The extracellular matrix is involved in the development of the mature osteoblast phenotype by controlling its proliferation and shutdown. In osteosarcoma cells the loss of growth regulation is associated with a disruption of this control (Lian, and Stein, 1992). Tumor cells invade bone tissue and affect bone growth, bone remodeling and the regeneration of injured

tissue. This deregulation induces the release of cytokines and growth factors trapped in the bone matrix, such as transforming growth factor-β (TGF-β), which leads to tumor progression (Lamora et al., 2016). Cancer cells can also produce soluble factors, such as cytokines including Interleukin-6 (IL-6), Interleukin-11 (IL-11), transforming growth factor-α (TNF-α), and Receptor activator of nuclear factor κB ligand (RANKL), that activate osteoclastogenesis, leading to bone degradation (Tanaka et al., 2014). Following bone resorption, growth factors trapped in the bone matrix, such as Insulin growth factor-1 (IGF-1) or TGF-β, are released in the bone microenvironment and stimulate tumor growth (Wittrant et al., 2004). Receptor activator of NF-κB (RANK) signaling was also found to promote the motility and anchorage-independent growth of osteosarcoma cells. Transgenic mouse models with alterations of RANKL have been reported to develop osteosarcoma (Adamopoulos et al., 2016). This could explain the heterogeneity of bone microarchitecture throughout the skeleton. This diverse heterogeneity renders osteoblasts difficult to uniformly characterize genetically for this disease which contributes to the challenges facing osteosarcoma research.

A regulator of osteoblast differentiation is Runt Related Transcription Factor 2 (runx2), that is part of the *runt* family of tumor suppressor genes. Runx2 is a transcription factor that provides instructions for making proteins involved in the development in bones, teeth, and cartilage. It is consistently disrupted in osteosarcoma cell lines and the ectopic expression of runx2 induces p27, inhibiting the activity of the S-phase cyclin complexes (Thomas, et al., 2004). This leads to the dephosphorylation of pRb and a G1 cell cycle arrest. The interaction between runx2 and the hypophosphorylated form of pRb leads to a cell cycle exit that promotes the increased

expression of the osteoblast phenotype. When p27 expression is lost this causes terminal cell cycle exit (G_0) in osteoblasts, which is consistent with the incompatibility of malignant transformation and permanent cell cycle exit. Loss of p27 correlates with dedifferentiation in human osteosarcoma that is consistent with the malignant transformation of tumors. The coupling of differentiation to cell cycle withdrawal is disrupted in osteosarcoma which is mediated through runx2 and p27 (Thomas, et al., 2004). A study by Dahlin et. al., in 1957 found that 81% of osteosarcoma tumors were undifferentiated or poorly differentiated, and in another study (Hopyan, et al., 1999) found that osteocalcin which is a late marker of osteogenic differentiation was not detected in greater than 75% of osteosarcomas. Thomas et al. (Thomas, et al., 2004) found that spontaneous and induced osteoblast differentiation were associated with increase p27 at the protein and mRNA levels. The expression of ectopic runx2 induced an Rb- and p27 dependent growth arrest which was mediated by the increased levels of p27.

Although osteosarcoma is most commonly found in the metaphysis of long bones it affects other bone types as well. Because the metaphysis is the primary area of bone cell growth during childhood bone elongation this region is likely more susceptible to osteosarcoma development. This differentiates osteosarcoma from other types of bone cancer (e.g. Ewing's sarcoma) because it is during development rather than genetic. Therefore, while there are genetic proponents to osteosarcoma these are not established as a driving factor for the disease like other cancers.

1.1.2 Stages and grades of osteosarcoma tumors

Osteosarcoma has two stages; localized or nonlocalized. About 80% of tumors are localized. These are classified as the primary tumor. Localized osteosarcoma is

further divided into subgroups known as resectable or non-resectable. The other stage is non-localized or metastatic osteosarcoma in which cancer cells have spread to other parts of the body. The primary site of metastasis is the lung with about 20% of patients presenting with metastasis upon diagnosis (Meyers et al., 2005), and it can also spread to other bones, the brain, or other organs (Saha, et al., 2013). Whether or not the tumor is localized or has metastasized is the main classification osteosarcoma. Another classification used is the Musculoskeletal Tumor Society (MSTS) Staging System. This system, also known as the Enneking system is based on the grade (G), extent (T), and metastasis (M) of the tumor. The grade of the tumor is a measure of how likely it is to grow and spread. This grading system is done by examining the tumor cells by microscopy to determine if it is low (G1) or high grade (G2). Low grade cells are phenotypically similar to normal cells, while high grade cells look more abnormal and show high proliferation. The extent of the primary tumor is classified as either intracompartmental (T1), or extracompartmental (T2). Intracompartmental means that it has remained within the bone and extracompartmental means that it has extended beyond the bone to nearby structures. Tumors that have not spread are classified as M0. Tumors that have spread are M1. These factors are used to give an overall stage using the Roman numerals I to III and stages I and II are further divided into A and B for the T classification (Gorlick, et al., 2016).

The site of the primary tumor is important. It is a prognostic tool for patients with localized osteosarcoma. When the tumor is localized to the extremity, the distal sites have more favorable outcomes than the proximal sites. Axial skeleton primary tumors are harder to completely resect with surgery and have the greatest risk of progression and death. Pelvic osteosarcomas make up 7% to 9% of tumors and have a

survival rate of 20% to 47% (Donati et al., 2004). Craniofacial osteosarcoma in the mandible and maxilla have a better prognosis than those in the head and neck (Jasnau et al., 2008). Extraskeletal osteosarcoma is rare and has a similar mortality rate as primary tumors of the bone (Goldstein-Jackson et al., 2005). Patients with localized osteosarcoma have much better outcomes than those with metastasis. Patients with *skip* metastasis (at least two discontinuous legions in the same bone) tend to have worse prognoses (Bacci et al., 2008). Metastasis across a joint might be a hematogenous spread and this might contribute to the lowered survival rate (Bacci et al., 2006).

1.1.3 Risk factors for osteosarcoma

The main risk factor in osteosarcoma is age. Currently, osteosarcoma is believed to be a developmental disease. While there are known genetic components, these are not used to define the disease. There is a bimodal distribution for risk for peak in adolescence and peak after 60 years (Mirabello, et al., 2009). Due to the increase in cellular division during growth, age is considered the main risk factor in osteosarcoma. This risk is highest for children at puberty and decreases around the age of 30 (Mirabello, et al., 2009). Risk increases again with older adults over the age of 60 probably due to other factors such as other bone diseases.

Other factors that influence the risk of osteosarcoma are height and gender. Children with osteosarcoma are usually tall for their age which suggests that the disease is related to rapid bone growth. Osteosarcoma is also more common in males. It is unclear why it occurs in males more; however, it could be due to an increased average height for males over females. Females also tend to develop osteosarcoma

earlier supporting the theory that it is due to bone development because females tend to reach their growth spurts earlier.

Cancer can develop from gene changes that are acquired during a person's lifetime. As cells proliferate mutations can occur spontaneously that are not always fixed and therefore cells accumulate mutations that can lead to cancer. Radiation therapy, used to treat another type of cancer can also cause secondary cancer because radiation can also damage DNA thereby causing mutations. Diseases such as Paget's disease and other types of bone cancer can lead to an increased risk of osteosarcoma for this reason. Normally gene changes occur in the cell due to the errors during mitosis and accumulate, leading to cancer. Besides radiation there currently are no known lifestyle-related or environmental causes of osteosarcoma like there are in other cancers. Lifestyle related risk factors like smoking, diet, body weight, and physical activity play an important role in many adult cancers, but these factors normally take years to have an influence in the risk of cancer and are not thought to play a role in osteosarcoma, especially in children (Lindsey, et al., 2016).

1.2 Treatment of Osteosarcoma

Osteosarcoma is treated with standard MAP chemotherapy before and after surgery (Sampson, et al., 2016). Drastic advances in treatment for osteosarcoma have been made in the past 50 years (Misaghi et al., 2018). Misaghi et al. state that the five-year survival rate in the 20th century was about 20%, however with the addition of adjuvant chemotherapy in the 1970s osteosarcoma survival rate increased to approximately 50%. By the 1990s the survival rate increased to approximately 65%. This rate however has plateaued, and the survival rate for patients with metastatic and

recurrence still remains low. Effective treatments need to be developed for patients with metastatic and recurrent disease.

1.2.1 Cytotoxic treatment

MAP chemotherapy is the first treatment given to a patient with osteosarcoma. For patients who relapse, these agents are ineffective. Other chemotherapeutic agents (gemcitabine, etoposide, ifosfamide, vincristine, cyclophosphamide, carboplatin) are used as salvage therapy, but the use of these is generally limited by high toxicities. Current research is being focused on other classes of drugs that show activity against osteosarcoma.

Cytotoxic agents induce DNA damage and inhibit DNA synthesis by activating the S-phase checkpoint which leads to apoptosis or cellular senescence (Luo, and Leverson, 2005). Chemotherapeutics have a multitude of mechanisms to cause DNA damage. For example, MAP therapy involves methotrexate which is an antimetabolite that blocks enzymes for DNA synthesis, doxorubicin intercalates DNA, and disrupts topoisomerase-II (Nitiss, 2009), and cisplatin is a platinum-based agent which forms DNA protein crosslinks as well as DNA intrastrand and interstrand crosslinks that restrict DNA repair (Noll, et al., 2006). Microtubule inhibitors such as vinca alkaloids and taxanes destabilize and stabilize microtubules respectively, which leads to the disruption of the mitotic spindle causing mitotic arrest (Mills et al., 2018). Although chemotherapy is highly toxic to the cell cycle, cancer cells may adapt or use DNA repair pathways and faulty checkpoints to escape damage. Cancer cells may reverse or avoid damage all together and may even evolve to a more aggressive and malignant phenotype making treatment less effective (Mills et al., 2018).

Necrosis is the only positive biomarker for osteosarcoma at this time. Patients with at least 90% of tumor necrosis after chemotherapy have a better prognosis (Bielack et al., 2002). Patients with less than 90% of tumor necrosis have a higher rate of recurrence within the first 2 years as well (Kim et al., 2007). The Pediatric Preclinical Testing Consortium (PPTC), originally known as the Pediatric Preclinical Testing Program (PPTP) has tested more than 50 drugs in their preclinical models of osteosarcoma using patient derived xenograft tumors (including Rapamycin, Sunitinib, Sorafenib). This group evaluates many agents that have been tested in clinical trials but many phase I and II trials have yet to advance to phase III trials. A review by Sampson et al., in 2013 summarizes the results of preclinical testing of agents in OS models conducted by the PPTP from the past 6 years before the review. Development of new therapies involves repurposing old agents, developing synthetic analogues of agents that are less toxic, as well as making orally administered agents. Although many new drugs have shown efficacy in some patients, combination studies with chemotherapy are warranted for better efficacy of single agent treatments. Phase I clinical trials determine if a new drug, treatment, or combination of treatments is safe for patients (toxicities). Phase II clinical trials determine drug safety and how well it works (i.e. targeted inhibition). The Children's Oncology Group (COG) and other independent investigators recognize the limitations (due to small patient sizes) of investigations of cytotoxic agents in varied schedule and dosages to treat tumors known for chemotherapy resistance.

High doses of radiation are needed to have an effect on osteosarcoma cells. This limits the use of radiation due to the harsh side effects. Newer radiation techniques such as intensity-modulated radiation therapy (IMRT) are promising since

these are designed to better target the tumor with an adjustable beam (Liao, et al., 2016). Another form of radiation is stereotactic radiosurgery (SRS) which gives a large amount of radiation to a small area with a tumor usually for only one session (Yu, et al., 2017). Conformal proton beam therapy uses the positive parts of atoms, but may be limiting due to availability and cost (Ciernik, et al., 2011). Carbon ions are heavier than protons and can do more damage to cancer cells and using them is relatively new so it is only available in a few places around the world. Radium-223 (Xofigo) is currently being used to treat osteosarcoma that has spread to other bones.

New imaging techniques allow a better understanding of the growth and spread of osteosarcoma allowing better surgical techniques to define how extensive the tumor is and how much it has spread through the body. This helps with planning surgeries to remove the cancer while sparring much more of the normal tissue than physicians were previously able to. As Misaghi et al. stated it was common for the limb to be amputated before the mid 1970s as treatment (Misaghi et al., 2018). Still, these techniques are limited and are ineffective when the tumor has already metastasized. Newer prosthetics have been able to help both with internal and external replacements of areas that need to be removed (Misaghi, et al., 2018).

1.2.2 Cytostatic treatment

Cytostatics are molecularly targeted agents that act on highly specific targets that are differentially expressed or activated in cancer cells. Targeted therapy uses drugs to attack specific cancer cells without affecting normal cells. There are many different types of targeted therapies currently in clinical trials such as kinase inhibitors which block kinases that couple extracellular signals with intracellular signaling pathways, which contribute to cancer cell survival An example of a drug that uses this

mechanism is sorafenib (Nexavar) which inhibits the tumor's ability to make new blood vessels and is used to treat recurrent osteosarcoma. Mammalian target of rapamycin (mTOR) inhibitors such as everolimus (Afinitor) blocks the mTOR signaling pathway. Blocking mTOR may prevent cancer cells from growing as well as angiogenesis, for the formation of new blood vessels that are needed for the tumors to grow.

Receptor tyrosine kinases (RTKs) are proteins that regulate cell proliferation, growth, and survival. Preclinical studies suggest that agents targeting multi-RTKs may be used for osteosarcoma tumors. RTKs like CD340 (cluster of differentiation 340 (ErbB2), IGF-1R, platlet derived growth factor receptor (PDGFR), vascular endothelial growth factor receptor (VEGFR), and downstream signal-transduction pathways can be potential targets (Pikman et al., 2012). Imatinib is a tyrosine kinase inhibitor that had success in some solid tumors, however it has not been very successful in osteosarcoma (Bond et al., 2008). VEGF inhibitors also have efficacy in a sub population. In a study by Yang et al., in 2011 VEGFA (6p21.1) was amplified in 25% of the osteosarcoma specimens, and the product of this protein promotes angiogenesis and blood vessel permeability in cancer (Harper, and Bates, 2008).

Targeting the immune system is immunotherapy. Immunotherapy drugs can be used with monoclonal antibodies made from a single type of immune system cell. These antibodies can bind to antigens on cancer cells or normal substances that may help the cancer cells grow. The antibodies bind and can prevent growth, spreading, and induce death. Monoclonal antibodies are given by infusion and can be used alone or to carry other drugs, toxins, or radioactive material directly to cancer cells.

