# DIFFERENTIATION OF PUTATIVE CANCER STEM CELLS AND ITS EFFECT ON TUMORIGENICITY IN OSTEOSARCOMA

By

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To my Mother

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DIFFERENTIATION OF PUTATIVE CANCER STEM CELLS AND ITS EFFECT ON TUMORIGENICITY IN OSTEOSARCOMA

By

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In previous work, our group had shown that a subpopulation of cells in osteosarcoma, a mesenchymal malignancy, have certain stem cell like properties. They expressed embryonic stem cell transcription genes essential in maintaining pluripotency (Oct-4 and Nanog), they were capable of growing as spherical colonies, and they had the capacity for self renewal *in vitro*. Additionally, some of our cell lines formed tumors when xenografted subcutaneously into non-obese diabetic/severe combined immune deficient (NOD/SCID) mice.

To investigate the relationship between "stemness" and tumorigenesis, a cell line (OS521Oct-4/GFP) was transfected with a reporter construct containing the human Oct-4 promoter linked to the gene for green fluorescent protein, and an independently regulated neomycin resistance gene. These transgenic cultures were observed to express GFP heterogeneously, as were the tumors that were formed from them. Using fluorescent activated cell sorting, the GFP+ cells were found to be 100 fold more tumorigenic than GFP- cells in terms of penetrance and kinetics, thus indicating that cells capable of expressing the Oct-4 gene had enhanced tumorigenic capacity.

To further investigate the putative osteosarcoma stem cell, we clonally expanded the Oct-4/GFP+ cells and characterized their capacity to form tumors following xenotransplantation in

NOD/SCID mice. Delivery of the cells yielded tumors that were heterogeneous for Oct-4 expression, showing that Oct-4/GFP+ cells can give rise to GFP- cells *in vivo*. We also demonstrated that subsequent passage of these cells gives rise to a population heterogeneous for Oct-4/GFP expression. This suggested that the loss of tumorigenicity was associated with cellular differentiation as indicated by the inability to activate the Oct-4 promoter.

Secondly, because differentiation of tumor cells has proven to be therapeutic in other forms of cancer, we wanted to examine the effect of induced differentiation of the putative osteosarcoma stem cell. OS521Oct-4/GFP clones in monolayer were incubated in commercially available osteogenic differentiation media for 21 days. We observed a 32% reduction in the proportion of cells expressing Oct-4, suggesting that certain tumor cells differentiated and no longer possessed a stem like phenotype.

The clonally derived tumor cells were then incubated in the presence of BMP4 or BMP 7 at 200>ng/ml. Flow cytometry showed a 58% and 36% reduction in GFP expression in the BMP 4 and BMP7 treated cells, respectively. These cells were then xenografted subcutaneously into NOD/SCID mice, and despite apparent differentiation, tumors still formed in both conditions. Analysis of the corresponding BMP4 tumors showed GFP expression at 10%, and the BMP 7 condition showed GFP expression at 70%.

These data suggest that a clonally derived population, homogeneous for Oct-4 expression, can undergo differentiation events leading to a loss of expression of the gene when passaged in vivo. The resulting tumors recapitulate the heterogeneity of the parental line and confirm the notion that GFP- cells arise from GFP+ cells. Further induced differentiation via BMP4/7 exposure can significantly decrease the proportion of Oct-4 expression, but not to the extent where tumor formation is completely inhibited.

# CHAPTER 1
# INTRODUCTION

Osteosarcoma (OS) is the most common primary bone malignancy of childhood and adolescence. Despite advances in surgery and chemotherapy, long-term survival rates have stagnated over the last 30 years, and still 40% of patients diagnosed with osteosarcoma die of their disease. The cancer stem cell theory, though, may account for the lack of effective chemotherapeutics in this and other types of cancer. Recent studies of leukemia, brain and breast cancer, suggest that within a tumor a subpopulation of cells with properties of stem cells, is responsible for tumorigenesis and malignancy while the remaining tumor mass is comprised of differentiated progeny of the tumor stem cell that are relatively benign. Thus, agents designed to eradicate the bulk of rapidly dividing cells within a tumor mass may not eliminate the actual tumorigenic cell. Following these reports, we initiated studies to explore the participation of stem-like cells in bone sarcomas. Identification of such a cancer stem cell could provide valuable insight into the process of tumorigenesis in osteosarcoma and perhaps a more effective target for therapeutic intervention.