Denosumab and dinutuximab (Unituxin) are monoclonal antibodies currently being studied for treatment of recurrent osteosarcoma. Dinutuximab attaches to GD2, a protein that is important for cancer cell growth (Sait, and Modak, 2017). Immune checkpoint inhibitors can be used to help the body's immune system recognize and attack cancer cells. These drugs have already been studied and shown to be helpful against many types of cancers and they are now being studied for osteosarcoma. Muramyl tripeptide (MTP or mifamurtide) is an experimental immune-modulating drug that is currently being studied that has been shown to help some patients when added to chemotherapy (Biteau, et al., 2016).

Targeting genes and miRNAs are Gene Therapy. The nuclear factor 1B (*NFIB)* gene affects osteosarcoma cell migration and proliferation and is associated with metastasis (Mirabello, et al., 2015). MicroRNAs have been used to indicate prognosis. Some microRNAs such as miR-214 are upregulated in osteosarcoma and are independently prognostic for progression-free survival and overall survival. A locus at 14q32 is associated with miR-382, miR-134, and miR-544, and these microRNAs all have an inverse correlation with how aggressive a tumor becomes and miRNA expression (Sarver, et al., 2013).

Although targeted therapies have been unsuccessful alone, the combination of selective inhibition with DNA damaging chemotherapy can be synergistic and enhance cell death. DNA lesions that promote nucleotide excision and stalled replication forks activate Ataxia telangiectasia mutated (ATM), Ataxia telangiectasia mutated (ATR), and checkpoint kinase 1 and 2 (CHK1 and CHK2), to halt cell cycle progression at G1 preventing cells from replicating DNA damage (Manic, et al.,

2015). Targeting these checkpoint kinases will cause cells to enter mitosis with DNA damage which can lead to mitotic catastrophe and cell death (Mills, et al., 2018).

Palbociclib is a CDK4/6 inhibitor in which the Rb status is critical for response (Cadoo et al., 2014). Loss of functioning Rb may facilitate the independence from CDK4/6 which would allow CDK2 to control proliferation allowing the cells to divide even in the presence of palbociclib. Elongation 2 factor (E2F) also has a significant role in modulating cellular response to palbociclib. Dean at al., in 2010 examined a downstream effect of CDK4/6 inhibition is the modulation of E2F targets. This deregulation can overcome the efficacy of palbociclib. The loss of Rb with the increase of E2F-target genes cyclins A and E provides a mechanism for the cells to gain independence from CDK4/6 (Dean et al., 2010).

Radiation-induced cytotoxicity is dependent on the cell cycle. The early G1 and late S-phases are resistant to radiation compared to the G1/S and G2/M transition phases (Johnson et al., 2010). This study demonstrated that cell lines dependent on CDK4/6 had a lengthened G1-phase when CDK4/6 are inhibited leading to an increase in radio-resistance, while for tumor models where CDK4/6 were not the main drivers of proliferation there was little effect. They also showed that CDK4/6 inhibitors could aid in the body's ability to tolerate radiation because the hematopoietic stem/progenitor cells are reversibly quiescent. Therefore, patients that lack the Rb protein could withstand higher doses of cytotoxic agents without sacrificing efficacy with the addition of CDK4/6 inhibitors. In 2010 Dean et al. tested this theory with palbociclib and carboplatin in mouse models with breast cancer. Single agent palbociclib inhibited tumor growth in Rb+ tumors, but not Rb- tumors. Combination therapy had myeloprotective effects without compromising efficacy in the mice that

were Rb+ in comparison to carboplatin alone. Rb- mice had no deleterious effect on tumor growth with the combination, but there was significant platelet protection. Taken together this suggests that palbociclib can act as an anti-proliferative agent in Rb-dependent tumors and as a myeloprotective agent that can enhance delivery of cytotoxic therapy in Rb-independent tumors.

Many chemotherapy agents rely on the proliferation of cells such as mercaptopurine and methotrexate so combining these drugs with a CDK4/6 inhibitor could be antagonistic. In 2012 Pikman et al., found palbociclib to be antagonistic with simultaneous and sequential administration of mercaptopurine, methotrexate, doxorubicin, or L-asparaginase in T-ALL cell lines. In 2012 McClendon et al., reported antagonism in breast cancer, however some drug combinations may enhance cell death such as in *MYCN*-amplified, *TP53* wildtype neuroblastoma (Gogolin et al., 2013). There are other examples of chemotherapy agents that have not been antagonistic with CDK4/6 inhibitors such as cytarabine in acute myeloid leukemia (AML) (Yang C, et al., 2011) and gemcitabine in a Calu-6 lung cancer xenograft model (Gelbert et al., 2014).

1.3 Cell Cycle

Alberts et al. state that the eukaryotic cell cycle is divided into four phases: gap 1(G1), DNA synthesis (S), gap 2 (G2), and mitosis (M). G1, S, and G2 are known as interphase (Alberts et al., 2002). A typical human cell will proliferate in culture in 24 hours where interphase normally occupies 23 hours, while the M-phase lasts for about 1 hour. The two gap phases monitor the internal and external environment to make sure conditions are suitable for DNA synthesis and division. G1 can also be delayed and the cells are able to exit the cell cycle in what is known as G zero (G_0) and enter

quiescence. Most cells remain in G_0 for extended periods of time, and it can even be years before proliferating again. This is where cancer treatment can be used, because if most normal cells are not dividing, then drugs that target checkpoint kinases at specific phases of the cell cycle in which the cells are proliferating will spare most of the normal cells in the body. Quiescence occurs due to lack of nutrition and growth factors whereas terminal cell cycle exit called senescence, takes place due to aging and high DNA damage. Cancer cells with DNA damage can also undergo senescence.

Small molecule inhibitors targeting cell cycle checkpoint kinases have been developed. Combining targeted therapies with chemotherapy allows for lesser side effects while maximizing efficacy. The cell cycle is able to be targeted because cancer proliferates even in the absence of growth factors, while most normal cells exit the cell cycle until there are signals for division. Various mitogenic signals cause human cells to enter the cell at G1. The Ras/Raf/MAPK pathway is one of these mechanisms that increases cyclin D expression. (Meloche, and Pouyssegur., 2007). Cyclin-dependent kinases (CDKs) regulate progression through the cell cycle, and multiple complexes can be formed. Osteosarcoma occurs mainly during bone growth spurts therefore cell cycle checkpoint kinases are being studied as possible targets.

1.3.1 Cell cycle defects in osteosarcoma

Alterations in p53 are invariable as well as alterations in the Rb pathway. Li-Fraumeni syndrome with germline p53 abnormalities and patients with hereditary retinoblastoma with germline Rb pathway alterations are predisposed to osteosarcoma which implies that these pathways are central to osteosarcomas pathogenesis. The *TP53* gene has been mapped to chromosome 17. p53 protein binds DNA and stimulates the production of p21 which interacts with a cell division-stimulating

protein (CDK2). When p21 is complexed with cdk2 the cell cannot progress to the next stage of cell division. Mutant p53 can no longer bind DNA in an effective way, therefore p21 protein is not able to stop cell division allowing cells to divide uncontrollably, and form tumors. One of the ways p53 expression is disrupted is by inactivation. This can happen at the posttranslational level and can also occur through regulation by the tumorigenic proteins. MDM2 is an oncoprotein that can inhibit p53 by promoting its degradation as well as the downregulation of its transcription. Amplification of MDM2 on chromosome 12q15 is infrequent in primary osteosarcoma with about 6.6% of tumors having this amplification, however amplification of MDM2 is more frequent in metastasis and recurrence with about 34% (Lonardo et al. in 1997). Because of the importance and involvement of so many cancers p53 is defined as guardian of genome.

pRb prevents cell cycle progression by binding to E2F transcription factor and inhibiting cell cycle progression from G1 to S until it is phosphorylated by the CDK4/cyclin D complex (Miller et al., 1996). *E2F* (6p22.3) is gained or amplified in 60% of osteosarcomas and encodes for the E2F3 transcription factor (Martin, et al., 2012). E2F is regulated by Rb, cyclins, CDKs, and CDKNsto stabilize the genome (Manning and Dyson, 2011).

1.3.2 p27 as a tumor suppressor and an oncogene

p27/kip1(p27) is a cell cycle inhibitor that accumulates during the G1 checkpoint of normal cells. This accumulation signals to the cell to halt so that the cell can be checked for damage. During this time the damage will be repaired or if the damage is too extensive other signals will be sent so that the cell can undergo cell death. CDKs are frequently upregulated in cancer. Inhibitors of CDKs like p27 induce

apoptosis of tumor cells. This defines the normal function of p27 as a tumor suppressor. p27 is also involved in non-CDK interactions in cancer and the protein is overexpressed. CDKs phosphorylate key regulatory substrates like the Rb protein. Moderate DNA damage activates p53, which is a transcription factor that stimulates expression of p21.This cyclin-kinase inhibitor binds and inhibits all CDK-cyclin complexes, causing arrest in G1 and G2. In response to extensive DNA damage, p53 activates genes that induce apoptosis.

Protein kinase B (PKB)/Akt contributes to resistance of antiproliferative signals in breast cancer. (Liang, et al., 2012) This study showed that this occurs by impairing the nuclear import and action of p27. Cells transfected to express Akt caused cytoplasmic p27 accumulation and resisted cytokine mediated G1 arrest. p27 contains an Akt consensus site at threonine 157 (T157A), that is phosphorylated by Akt and impairs the nuclear export *in vitro* (Liang, et al., 2012). Liang, et al., also found that p27WT mislocalized to the cytoplasm, but the p27T157A was in the nucleus. Cells with Akt, p27WT failed to cause G1 arrest, but the antiproliferative effect of p27T157A was not impaired. Cytoplasmic p27 was seen in 41% (52 of 12) primary human breast cancers in conjunction with Akt activation and correlated with poor patient prognosis (Liang, et al., 2012).

Following anti-mitogenic signals or DNA damage, p27 interacts with cyclin-CDK complexes to inhibit catalytic activity and induce cell-cycle arrest. This phosphorylates the cyclin-CDK complex, inactivating it which downregulates Rb. The accumulation of p27 in the nucleus acts as a tumor suppressor by facilitating the repair of DNA damage, but if the damage is too extensive it will trigger the apoptotic pathway. When p27 is upregulated in the cytoplasm it functions as an oncogene by

binding to proteins that allow osteosarcoma cells to proliferate and evade cell death. Normally a tumor suppressor like p27 follows what is known as a two-hit hypothesis. This implies that both alleles must be affected before the effect occurs. This is because even if one allele is damaged the other will still be able to produce the protein needed. Normally mutant tumor suppressors are recessive while oncogenes are dominant. p53 is an exception to this rule and can function as a dominant negative so if one becomes mutated or fails it increases risk for cancer. In addition to the CDK-binding site, p27 has other known binding sites including caspase-3 site where it can be cleaved, and a nuclear localizing sequence allowing it to be exported from the nucleus. Importantly, Dean et al., 2010 showed that loss of inhibitory proteins like p27 has been associated with poor prognosis in breast cancer and that these features of p27 may be predictive of poor response to CDK4/6 inhibitors.

1.3.3 p27 interactions with STMN1

Non-canonical p27 functions are observed in cancer cells, where p27 is exported to the cytoplasm and binds cytoplasmic proteins e.g. stathmin 1 (STMN1), to promote cell migration and invasion (Currier et al., 2019). STMN1 is a microtubule destabilizing protein that impairs microtubule formation by sequestering β-tubulin monomers. This protein is important for the disintegration of the mitotic spindle following mitosis and promotes cytokinesis. When p27 binds to STMN1 it inhibits STMN1 function. When p27 and STMN1 are upregulated, this leads to the stabilization of the microtubules and the spindles allowing cancer cells to proliferate. This interaction and other studies suggest that both p27 and STMN1 switch from tumor suppressor to oncogenic activity.

1.4 Hypothesis and experimental aims

p27 can be studied as a predictive biomarker in osteosarcoma. It is constitutively present in the cytoplasm of some tumors and acts as an oncoprotein. I hypothesize that: *Nuclear p27 acts a tumor suppressor, while cytoplasmic p27 acts as an oncoprotein that decreases the sensitivity of osteosarcoma cells to drug treatment.* I predict that combining targeted therapy with chemotherapy will lead to a change in expression of p27, and a decreased cell viability in osteosarcoma cells. To test this prediction, I will accomplish the following aims:

1. Confirm the presence of p27 in the nucleus and cytoplasm of human osteosarcoma cell lines.

2. Assess the viability of osteosarcoma cells treated with gemcitabine and palbociclib.

3. Determine the effects of single agent and combination drug treatments in relation to p27 expression.

4. Determine the binding proteins of p27 in osteosarcoma cell lines.

Chapter 2

MATERIALS AND METHODS

2.1 Tissue culture

Human osteosarcoma cell lines 143B, HOS and human osteoblasts (CRL-11372) were purchased from ATCC (Manassas, VA). HOS and 143B cell lines were cultured in Modified Eagle's Medium (MEM), CRL-11372 was cultured in HAM's F12 Medium. Media were supplemented with 10% fetal bovine serum (FBS), 2 mmol/L l-glutamine, 25 U/mL penicillin, and 25 µg/mL streptomycin. Cells were maintained at 37 °C in humidified incubators in an atmosphere of 5% CO_2.

2.2 Cell viability assay

Cells were seeded in 96-well plates at ($2x10^3$ cells/well) in medium without antibiotics and grown overnight. Cells were then treated with gemcitabine (GEM) at 45nM, palbociclib (PAL) at 5µM corresponding to IC50 concentrations. Gemcitabine and palbociclib were administered the same time, or sequentially; gemcitabine before palbociclib and palbociclib before gemcitabine. Untreated cells (C) were grown for 48 hours (as stated in figure 1A), single agent treatments and the GEM+PAL combination, cells received media only for 24 h, followed by respective treatment for 24 h. The sequence combinations received one agent for 24 h followed by a wash with phosphate-buffered saline (PBS), then incubated with the next drug for 24 h. Viable cells were measured after 48 h with cell titer blue (Promega, Madison, WI) reagent. After incubation at 37°C for 4 hours, the absorbance was read at 570 nm in the Victor4 plate reader (Perkin Elmer, Waltham, MA). Data are represented as the mean of 6 measurements ± SE. Cell viability and t-test statistical analysis were assessed with Excel.

2.3 Real Time-quantitative PCR

Total RNA was isolated from 143B cells using Trizol reagent (Invitrogen, Carlsbad, CA) following treatment with gemcitabine, or palbociclib, or the combination of the two drugs for 24 h. First strand cDNA was created from RNA using the High Capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). TaqMan Gene Expression assays for *CDKN1B, GAPDH,* and *STMN1*, were purchased from Life Technologies (Carlsbad, CA). The ABI 7900 HT Real-Time (Applied Biosystems) equipment was used to detect mRNA levels. GAPDH was used as a normalizing control. Gene amplification was determined using the ΔΔCt method of relative quantification. Data are presented as mean ± SE of three measurements.

2.4 Cytoplasmic and nuclear fractionation

Lysates were prepared using the Cell Fractionation Kit (Abcam, Cambridge, MA). Lysates were resuspended in fractionation buffer and centrifuged at 3,000 rpm for 5 m. The supernatant contained the cytoplasmic fraction. The nuclear pellet was then washed with fractionation buffer centrifuged again at 3,000 rpm for 10 m and the pellet containing the nuclei was resuspended in TBS with 0.1% SDS and sonicated to shear genomic DNA and homogenize the lysate. The cytoplasmic fraction was then centrifuged, and the supernatant contained the cytoplasm and membrane fraction.

2.5 Western blot analysis

After 48 h drug treatments (Section 2.1.2) cells were washed with PBS and lysed in radioimmunoprecipitation assay (RIPA) buffer (50 mmol/L Tris-HCL pH 7.5, 150 mmol/L NaCl, Triton X-100, 0.1% SDS, and 1% sodium deoxycholate, Invitrogen), containing protease and phosphatase inhibitors (Thermo Fisher Scientific,

Carlsbad, CA). The lysates were sonicated and cleared of cell debris by centrifugation. The supernatants were used to determine protein concentration using a BCA assay (Invitrogen). Samples containing approximately 25 μg of protein and Laemlli 4x sample buffer (Bio-Rad, Hercules, CA) with β-mercaptoethanol (Thermo Fisher Scientific, Carlsbad, CA) were heated at 100 °C for 5 m. Proteins (approximately 25 μg per lane) were resolved by SDS-PAGE on 10% Tris-glycine gels, transferred to nitrocellulose membranes and blocked for 1 hour at room temperature in 5% milk in PBST (Phosphate buffered saline containing 0.03% Tween-20). The membranes were then incubated overnight at 4 °C in primary antibody diluted in buffer containing 5% BSA. Membranes were then washed in PBST and incubated for 1 h at room temperature with the appropriate horseradish peroxidase-conjugated secondary antibody (Cell Signaling) in 5% milk/PBST. After secondary incubation the membranes were washed with PBST and developed using chemiluminescent substrate (Bio-Rad, Hercules, CA). GAPDH antibody was used as the loading control. Western blot band intensity was determined using the LI-COR (Lincoln, NE). Band intensity was calculated using Image J (NIH, Bethesda, MD). Students t-test analysis was performed to determine statistical significance.

2.6 Immunostaining and Immunofluorescence

143B cells ($2x\ 10^3$) were grown on glass coverslips (precoated with 0.01% w/v poly-L-lysine) in 12-well plates and were treated with the appropriate drug in the same manner as stated previously. After 48 hours cells were fixed with 4% paraformaldehyde and permeabilized using 2% Triton X-100 in PBS and blocked with 5% horse serum. Cells were immunostained with anti-p27 rabbit monoclonal antibody (1:100 dilution) anti-STMN1 rabbit monoclonal antibody (1:100 dilution) overnight at

4 °C, followed by an Alexa Fluor 488 anti-rabbit conjugated secondary antibody (1:1000 dilution, Thermo Fisher Scientific, Rockford, IL) and Alexa Fluor 647 anti-mouse conjugated secondary antibody (1:1000 dilution, Thermo Fisher Scientific, Rockford, IL) for 1 h at room temperature. Nuclei were counterstained with Hoechst 33342 (Invitrogen, CA). The coverslips were mounted using SlowFade Gold Anti-Fade Reagent (Thermo Fisher Scientific, Rockford, IL). Images were obtained using a confocal microscope (Zeiss).

2.7 Immunoprecipitation

Cell lysates were incubated with anti-p27 antibody overnight for 24 h at 4 °C. Samples were then incubated with Protein-G beads (Invitrogen, CA) for 2 h at 4 °C and precipitated by centrifugation. Immunoprecipitated complexes were then washed with PBST for analysis by mass spectrometry or immunoblot.

2.8 Mass Spectrometry

Following immunoprecipitation (Section 2.1.7), samples were resuspended in 50mM Tris-HCl pH 8.0 (Thermo Fisher Scientific). pH was adjusted to a (Thermo Fisher Scientific). Dithiothreitol (DTT, Thermo Fisher Scientific) was then added at a 1:25 dilution and samples were incubated at 60 °C for 1 h. Indole-3-acetic acid (IAA, Thermo Fisher Scientific) was then added at a 1:25 dilution and samples were incubated for 30 m at room temperature in the dark. Following incubation trypsin (Thermo Fisher Scientific) was added and samples were incubated overnight (at least 4 h) at 37 °C. Following overnight trypsinization, 50 mM Tris-HCL was added, and samples were centrifuged for 1 m. After trypsinization a sample buffer containing 2% Trifluoroacetic acid (TFA, Thermo Fisher Scientific), and 20% Acetonitrile (ACN,

Thermo Fisher Scientific) was added to each sample yielding a final sample concentration of 0.5% TFA in 5% ACN. The C18 spin column was then placed in a receiver tube and the "wet" (activation) solution containing 0.1% TFA and 50% ACN was used to rinse the resin off the walls of the column. The columns were then centrifuged at 1,500 x g for 1 m. After column activation the Equilibrate Solution (0.1% TFA, 5% ACN) was added to the columns and centrifuged. The supernatants were then loaded on top of the resin bed, centrifuged and Equilibrate Solution was used to wash the column. The columns were then placed into new receiver tubes and 20 µL of Elution Buffer (0.1% TFA, 70% ACN) was added to the top of the resin bed. Following the Elution Buffer, the spin columns were centrifuged the samples were dried in a vacuum evaporator. Samples were submitted to the Mass Spectrometry Facility (University of Delaware) to be analyzed through the Thermal Q-Exactive Orbitrap (Thermo Fisher Scientific). Data analysis was then performed by Bioinformatics Core using uniprot (www.uniprot.org).

Chapter 3

RESULTS

3.1 p27 is located in the nucleus and cytoplasm in human osteosarcoma cells

Several studies demonstrate a small population of osteosarcoma cells is able to evade treatment by using the cell's compensatory mechanisms to reverse damage and continue to proliferate (Sampson, et al., 2016, Kreahline et al., 2014). Our work, as well as there's has shown this involves the aberrant localization of p27 to the cytoplasm (Zhao et al., 2014). To determine the subcellular localization of p27 in osteosarcoma, I subjected 143B human osteosarcoma cells to immunofluorescence staining with antibodies against p27. Figure 3.1A shows prominent nuclear and cytoplasmic staining of the p27 protein in 143B cells. I also examined the presence of p27 in nuclear and cytoplasmic fractions isolated from 5 osteosarcoma cell lines (SaOS, 143B, HOS, U2OS, MG63), by immunoblot analysis. Figure 3.1 B shows the presence of p27 protein in the cytoplasmic fractions of osteosarcoma cell lines, but not in control cell lines of CRL-11372, an osteoblast cell line and HFL1, a fibroblast cell line. This data demonstrates that p27 is in the nucleus and cytoplasm of osteosarcoma cells.

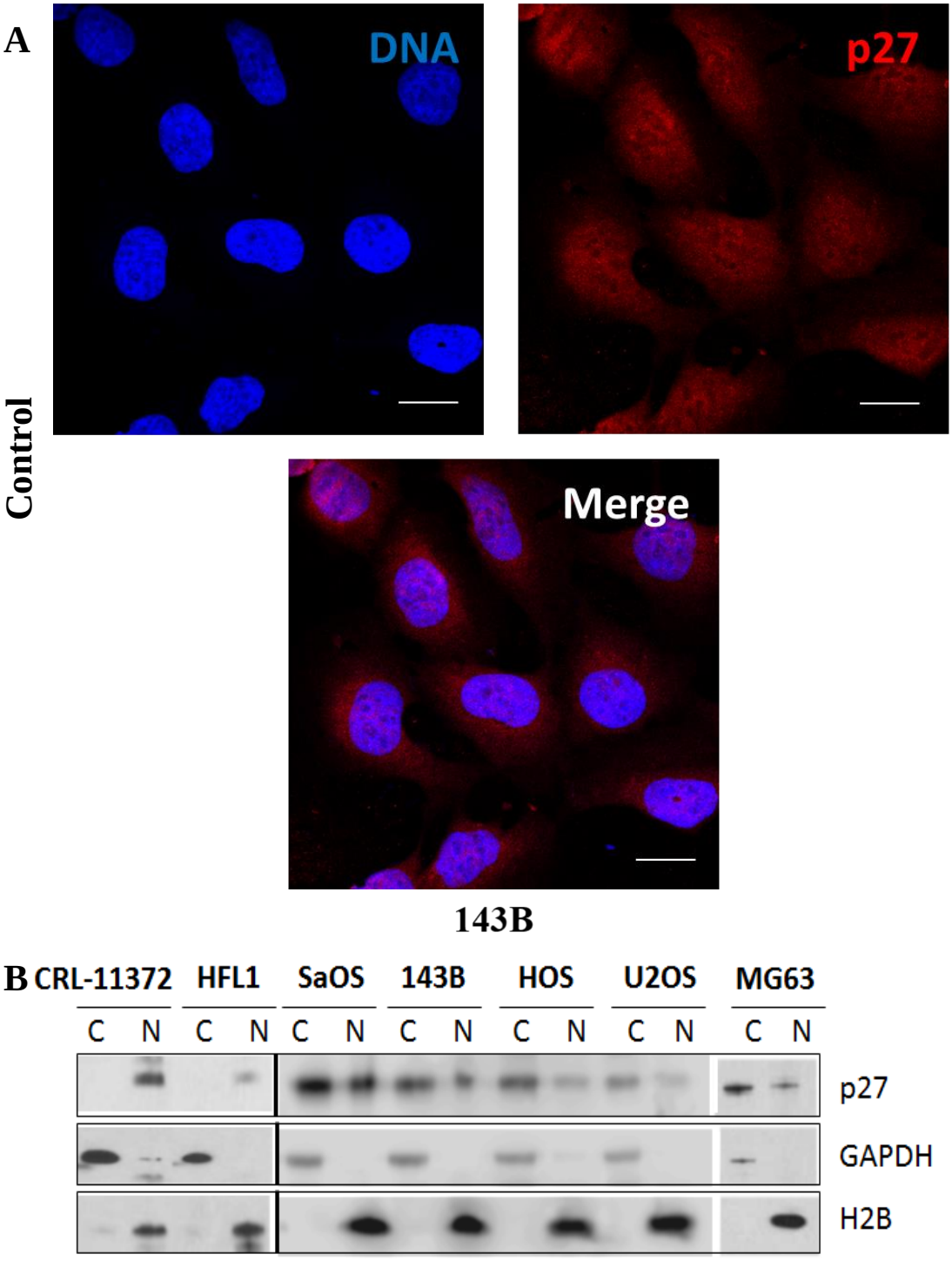


Figure 3.1 **p27 is expressed in the cytoplasm and nucleus of osteosarcoma.** (A) Osteosarcoma cells were subjected to immunofluorescence staining using anti-p27 antibodies. Representative images are shown. p27 is colored red; nuclei were stained with Hoechst dye and are colored blue. Magnification is 63x; scale bars = 25 µm

(B) Nuclear and cytoplasmic lysates were extracted from osteoblasts (CRL-11372), lung fibroblasts (HFL1) and SaOS, 143B, HOS, U2OS, MG63 human osteosarcoma cell lines. Lysates were examined by immunoblot analysis with anti-p27 antibody. GAPDH was the cytosolic marker and H2B was the nuclear marker.

3.2 Gemcitabine and palbociclib alone and in combination decrease cell viability of osteosarcoma cells

I next examined the efficacy of targeting CDK4/6 in osteosarcoma. It was previously shown that gemcitabine decreased cell viability in human osteosarcoma cell lines, (Kreahling et al., 2013). I examined if combining gemcitabine with palbociclib, could have an additive effect. To test this, I used the HOS and 143B human osteosarcoma cell lines. HOS and 143B are an isogenic pair derived from a 13-year-old Caucasian female. 143B is a metastatic cell line that is Thymidine Kinase negative (TK-) and is derived from HOS which is TK+. Drug administration is shown in figure 3.2 A. Figure 3.2 B and C show that the highest drug toxicity was achieved for the specific sequence of gemcitabine administered before palbociclib in 143B and HOS cells respectively, in comparison to untreated controls. Palbociclib administered before gemcitabine (data not shown) did not significantly decrease cell viability, and was less potent than the single agents alone (figure 3.2 B, C). These results suggest that chemotherapy enhances the effect of cell cycle inhibition in osteosarcoma cells.