In previous and ongoing work we have demonstrated that a subpopulation of cells in bone sarcomas share attributes with certain stem-, or stem-like cells: 1) They demonstrated the capacity for self-renewal *in vitro* in clonal expansion assays (they formed sarcospheres in anchorage-independent serum starved conditions), and *in vivo* following serial xenotransplantation in immunocompromised mice; 2) cells from tumors were capable of being induced to differentiate along mesenchymal lineages, and 3) they differentially expressed the embryonic stem (ES) cell-specific genes Oct-4 and Nanog. We saw a direct correlation between the expression of Oct-4 and the functional behavior of cells isolated from bone sarcomas. We found that *in vitro*, as our sphere cultures enlarged and differentiated, the percentage of cells

expressing these genes decreased. In at least one patient derived osteosarcoma cell line (OS521Oct-4/GFP), tumorigenesis, as indicated by the capacity to form tumors following xenotransplantation in immunocompromised mice, appeared to be functionally linked to the ability of the cells to express an exogenous reporter construct. This construct contained the full length Oct-4 promoter linked to the coding region for green fluorescent protein GFP) and an independent SV40-promoter driven neomycin resistance cassette. This allowed positive selection of cells that acquired the plasmid, irrespective of their capacity to transcribe the Oct-4 promoter. Thus, in medium supplemented with G418, all surviving cells would contain the plasmid and express the neomycin resistance gene; however, only the subset of cells that were capable of activating the Oct-4 promoter sequence would fluoresce green. We found that following stable transfection of the osteosarcoma line, expression of Oct4 was heterogeneous. Only about 24% of the G418 resistant cells were GFP+. This subpopulation of GFP+ cells had greater than 100 fold enhanced tumorigenicity relative to populations that did not transcribe the Oct-4 promoter. Those cells that *in vivo* spontaneously lost the capacity to express the Oct-4 reporter construct also lost their ability to form tumors following xenotransplantation.

These data suggest that the tumorigenic cells in osteosarcoma, a somatic malignancy, have been reprogrammed to an “ES cell-like” phenotype and that this phenotype drives tumorigenicity.

The goal of my research was to investigate the relationship between cellular differentiation and tumorigenic capacity in osteosarcoma. We hypothesized that a subset osteosarcoma cells, which expressed the embryonic transcription factor Oct-4, retained a stem cell like phenotype; and because of this, these cells were responsible for tumor initiation. Cellular differentiation of these cells should silence the expression of this gene, resulting in a

loss of their stem cell like qualities, thereby negatively impacting their tumorigenic capacity. We addressed this hypothesis through two specific aims.

**Aim 1: To Identify the Origin of the Heterogeneous Expression Of Oct-4/GFP in the OS521Oct-4GFP Cell Line**

We isolated and expanded individual GFP+clones from xenograft tumors to determine if the heterogeneity in Oct-4/GFP expression observed *in vivo* (e.g. 67% Oct-4/GFP+ vs. 33% GFP-) arose from expansion of pre-existing GFP+ and GFP- cell populations in the cell inoculums, or represented changes in expression of the Oct-4/GFP reporter arising from proliferation/differentiation of Oct-4/GFP+ tumor initiating cells. We have previously shown that activity of the Oct-4/GFP reporter identifies a highly tumorigenic sub-population of cells in osteosarcoma tumors. We reasoned that any changes in expression of the exogenous Oct-4 promoter could be attributed to changes in the biology of the cells and not to pre-existing heterogeneity in the injected cell population.

**Aim 2: To Address the Role of Differentiation on Tumorigenic Capacity of OS521Oct-4/GFP Clones**

We examined the ability of culture conditions and agents that induce cellular differentiation of stem cells to alter expression of the Oct-4/GFP reporter *in vitro,* and inhibit tumorigenesis *in vivo*. As indicated in Aim 1, we hypothesized that change in expression of the Oct-4/GFP reporter and loss of tumorigenic potential *in vivo* are the results of proliferation/differentiation of Oct-4/GFP+ tumor initiating cells.