A

Treatment	Control	Gemcitabine	Palbociclib	GEM+PAL	GEM/PAL
Day 1	Seed cells	Seed cells	Seed cells	Seed cells	Seed cells
Day 2	Media only	Media only	Media only	Media only	Gemcitabine
Day 3	Media only	Gemcitabine	Palbociclib	GEM+PAL	Palbociclib
Day 4	Cell Titer Blue	Cell Titer Blue	Cell Titer Blue	Cell Titer Blue	Cell Titer Blue

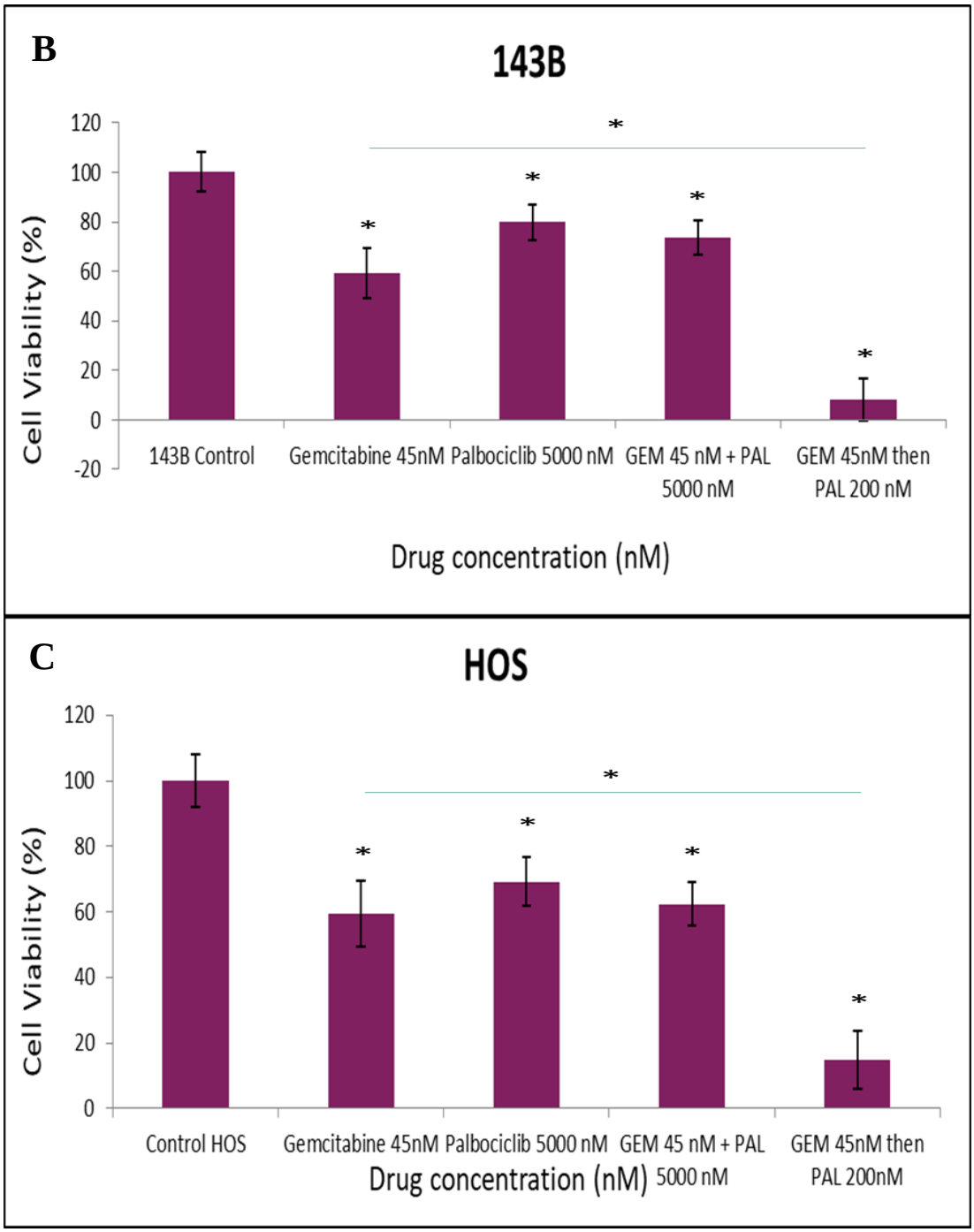


Figure 3.2 **Gemcitabine and palbociclib alone and in combination decrease cell viability of osteosarcoma cells.** (A) Table of dosing sequence for drug treatments. (B, C) Cell viability plots of osteosarcoma cell lines 143B and HOS respectively treated with gemcitabine or palbociclib alone, in combination, and sequentially.

At least 3 independent experiments were performed. Each bar represents the mean of 6 replicates. Error bars represent SD (*p<0.05).

3.3 Gemcitabine and palbociclib induce different changes in p27 expression in osteosarcoma cells

In our previous studies (Currier et al., 2019), we found that p27 was upregulated in cells treated with cell cycle checkpoint inhibitors and induced cell cycle arrest. I examined the protein expression of p27 in 143B, and HOS cells after treatment with gemcitabine and palbociclib using Western blot analysis. Figure 3.3 A shows that in HOS cells, gemcitabine significantly decreased p27 levels. In contrast, p27 was significantly increased when the cells were treated with palbociclib alone. The simultaneous combination of the two drugs increased the levels of p27 in the cells to about the same degree as palbociclib alone, and when palbociclib is administered before gemcitabine. However, when the cells are sequentially treated with gemcitabine first, and then palbociclib the biggest decrease in p27 was observed. Figure 3.3 B is the relative quantification of p27 expression using Image J software. Further, other studies (Eymin et al., 1999) suggest that p27 can be cleaved by caspase, and cleavage leads to the activation of the proapoptotic pathway. Figure 3.3 C shows that in 143B cells increases in cleaved p27 were also observed following drug treatment suggesting that cleaved p27 correlates with drug toxicity. The osteoblast cell line, CRL-11372, displayed no constitutive p27 expression (figure 3.3 C). Figure 3.3 D is the relative quantification of the cleaved p27 bands using Image J software.

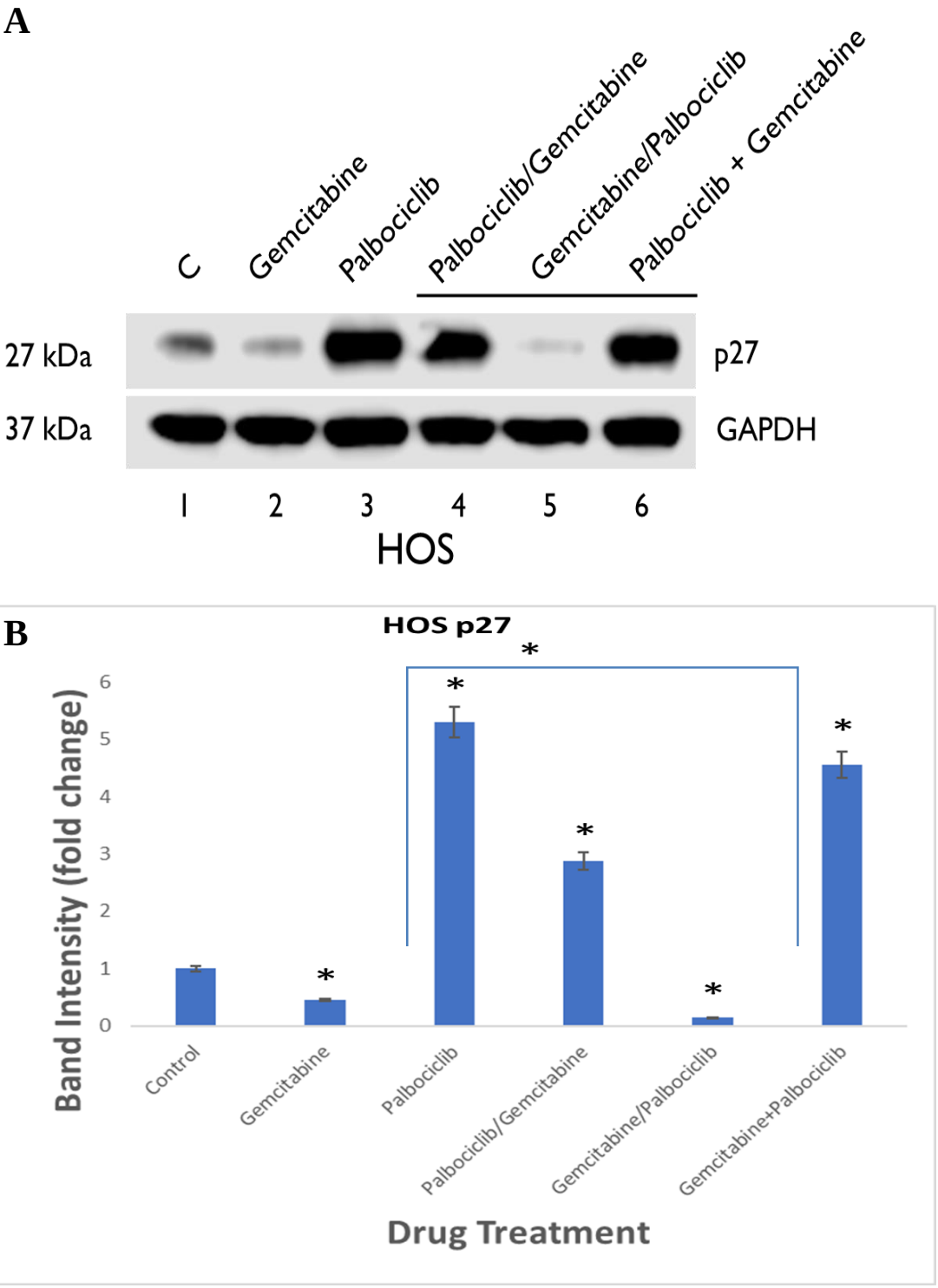
mRNA gene expression of *CDKN1B* and *STMN1* was also assessed in 143B cells following treatments. Gemcitabine decreased expression of *CDKN1B*, while palbociclib, and the combination of gemcitabine and palbociclib increased expression of *CDKN1B* (figure 3.3 E). These mRNA changes for *CDKN1B* correlate with the p27

protein changes in figure 3.3 A. Figure 3.3 F shows significant increases for *STMN1* were observed for palbociclib alone and the combination of gemcitabine and palbociclib together.

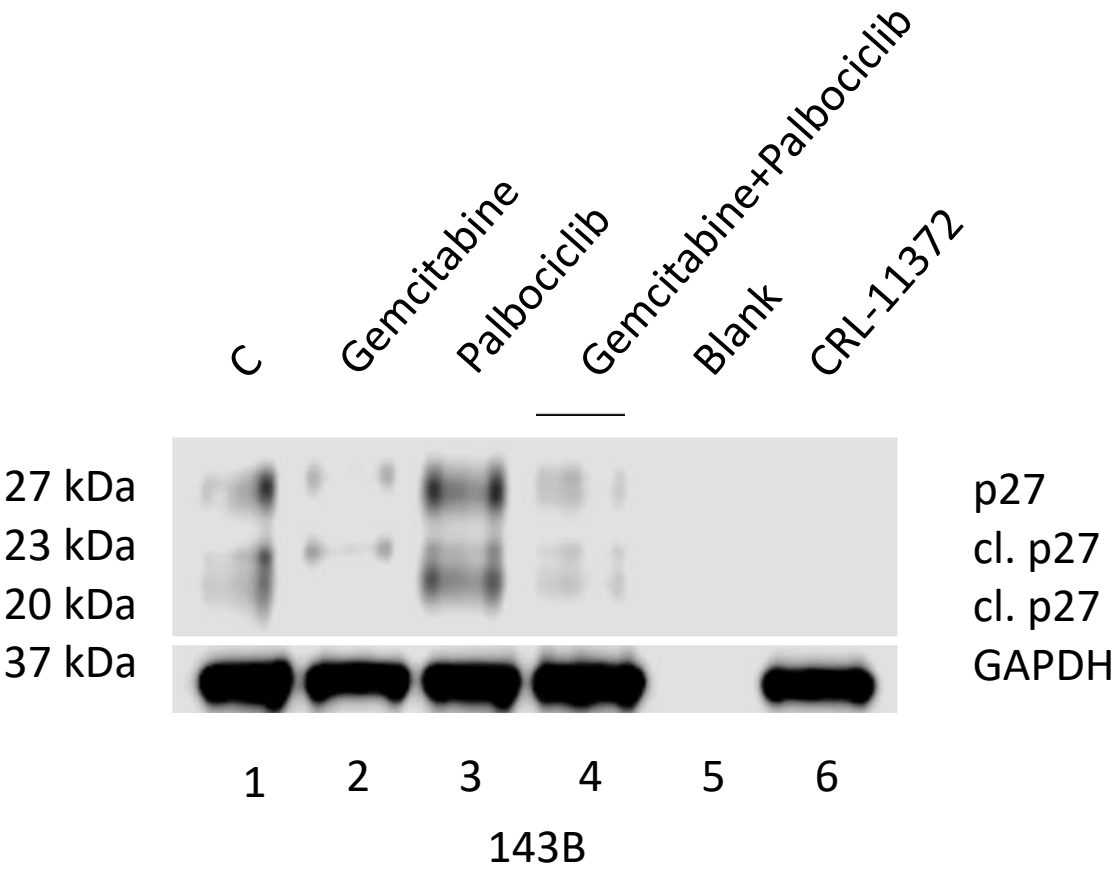
To determine the subcellular effects of drug treatments on p27 I then subjected 143B cells to immunofluorescence staining with antibodies against p27, STMN1, β-tubulin. Figure 3.3 G shows treatment with palbociclib resulted in enlarged cells and an increased intensity of nuclear p27 staining. Stathmin is a microtubule destabilizing protein that impairs microtubule formation by sequestering tubulin activity. p27 binds to STMN1 and inhibits STMN1's function leading to stabilization of the microtubules and the spindles allowing cancer cells to proliferate. STMN1 is located in the cytoplasm and colocalizes with p27 (figure 3.3 G).

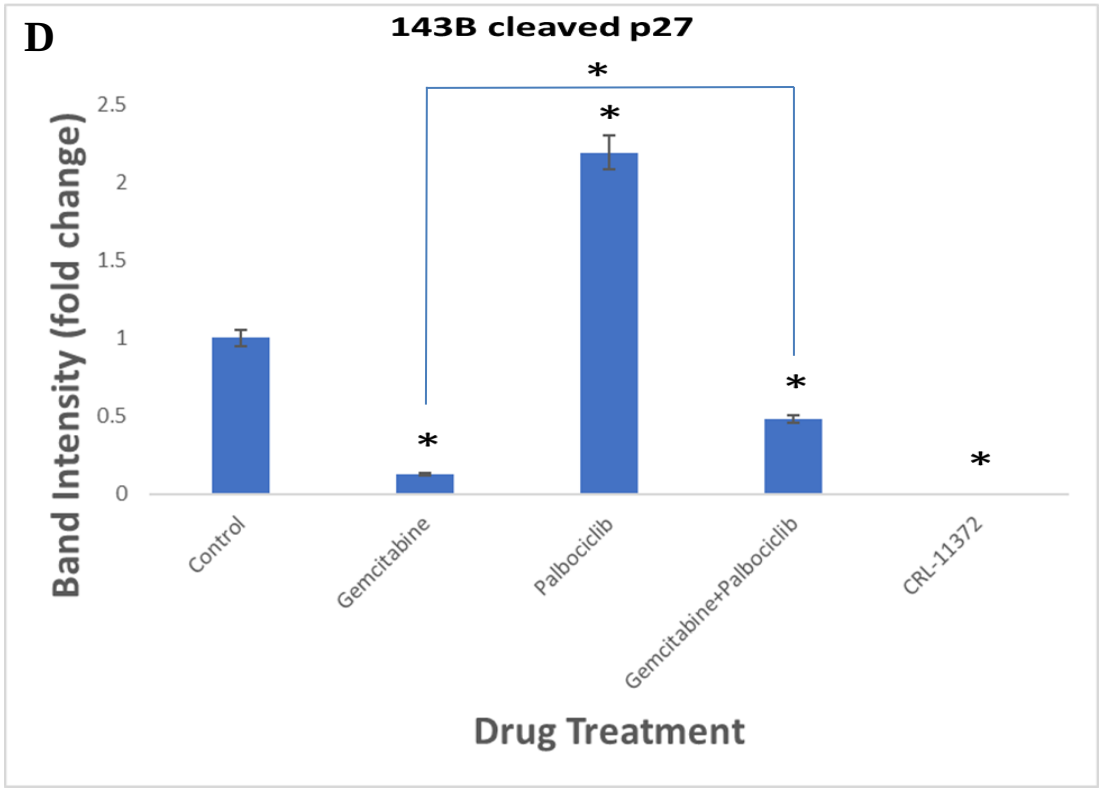
I also investigated the effects of gemcitabine and palbociclib on the cytoskeleton using immunofluorescence staining of β-tubulin. Figure 3.3 H shows that palbociclib showed low levels of disruption of the cytoskeleton. In contrast gemcitabine before palbociclib resulted in decreased expression of p27, fewer cells, and greater disruption of the cytoskeleton which is an indicator of cellular catastrophe.

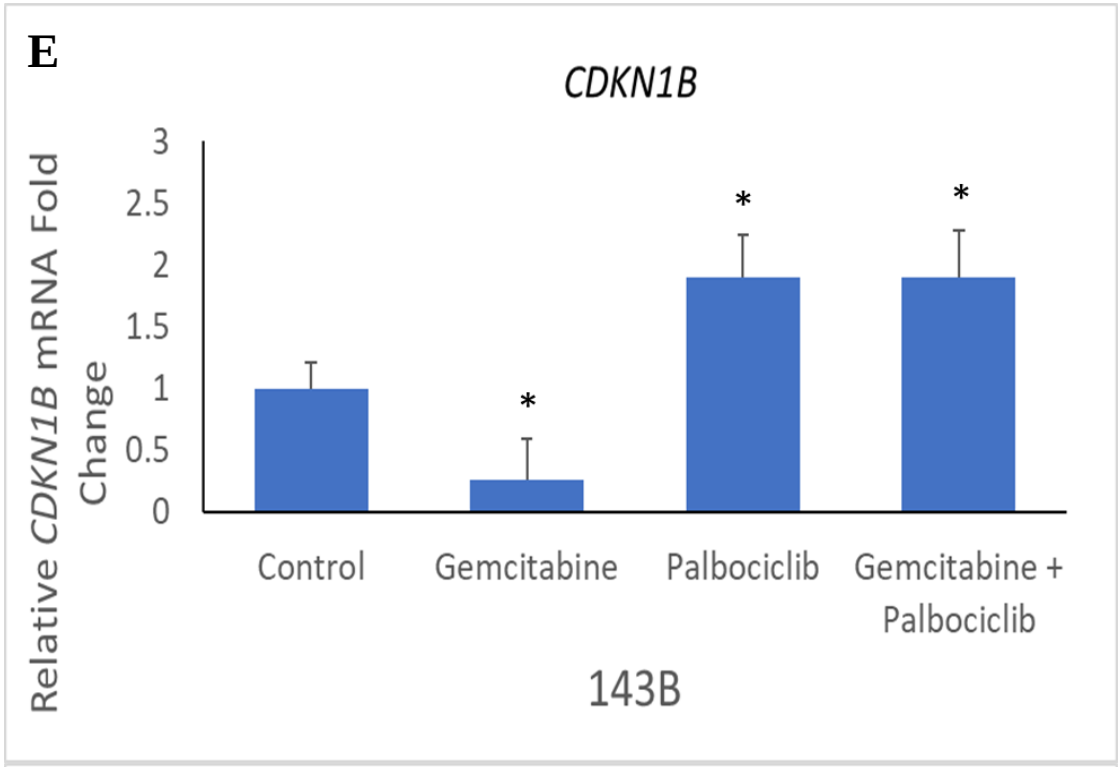
Taken together, this data supports the hypothesis that the effect of palbociclib is enhanced by gemcitabine *in vitro* and that p27 expression and localization by drug treatments may predict osteosarcoma cell death.

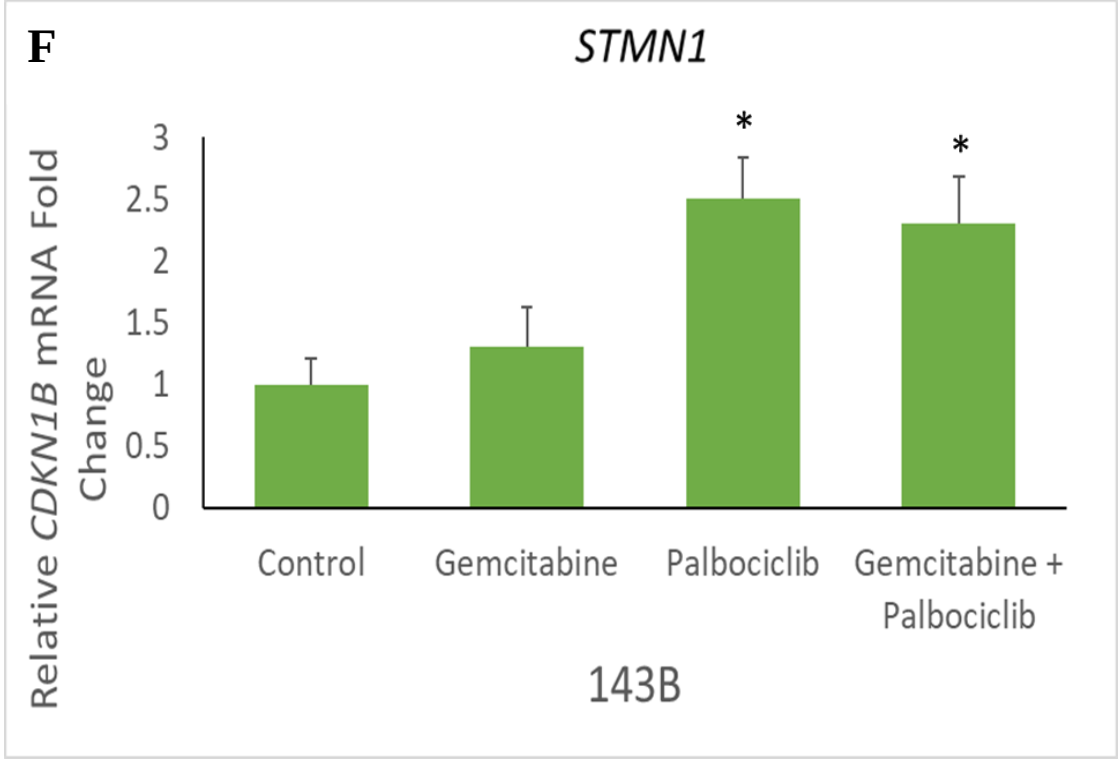

A
C
Gemcitabine
Palbociclib
Palbociclib/Gemcitabine
Gemcitabine/Palbociclib
Palbociclib + Gemcitabine
27 kDa
p27
37 kDa
GAPDH
1
2
3
4
5
6
HOS
B
HOS p27
Band Intensity (fold change)
0
1
2
3
4
5
6
Control
Gemcitabine
Palbociclib
Palbociclib/Gemcitabine
Gemcitabine/Palbociclib
Gemcitabine+Palbociclib
Drug Treatment

C

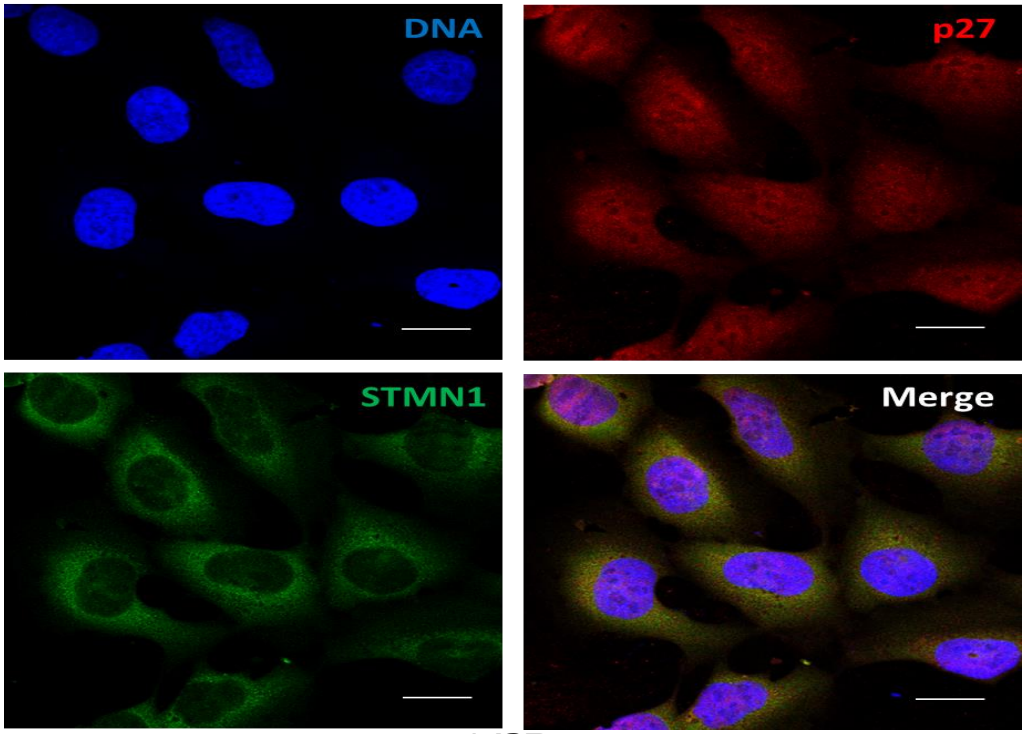

C
Gemcitabine
Palbociclib
Gemcitabine+Palbociclib
Blank
CRL-11372
27 kDa
23 kDa
20 kDa
37 kDa
p27
cl. p27
cl. p27
GAPDH
1
2
3
4
5
6
143B

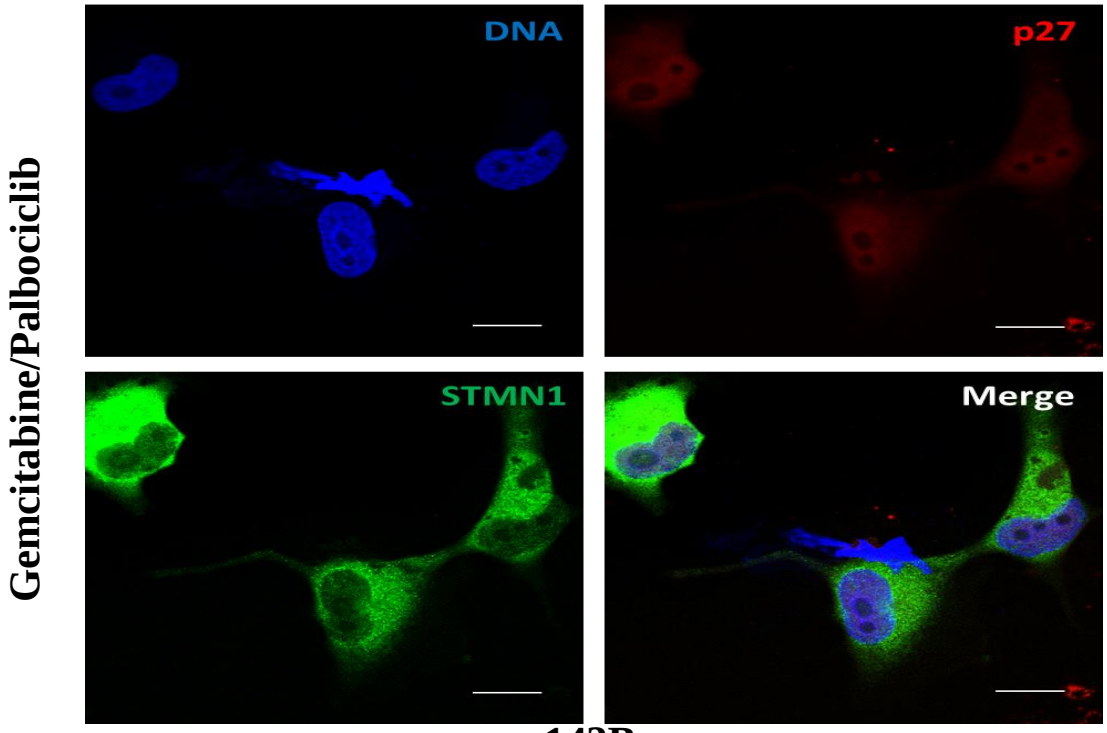

D
143B cleaved p27
Band Intensity (fold change)
2.5
2
1.5
1
0.5
0
Control
Gemcitabine
Palbociclib
Gemcitabine+Palbociclib
CRL-11372
Drug Treatment
*
*
*
*
*


E
CDKN1B
Relative CDKN1B mRNA Fold Change
3
2.5
2
1.5
1
0.5
0
*
*
*
Control
Gemcitabine
Palbociclib
Gemcitabine + Palbociclib
143B

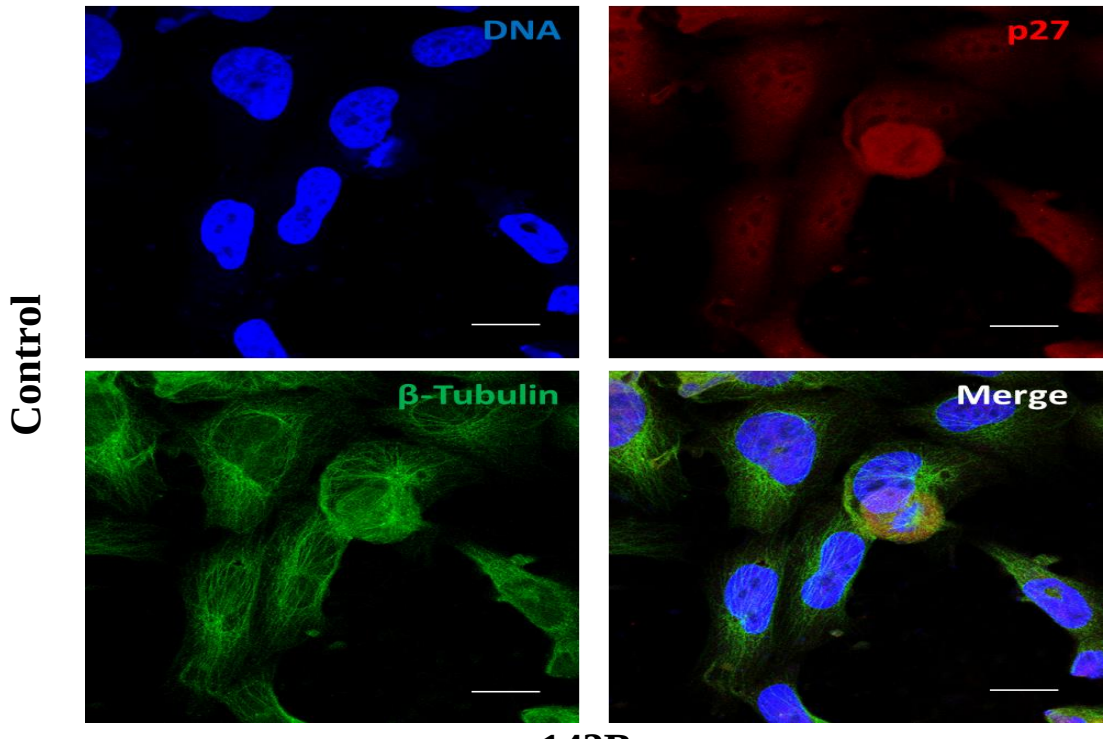

F
STMN1
Relative CDKN1B mRNA Fold Change
3
2.5
2
1.5
1
0.5
0
*
*
Control
Gemcitabine
Palbociclib
Gemcitabine + Palbociclib
143B

G


Control
DNA
p27
STMN1
Merge
143B
Palbociclib
DNA
p27
STMN1
Merge
143B


Gemcitabine/Palbociclib
DNA
p27
STMN1
Merge
143B

H


Control
DNA
p27
β-Tubulin
Merge
143B

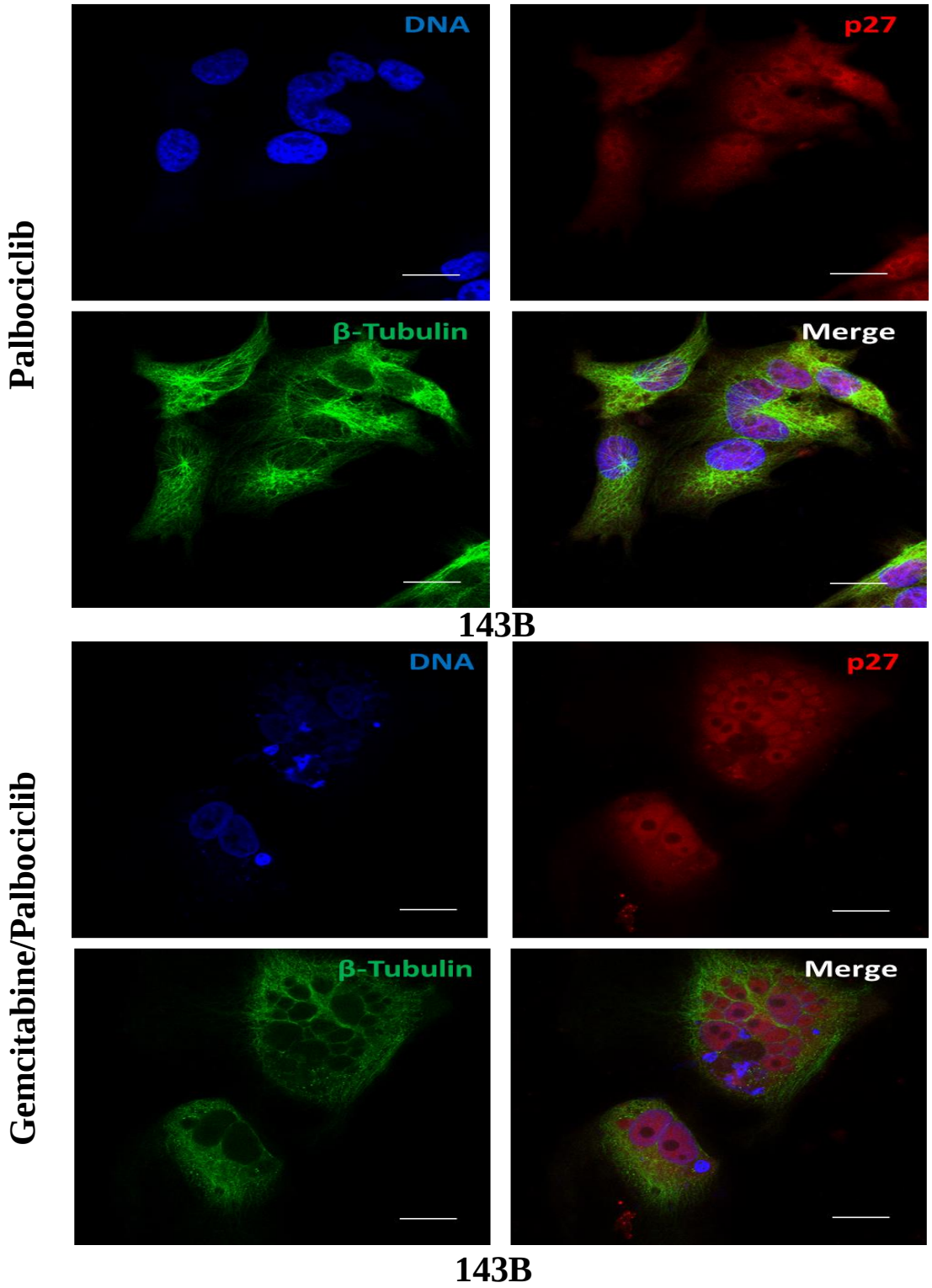

Palbociclib
DNA
p27
β-Tubulin
Merge
143B
Gemcitabine/Palbociclib
DNA
p27
β-Tubulin
Merge
143B

Figure 3.3 **p27 expression changes in osteosarcoma cells following treatment with gemcitabine and palbociclib.** (A) Lysates were analyzed by immunoblotting with antibodies against p27. GAPDH was the protein loading control. At least 3 independent experiments were performed. Error bars represent SD (* $p<0.05$). (B) Band intensity was plotted based on band intensity using image J software normalized to GAPDH as control. At least 3 independent experiments were performed. Error bars represent SD (* $p<0.05$). (C) Lysates were analyzed by immunoblotting with antibodies against p27. GAPDH was the protein loading control. At least 3 independent experiments were performed. Error bars represent SD (* $p<0.05$). (D) Band intensity was plotted based on band intensity using image J software normalized to GAPDH as control. At least 3 independent experiments were performed. Error bars represent SD (* $p<0.05$) (E, F) 143B cells were treated with gemcitabine, and palbociclib alone as indicated in the graph above. Expression of *CDKN1B* and *STMN1* were evaluated. mRNA expression was quantified relative to control osteoblasts, CRL-11372 (RQ = 1). Fold changes in mRNA levels are normalized to the house keeping gene GAPDH. Error bars represent SD. (* $p < 0.05$). (G, H) 143B osteosarcoma cells, and osteosarcoma cells treated with gemcitabine and palbociclib were subjected to immunofluorescence staining using anti-p27 antibodies, anti-STMN1 antibodies, and anti-β-tubulin antibodies. Representative images are shown. p27 is colored red, STMN1 and β-tubulin are colored green respectively; nuclei were stained with Hoechst dye and are colored blue. Magnification is 63x; scale bars = 25 μm.

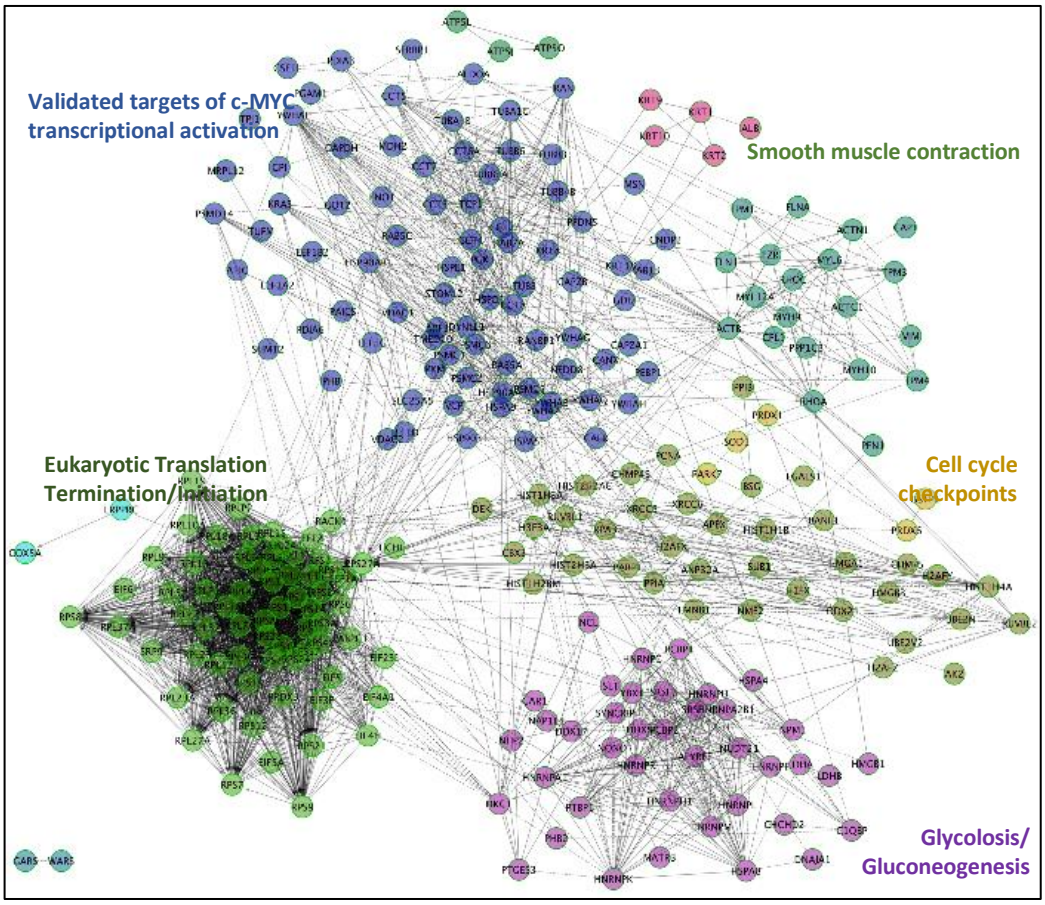
3.4 p27 interacting proteins in Osteosarcoma

The current data suggest p27 is a key component in the protein regulatory network of osteosarcoma. The function of p27 seems to rely on its localization and its binding proteins. To determine whether p27 interacting proteins can be used as drug targets, the interactome of p27 was studied. Interacting proteins can be targeting to increase cell death in osteosarcoma cells. In order to identify cytoplasmic and nuclear binding proteins of p27, I immunoprecipitated p27 protein in 143B osteosarcoma cells using anti-p27 antibody. The immunoprecipitated proteins were subjected to mass spectrometry (MS/MS) analysis to identify the proteins that were bound to p27. A network of these binding proteins and the cellular processes for which they are involved was established (figure 3.4 A). The percent of coverage was calculated by

dividing the number of amino acids in all peptides by the total number of amino acids in the entire protein sequence. Based on coverage, I identified 154 interacting proteins. These p27 interactions were used to determine disease network components in osteosarcoma cell lines (figure 3.4 B). The table shown below (figure 3.4 C) indicates proteins with the highest coverage. These fifteen proteins are localized in the cytoplasm, indicated by the red outlines, or in the nucleus, indicated by the gray outlines. The cytoskeletal proteins (actin, vimentin, tubulin) are known p27 interacting proteins (Fife et al., 2014) that are associated with the regulation of microtubules and the actin cytoskeleton. These were among the highly scored p27 interactors. The physiological interaction with actin, vimentin and tubulin can enhance cell migration.

Three additional proteins (β-integrin, actinin, and exportin) were chosen to confirm their interaction with p27 (figure 3.4 C). Western blot analysis of p27 immunoprecipitates with anti-β-Integrin, anti-actinin, and anti-exportin antibodies show that these proteins co-immunoprecipitated with p27, and validated the mass spectrometry data (figure 3.4 D). Interaction with β-Integrin and actinin are associated with focal adhesion and interaction with exportin allows for nuclear export of p27. Further analysis of this data will identify crosstalk between different pathways to further build the p27 network and provide a clearer understanding of how p27 controls the molecular mechanisms and progression of osteosarcoma tumors.

A



Pathway	No. of Proteins	Selected Genes
PI3K	7	YWHAE,YWHAB
mTOR	9	EIF4A1,EEF2, KRAS
c-Myc	16	HSP90AA1,ENO1, NPM1
Metastatic	8	VIM, CFL1, TAGLN2,TUBB3
NMD	6	RLP4, RLP5, RLP30

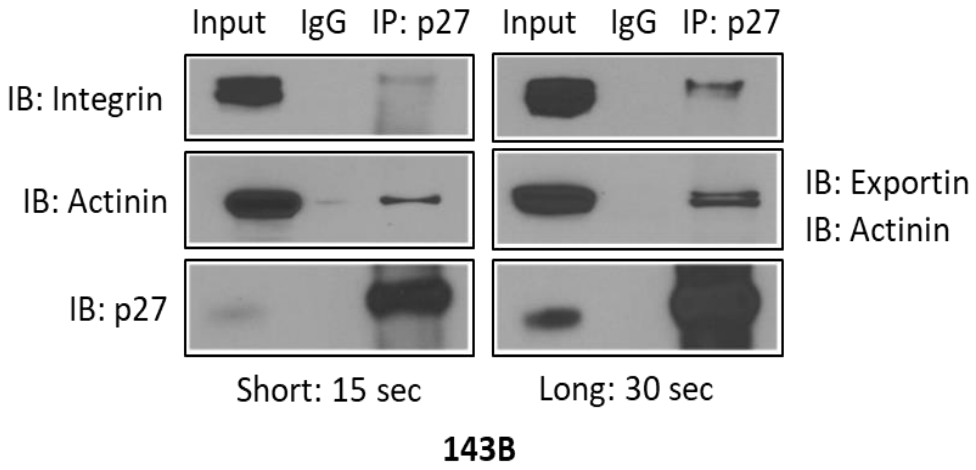
B

Accession	Description	Coverage
P06733	Alpha-enolase OS=Homo sapiens OX=9606 GN=ENO1 PE=1 SV=2 - [ENOA_HUMAN]	87.33
P60709	Actin, cytoplasmic 1 OS=Homo sapiens OX=9606 GN=ACTB PE=1 SV=1 - [ACTB_HUMAN]	79.20
P05387	60S acidic ribosomal protein P2 OS=Homo sapiens OX=9606 GN=RPLP2 PE=1 SV=1 - [RLA2_HUMAN]	78.26
P08670	Vimentin OS=Homo sapiens OX=9606 GN=VIM PE=1 SV=4 - [VIME_HUMAN]	76.61
P62937	Peptidyl-prolyl cis-trans isomerase A OS=Homo sapiens OX=9606 GN=PPIA PE=1 SV=2 - [PPIA_HUMAN]	75.15
P62805	Histone H4 OS=Homo sapiens OX=9606 GN=HIST1H4A PE=1 SV=2 - [H4_HUMAN]	72.82
P37802	Transgelin-2 OS=Homo sapiens OX=9606 GN=TAGLN2 PE=1 SV=3 - [TAGL2_HUMAN]	72.36
P22392	Nucleoside diphosphate kinase B OS=Homo sapiens OX=9606 GN=NME2 PE=1 SV=1 - [NDKB_HUMAN]	71.71
P07355	Annexin A2 OS=Homo sapiens OX=9606 GN=ANXA2 PE=1 SV=2 - [ANXA2_HUMAN]	70.80
P61604	10 kDa heat shock protein, mitochondrial OS=Homo sapiens OX=9606 GN=HSPE1 PE=1 SV=2 - [CH10_HUMAN]	69.61
P07437	Tubulin beta chain OS=Homo sapiens OX=9606 GN=TUBB PE=1 SV=2 - [TBB5_HUMAN]	69.59
P60174	Triosephosphate isomerase OS=Homo sapiens OX=9606 GN=TPI1 PE=1 SV=3 - [TPIS_HUMAN]	69.58
P07737	Profilin-1 OS=Homo sapiens OX=9606 GN=PFN1 PE=1 SV=2 - [PROF1_HUMAN]	69.29
P62258	14-3-3 protein epsilon OS=Homo sapiens OX=9606 GN=YWHAE PE=1 SV=1 - [1433E_HUMAN]	67.84
P63241	Eukaryotic translation initiation factor 5A-1 OS=Homo sapiens OX=9606 GN=EIF5A PE=1 SV=2 - [IF5A1_HUMAN]	66.88

P16949	Stathmin OS=Homo sapiens OX=9606 GN=STMN1 PE=1 SV=3 - [STMN1_HUMAN]	28.19
P12814	Alpha-actinin-1 OS=Homo sapiens OX=9606 GN=ACTN1 PE=1 SV=2 - [ACTN1_HUMAN]	41.03
P55060	Exportin-2 OS=Homo sapiens OX=9606 GN=CSE1L PE=1 SV=3 - [XPO2_HUMAN]	21.11

C

D



143B

Figure 3.4 **Mass spectrometry determination of p27 binding proteins.** (A) 143B cells were immunoprecipitated with p27 antibody and subjected to mass spectrometry analysis and the interacting proteins were used to establish a connectivity map of p27 interactome. Blue represents the validated targets of c-MYC transcriptional activation, light green represents smooth muscle contraction, dark green represents Eukaryotic translation termination/initiation, yellow represents cell cycle checkpoints, and purple represents glycolysis/gluconeogenesis (B) p27 interactions identify disease network components in osteosarcoma cell lines. (C) Table of binding proteins of p27 were acquired after mass spectrometry analysis. The proteins outlined in red are located in the cytoplasm whereas the proteins that are outlined in gray localized to the nucleus. Three proteins (bottom) were chosen to confirm p27 binding (D) p27 was immunoprecipitated from 143B cell lysates. Immunoprecipitates were separated using Western blot analysis using antibodies against β-Integrin, actinin, and exportin. Images show short and long exposure times.

Chapter 4

DISCUSSION AND FUTURE DIRECTIONS

Osteosarcoma tumors have highly variable biological and genetic features and no mutation is frequent enough to target for therapy. Furthermore, tumors can evolve into drug resistant phenotypes following drug treatments. This highlights the clinical need for new treatment strategies. Despite promising preclinical data, there has been limited clinical successes with new drugs that have been tested. Understanding the underlying biology of tumors will aid the development of new therapies. The cell cycle is a common target for anti-cancer drugs because tumor cells proliferate when most cells in the body enter G_0 and exit the cell cycle.

Increased signaling through CDKs is frequently observed in cancer. In the past year, increasingly specific, orally administered small molecule cell cycle inhibitors directed to CDK4/6 (palbociclib, abemaciclib, ribociclib) have been approved for treatment of advanced breast cancer, generating renewed interest in cell cycle inhibition as a therapeutic strategy. These agents inhibit the activity of key checkpoint controls that halt cell cycle progression and induce cell cycle arrest. However, the early results of clinical testing suggest these inhibitors are not highly effective as monotherapies and should be studied in combination strategies. Further, predictive markers could help stratify responders. Therefore, understanding the role of p27 in osteosarcoma may provide opportunities for the development of predictive markers and new treatments.

In this study, I examined p27 expression and localization in osteosarcoma cells. I showed that p27 is present in the nucleus as well as the cytoplasm. Further, cytoplasmic p27 colocalizes with STMN1 protein. In normal cells, STMN1 binds β-

tubulin monomers to destabilize microtubules. Therefore, STMN1 association with p27 can prevent microtubule destabilization, suggesting that p27 functions in the regulation of microtubule dynamics in osteosarcoma. This is supported by the work of other labs (Baldassarre et al., 2005) that demonstrates a role for p27 in cell migration and invasion in cancer cells. Collectively, these results suggest that p27 could act as an oncogene, through non-CDK interactions in the cytoplasm.

I also examined how p27 affects the response of osteosarcoma cells to the chemotherapeutic agent gemcitabine and CDK4/6 inhibition therapy. Data from Western blots and immunofluorescence staining show gemcitabine reduced p27 expression in osteosarcoma cell lines while palbociclib treatment increased p27 levels in the nucleus and increased the tumor suppressor function by arresting the cells, presumably in G1-phase. When both drugs were combined, the cell response is dependent on the sequence of drug administration. When palbociclib was administered before gemcitabine, the decrease in cell viability was not significant because it acts antagonistically, likely because palbociclib arrested the cells in G1 which blocks progression to S-phase. This suggests that pretreatment of cells with palbociclib inhibits the activity of gemcitabine. In contrast, when gemcitabine was administered before palbociclib, cells with DNA damage that are able to evade cell death cycle to G1 and undergo cell cycle arrest mediated by palbociclib. This effectively causes cells with DNA damage from gemcitabine to be checked further for damage leading to apoptosis. Taken together this data suggests that the dosing sequencing of combining cell cycle inhibitors with chemotherapy agents increases toxicity, and that the order of dosing sequencing is significant.

We propose the use of p27 as a prognostic marker and suggest that that cleaved p27 could also be leveraged to inform the efficacy of treatments. *In vivo* studies using preclinical osteosarcoma models will be necessary to further examine these mechanisms before translation to clinical trials. These data provide insight to mechanisms of how drugs interact with and affect p27 leading to a decrease in cell viability for osteosarcoma cells.

Mass spectrometry analysis has identified several components of the actin cytoskeleton and microtubules as p27 interacting proteins (actin, vimentin and tubulin). These data support the role of p27 in regulating migration and organization of the cytoskeleton. As several p27 interacting proteins are essential for cell processes including proliferation and microtubule spindle stability, these results provide opportunities for identification of small molecules that target these proteins and disrupt these interactions. An *in silico* analysis suggests the class of microtubule targeting agents may be developed to treat osteosarcoma. Several microtubule stabilizing (paclitaxel and docetaxel) as well as destabilizing (vincristine and eribulin) agents have been clinically tested in osteosarcoma patients. The frequent acquisition of drug resistant phenotypes as well as high toxicities are a major setback for the development of these therapies. Integration of other p27 interactors may lead to the development of novel p27-based combination treatments that may increase potency and efficacy of these agents against osteosarcoma.

Mass spectrometry analysis also demonstrated PI3K, mTOR and c-MYC signaling pathways were enriched for highly scored p27 interactors. Activation of the PI3K/AKT pathway leads to increased proliferation, transcriptional activity, inhibition of apoptosis and renders tumors refractory to therapy. Targeting mTOR with single-

agents (rapamycin, ridaforolimus, everolimus) and combination strategies (with cyclophosphamide or sorafenib) have been tested in clinical trials for patients with osteosarcoma, and suggest that there are clinical benefits and the potential for progression free survival rates in targeting this pathway. In addition, AKT mediates C-terminal p27 phosphorylation that drives nuclear to cytoplasmic localization. The ability to identify new interacting proteins provides opportunities for developing new therapeutic strategies.

In summary, these results have potential implication for the clinical use of cell cycle inhibitors to enhance chemotherapy responses for patients with osteosarcoma. However, early clinical trials suggest these agents will be most effective when combined with chemotherapy and administered in the most effective dosing sequence to increase long-term patient survival. We further suggest that p27 may serve as a predictive marker for drug treatments by decreasing cytoplasmic p27 that causes p27-dependent osteosarcoma cell survival. This work provides a better understanding of the complex nature of p27 biological activity that can aid the strategic design of new therapies for osteosarcoma patients.

REFERENCES

A. B. Mohseny, K. Szuhai, S. Romeo et al., (2009). “Osteosarcoma originates from mesenchymal stem cells in consequence of aneuploidization and genomic loss of Cdkn2,” Journal of Pathology, vol. 219, no. 3, pp. 294–305.

A. L. Manning and N. J. Dyson. (2011). “PRB, a tumor suppressor with a stabilizing presence,” Trends in Cell Biology, vol. 21, no. 8, pp. 433–441.

Adamopoulos, C., Gargalionis, A. N., Basdra, E. K., & Papavassiliou, A. G. (2016). Deciphering signaling networks in osteosarcoma pathobiology. Experimental biology and medicine (Maywood, N.J.), 241(12), 1296–1305. doi:10.1177/1535370216648806

Alberts B, Johnson A, Lewis J, et al. (2002). Molecular Biology of the Cell. 4th edition. New York: Garland Science; An Overview of the Cell Cycle. Available from: https://www.ncbi.nlm.nih.gov/books/NBK26869/

Allison, D. C., Carney, S. C., Ahlmann, E. R., Hendifar, A., Chawla, S., Fedenko, A., Menendez, L. R. (2012). A meta-analysis of osteosarcoma outcomes in the modern medical era. Sarcoma, 2012, 704872. doi:10.1155/2012/704872

Bacci G, Fabbri N, Balladelli A, et al. (2006).: Treatment and prognosis for synchronous multifocal osteosarcoma in 42 patients. J Bone Joint Surg Br 88 (8): 1071-5.

Bacci G, Rocca M, Salone M, et al. (2006).: High grade osteosarcoma of the extremities with lung metastases at presentation: treatment with neoadjuvant chemotherapy and simultaneous resection of primary and metastatic lesions. J Surg Oncol 98 (6): 415-20.

Baldassarre G, et al. (2005). p27(Kip1)-stathmin interaction influences sarcoma cell migration and invasion. Cancer Cell. 7:51–63. doi: 10.1016/j.ccr.2004.11.025.

Bielack SS, Kempf-Bielack B, Delling G, et al. (2002).: Prognostic factors in high-grade osteosarcoma of the extremities or trunk: an analysis of 1,702 patients treated on neoadjuvant cooperative osteosarcoma study group protocols. J Clin Oncol 20 (3): 776-90.

Biteau, K., Guiho, R., Chatelais, M., Taurelle, J., Chesneau, J., Corradini, N., Redini, F. (2016). L-MTP-PE and zoledronic acid combination in osteosarcoma: preclinical evidence of positive therapeutic combination for clinical transfer. American journal of cancer research, 6(3), 677–689.

Bond M, Bernstein ML, Pappo A, et al. (2008). A phase II study of imatinib mesylate in children with refractory or relapsed solid tumours: a Children's Oncology Group study. Pediatr Blood Cancer. 50:254–8.

Bosse, K. R., & Maris, J. M. (2016). Advances in the translational genomics of neuroblastoma: From improving risk stratification and revealing novel biology to identifying actionable genomic alterations. Cancer, 122(1), 20–33. doi:10.1002/cncr.29706

C. W. Miller, A. Aslo, A. Won, M. Tan, B. Lampkin, and H. P. Koeffler. (1996). "Alterations of the p53, Rb and MDM2 genes in osteosarcoma," Journal of Cancer Research and Clinical Oncology, vol. 122, no. 9, pp. 559–565.

Cadoo, K. A., Gucalp, A., & Traina, T. A. (2014). Palbociclib: an evidence-based review of its potential in the treatment of breast cancer. Breast cancer (Dove Medical Press), 6, 123-33. doi:10.2147/BCTT.S46725

Calvert, G. T., Randall, R. L., Jones, K. B., Cannon-Albright, L., Lessnick, S., & Schiffman, J. D. (2012). At-risk populations for osteosarcoma: the syndromes and beyond. Sarcoma, 2012, 152382. doi:10.1155/2012/152382

Chen, X., Wang, Z., Duan, N., Zhu, G., Schwarz, E. M., & Xie, C. (2018). Osteoblast-osteoclast interactions. Connective tissue research, 59(2), 99–107. doi:10.1080/03008207.2017.1290085

Ciernik, I. F., Niemierko, A., Harmon, D. C., Kobayashi, W., Chen, Y. L., Yock, T. I., Delaney, T. F. (2011). Proton-based radiotherapy for unresectable or incompletely resected osteosarcoma. Cancer, 117(19), 4522–4530. doi:10.1002/cncr.26037

Currier, A. W., Kolb, E. A., Gorlick, R. G., Roth, M. E., Gopalakrishnan, V., & Sampson, V. B. (2019). P27/Kip1 functions as a tumor suppressor and oncoprotein in osteosarcoma. from https://www.nature.com/articles/s41598-019-42450-0#article-info

Dean JL, Thangavel C, McClendon AK, Reed CA, Knudsen ES. (2010). Therapeutic CDK4/6 inhibition in breast cancer: key mechanisms of response and failure. Oncogene. 29(28):4018–4032.

Donati D, Giacomini S, Gozzi E, et al. (2004). Osteosarcoma of the pelvis. Eur J Surg Oncol 30 (3): 332-40.

Eymin B, et al. Caspase-induced proteolysis of the cyclin-dependent kinase inhibitor p27Kip1 mediates its anti-apoptotic activity. Oncogene. 1999;18:4839–4847. doi: 10.1038/sj.onc.1202860.

F. Lonardo, T. Ueda, A. G. Huvos, J. Healey, and M. Ladanyi (1997). “p53 and MDM2 alterations in osteosarcomas: correlation with clinicopathologic features and proliferative rate,” Cancer, vol. 79, no. 8, pp. 1541–1547

Fife, C. M., McCarroll, J. A., & Kavallaris, M. (2014). Movers and shakers: cell cytoskeleton in cancer metastasis. British journal of pharmacology, 171(24), 5507–5523. doi:10.1111/bph.12704

Gelbert LM, Cai S, Lin X, Sanchez-Martinez C, del Prado M, Lallena MJ, et al. (2014). Preclinical characterization of the CDK4/6 inhibitor LY2835219: in-vivo cell cycle-dependent/independent anti-tumor activities alone/in combination with gemcitabine. Invest New Drugs. 32:825–37.

Geller DS, Gorlick R. (2010). Osteosarcoma: a review of diagnosis, management, and treatment strategies. Clin Adv Hematol Oncol. 8:705–18.

Gogolin S, Ehemann V, Becker G, Brueckner LM, Dreidax D, Bannert S,et al. (2013). CDK4 inhibition restores G1–S arrest in MYCN - amplified neuroblastoma cells in the context of doxorubicin-induced DNA damage. Cell Cycle. 12:1091–104.

Goldstein-Jackson SY, Gosheger G, Delling G, et al. (2005).: Extraskeletal osteosarcoma has a favourable prognosis when treated like conventional osteosarcoma. J Cancer Res Clin Oncol 131 (8): 520-6.

Gorlick R, Janeway K, Marina N. Chapter 34: Osteosarcoma. (2016). In: Pizzo PA, Poplack DG, eds. Principles and Practice of Pediatric Oncology. 7th ed. Philadelphia Pa: Lippincott Williams & Wilkins.

Hopyan, S., N. Gokgoz, R.S. Bell, I.L. Andrulis, B.A. Alman, and J.S. Wunder. (1999). Expression of osteocalcin and its transcriptional regulators core-binding factor alpha 1 and MSX2 in osteoid-forming tumors. J. Orthop. Res. 17:633–638.

J. A. López-Guerrero, C. López-Ginés, A. Pellín, C. Carda, and A. Llombart-Bosch. (2004). "Deregulation of the G1 to S-phase cell cycle checkpoint is involved in the pathogenesis of human osteosarcoma," Diagnostic Molecular Pathology, vol. 13, no. 2, pp. 81–91.

J. W. Martin, M. Yoshimoto, O. Ludkovski et al., (2010). "Analysis of segmental duplications, mouse genome synteny and recurrent cancer-associated amplicons in human chromosome 6p21-p12," Cytogenetic and Genome Research, vol. 128, no. 4, pp. 199–213.

J. Yang, D. Yang, Y. Sun et al., (2011). "Genetic amplification of the vascular endothelial growth factor (VEGF) pathway genes, including VEGFA, in human osteosarcoma," Cancer, vol. 117, no. 21, pp. 4925–4938.

Jasnau S, Meyer U, Potratz J, et al. (2008). Craniofacial osteosarcoma Experience of the cooperative German-Austrian-Swiss osteosarcoma study group. Oral Oncol 44 (3): 286-94.

Jeff W. Martin, Jeremy A. Squire, and Maria Zielenska, (2012). "The Genetics of Osteosarcoma," Sarcoma, vol. 2012, Article ID 627254, 11 pages, https://doi.org/10.1155/2012/627254.

Jiyong Liang, Judit Zubovitz, Teresa Petrocelli, Rouslan Kotchetkov, Michael K. Connor, Kathy Han, Jin-Hwa Lee, Sandra Ciarallo, Charles Catzavelos, Richard

Beniston, Edmee Franssen, Joyce M. Slingerland. (2002). Nature Medicine, Volume 8, Number 10, Page 1153

Johnson, S. M., Torrice, C. D., Bell, J. F., Monahan, K. B., Jiang, Q., Wang, Y., … Sharpless, N. E. (2010). Mitigation of hematologic radiation toxicity in mice through pharmacological quiescence induced by CDK4/6 inhibition. The Journal of clinical investigation, 120(7), 2528–2536. doi:10.1172/JCI41402

Kager L, Zoubek A, Kastner U, Kempf-Bielack B, Potratz J, Kotz R et al. (2006). Skip metastases in osteosarcoma: experience of the Cooperative Osteosarcoma Study Group. J Clin Oncol. 24: 1535–1541.

Kansara M, Thomas DM. Molecular pathogenesis of osteosarcoma. (2007). DNA Cell Biol; 26: 1–18.

Kim MS, Cho WH, Song WS, et al. (2007).: time dependency of prognostic factors in patients with stage II osteosarcomas. Clin Orthop Relat Res 463: 157-65.

Kreahling, J. M., Foroutan, P., Reed, D., Martinez, G., Razabdouski, T., Bui, M. M., … Altiok, S. (2013). Wee1 inhibition by MK-1775 leads to tumor inhibition and enhances efficacy of gemcitabine in human sarcomas. PloS one, 8(3), e57523. doi:10.1371/journal.pone.0057523.

Lamora, A., Talbot, J., Mullard, M., Brounais-Le Royer, B., Redini, F., & Verrecchia, F. (2016). TGF-β Signaling in Bone Remodeling and Osteosarcoma Progression. Journal of clinical medicine, 5(11), 96. doi:10.3390/jcm5110096

Lian, J. B., & Stein, G. S. (1992). Concepts of Osteoblast Growth and Differentiation: Basis for Modulation of Bone Cell Development and Tissue Formation. Critical Reviews in Oral Biology & Medicine, 3(3), 269–305. https://doi.org/10.1177/10454411920030030501

Liao, L. Q., Yan, H. H., Mai, J. H., Liu, W. W., Li, H., Guo, Z. M., … Liu, X. K. (2016). Radiation-induced osteosarcoma of the maxilla and mandible after radiotherapy for nasopharyngeal carcinoma. Chinese journal of cancer, 35(1), 89. doi:10.1186/s40880-016-0153-8.

Lindsey, B. A., Markel, J. E., & Kleinerman, E. S. (2016). Osteosarcoma Overview. Rheumatology and therapy, 4(1), 25–43. doi:10.1007/s40744-016-0050-2.

Luo Y, Leverson JD. (2005). New opportunities in chemosensitization and radiosensitization: modulating the DNA-damage response. Expert Rev Anticancer Ther. 5(2):333–42.

Manic G, Obrist F, Sistigu A, Vitale I. (2015). Trial Watch: Targeting ATM-CHK2 and ATR-CHK1 pathways for anticancer therapy. Mol Cell Oncol. 2(4):e1012976.

McClendon AK, Dean JL, Rivadeneira DB, Yu JE, Reed CA, Gao E, et al. (2012). CDK4/6 inhibition antagonizes the cytotoxic response to anthracycline therapy. Cell Cycle 2012;11:2747–55.

McIntyre JF, Smith-Sorensen B, Friend SH, et al. (1994). Germline mutations of the p53 tumor suppressor gene in children with osteosarcoma. J Clin Oncol. 12(5):925–930.

Meloche S, Pouyssegur J. (2007). The ERK1/2 mitogen-activated protein kinase pathway as a master regulator of the G1- to S-phase transition. Oncogene. 26(22):3227–39.

Meyers PA, Schwartz CL, Krailo M, et al.: Osteosarcoma: a randomized, prospective trial of the addition of ifosfamide and/or muramyl tripeptide to cisplatin, doxorubicin, and high-dose methotrexate. J Clin Oncol 23 (9): 2004-11, 2005.

Mills, C. C., Kolb, E. A., & Sampson, V. B. (2018). Development of Chemotherapy with Cell-Cycle Inhibitors for Adult and Pediatric Cancer Therapy. Cancer research, 78(2), 320–325. doi:10.1158/0008-5472.CAN-17-2782.

Mirabello, L., Koster, R., Moriarity, B. S., Spector, L. G., Meltzer, P. S., Gary, J., … Savage, S. A. (2015). A Genome-Wide Scan Identifies Variants in NFIB Associated with Metastasis in Patients with Osteosarcoma. Cancer discovery, 5(9), 920–931. doi:10.1158/2159-8290.CD-15-0125.

Mirabello, L., Troisi, R. J., & Savage, S. A. (2009). International osteosarcoma incidence patterns in children and adolescents, middle ages and elderly persons. International journal of cancer, 125(1), 229–234. doi:10.1002/ijc.24320.

Mirabello, L., Yeager, M., Mai, P. L., Gastier-Foster, J. M., Gorlick, R., Khanna, C., Savage, S. A. (2015). Germline TP53 variants and susceptibility to osteosarcoma. Journal of the National Cancer Institute, 107(7), djv101. doi:10.1093/jnci/djv101.

Misaghi, A., Goldin, A., Awad, M., & Kulidjian, A. A. (2018). Osteosarcoma: a comprehensive review. SICOT-J, 4, 12. doi:10.1051/sicotj/2017028.

Nitiss JL. Targeting DNA topoisomerase II in cancer chemotherapy. Nat Rev Cancer. 2009;9(5):338–50.

Noll DM, Mason TM, Miller PS. Formation and repair of interstrand cross-links in DNA. Chem Rev. 2006;106(2):277–301.

Pikman, Y., Alexe, G., Roti, G., Conway, A. S., Furman, A., Lee, E. S., Place, A. E., Kim, S., Saran, C., Modiste, R., Weinstock, D. M., Harris, M., Kung, A. L., Rettew A. N., Young E. D., Lev D. C., Kleinerman E. S., Abdul-Karim F. W., Getty P. J., et al. (2012). Multiple receptor tyrosine kinases promote the in vitro phenotype

of metastatic human osteosarcoma cell lines. Oncogenesis 1, e34.10.1038/oncsis.2012.34

S. J. Harper and D. O. Bates, “VEGF-A splicing: the key to anti-angiogenic therapeutics?” Nature Reviews Cancer, vol. 8, no. 11, pp. 880–887, 2008.

Saha, D., Saha, K., Banerjee, A., & Jash, D. (2013). Osteosarcoma relapse as pleural metastasis. South Asian journal of cancer, 2(2), 56. doi:10.4103/2278-330X.110483

Sait, S., & Modak, S. (2017). Anti-GD2 immunotherapy for neuroblastoma. Expert review of anticancer therapy, 17(10), 889–904. doi:10.1080/14737140.2017.1364995.

Sampson, V. B., Gorlick, R., Kamara, D., & Anders Kolb, E. (2013). A review of targeted therapies evaluated by the pediatric preclinical testing program for osteosarcoma. Frontiers in oncology, 3, 132. doi:10.3389/fonc.2013.00132.

Sampson, V. B., Vetter, N. S., Zhang, W., Patil, P. U., Mason, R. W., George, E., … Kolb, E. A. (2016). Integrating mechanisms of response and resistance against the tubulin binding agent Eribulin in preclinical models of osteosarcoma. Oncotarget, 7(52), 86594–86607. doi:10.18632/oncotarget.13358.

Sarver AL, Thayanithy V, Scott MC, Cleton-Jansen AM, Hogendoorn PC, Modiano JF, Subramanian S. Micrornas at the human 14q32 locus have prognostic significance in osteosarcoma. Orphanet J Rare Dis. 2013;8.

Sayles, L.C., Breese, M.R., Koehne, A.L. et al. Genome-informed targeted therapy for osteosarcoma. ([Epub ahead of print]) Cancer Discov. 2018 Sep 28;https://doi.org/10.1158/2159-8290.CD-17-1152.

Sharon A. Savage and Lisa Mirabello, "Using Epidemiology and Genomics to Understand Osteosarcoma Etiology," Sarcoma, vol. 2011, Article ID 548151, 13 pages, 2011. https://doi.org/10.1155/2011/548151.

Tanaka, T., Narazaki, M., & Kishimoto, T. (2014). IL-6 in inflammation, immunity, and disease. Cold Spring Harbor perspectives in biology, 6(10), a016295. doi:10.1101/cshperspect.a016295.

Taran, S. J., Taran, R., & Malipatil, N. B. (2017). Pediatric Osteosarcoma: An Updated Review. Indian journal of medical and paediatric oncology : official journal of Indian Society of Medical & Paediatric Oncology, 38(1), 33–43. doi:10.4103/0971-5851.203513.

Thomas, D. M., Johnson, S. A., Sims, N. A., Trivett, M. K., Slavin, J. L., Rubin, B. P., Hinds, P. W. (2004). Terminal osteoblast differentiation, mediated by runx2 and p27KIP1, is disrupted in osteosarcoma. The Journal of cell biology, 167(5), 925–934. doi:10.1083/jcb.200409187.

Wang LL. Biology of osteogenic sarcoma. Cancer J 2005; 11: 294–305.

Wittrant Y., Théoleyre S., Chipoy C., Padrines M., Blanchard F., Heymann D., Rédini F. RANKL/RANK/OPG: New therapeutic targets in bone tumours and associated osteolysis. Biochim. Biophys. Acta. 2004;1704:49–57. doi: 10.1016/j.bbcan.2004.05.002.

Yang C, Boyson CA, Di Liberto M, Huang X, Hannah J, Dorn DC, et al. (2015). CDK4/6 Inhibitor PD 0332991 Sensitizes Acute Myeloid Leukemia to Cytarabine-Mediated Cytotoxicity. Cancer Res;75:1838–45.

Yang, R. K., & Sondel, P. M. (2010). Anti-GD2 Strategy in the Treatment of Neuroblastoma. Drugs of the future, 35(8), 665.

Yu, W., Liu, Z., Tang, L., Lin, F., Yao, Y., & Shen, Z. (2017). Efficacy and safety of stereotactic radiosurgery for pulmonary metastases from osteosarcoma: Experience in 73 patients. Scientific reports, 7(1), 17480. doi:10.1038/s41598-017-14521-7.

Zhao, H., Faltermeier, C. M., Mendelsohn, L., Porter, P. L., Clurman, B. E., & Roberts, J. M. (2014). Mislocalization of p27 to the cytoplasm of breast cancer cells confers resistance to anti-HER2 targeted therapy. Oncotarget, 5(24), 12704–12714. doi:10.18632/oncotarget.2871.