# CHAPTER 2
# METHODS

## Derivation of Osteosarcoma Cell Lines from Patient Biopsies and Description of phOct-4/GFP

For each tumor sample, cells were isolated from ~1 cubic cm of tumor from an open biopsy taken from patients that have not previously received any form of chemotherapy. Histologic analysis of a frozen section taken from an adjacent area of the biopsy specimen was used to confirm the diagnosis of osteosarcoma prior to cell culture preparation. From the biopsy, the tissue matrix was enzymatically digested with 0.06g each of collagenase type I and II, and 0.3μg of neutral protease for thirty minutes under agitation. The isolated cells were then expanded in monolayer culture. To assess the tumorigenicity of the respective osteosarcoma cell lines, the cells were first trypsinized, washed and counted. The cells were resuspended in a minimal volume of saline solution, and $3x10^4$ cells were injected subcutaneously into the backs of separate groups of 6 NOD/SCID mice. (From previous experience with this model system we have found that the incidence of tumor formation does not increase significantly at cell doses beyond $3 \times 10^4$/mouse.) The animals were then be monitored for tumor growth over a period of 8 weeks. For this initial screen we worked to identify cell lines that formed tumors in at least 3 animals/group at 6 weeks post-injection. From cell lines that were capable of tumorigenesis at a sufficient frequency, the resulting tumors were harvested, enzymatically digested and the isolated cells pooled.

The tumor cultures were then expanded and split into thirds. One portion of the cells were frozen in liquid nitrogen and archived. The second was reseeded into culture and remained unmodified. The third portion was transfected with the phOct-4/GFP construct, and 24 hours later seeded into media containing G418 to positively select for cells containing the plasmid. Following the initial selection (typically 70-80% survive), the transfected cells were then split on

alternate days for 14-21 days at a ratio of 1:3 to ensure stable transfection. The cells in culture were then expanded to obtain sufficient numbers for evaluation of the relative tumorigenicity of GFP-enriched and GFP-depleted cell populations in NOD/SCID mice. These cells were also used to establish clonal populations.

**Xenotransplantation and Tumorigenicity Assays**

The animals used in this project were maintained according to University of Florida IACUC guidelines for tumor studies in experimental animals. All animal studies were performed according to IUCUC protocol number D780.

Cells to be xenografted in to NOD/SCID mice from monolayer were first trypsinized, washed and counted, then resuspended in Optimem. The desired concentration of cells was calculated so that injection volumes equaled 100µL/mouse.

Fresh tumors, from which cells were to be xenografted into NOD/SCID mice, were first enzymatically digested as described above. The isolated cells were then expanded for a period of 24 hours in monolayer with standard culture media (DMEM/F-12, 500µg/ml G418, 10% fetal bovine serum, 3% penstrep). Preparation of these cells for injection then followed monolayer preparation protocol.

Injection protocols were as follows. Mice were lightly anesthetized via isoflourane inhalation and, using 1ml insulin syringes, were subcutaneously inoculated on the dorsal side between the scapulas. Mice recovered from anesthesia on their own, and were then monitored daily for tumor formation. The skin of the back was palpated for tumor growth, and the numbers of animals with tumors, days to tumor onset, and tumor size was scored for tumorigenicity assays. When tumors reached ≥ 1.0 cm in diameter or 12 weeks post injection, they were harvested and prepared as necessary for desired assays. The lungs of all animals with tumors were also harvested and examined for metastases.

## Fluorescence Activated Cell Sorting and Flow Cytometry

Cells to be analyzed via FACS and/or flow cytometry were first plated at a density of $1x10^6$ cells in Corning $25cm^2$ flasks, 18-24 hours prior to the assay. The cells were allowed to expand in 7.5ml of Gibco DMEM F-12 media, with the addition of 500µg/ml G4-18. Directly before analysis, the cells were trypsinized and washed two times in a freshly prepared 0.05% PBS/BSA solution. Cells were recovered by centrifugation at 1,000RPM for 4 minutes and resuspended at a density of $1x10^5$ cells per 500µl of the PBS/BSA solution. Analysis was then performed on the BD FACS ARIA© for sorting, or the BD LSR II© Flow Cytometer to determine the number of cells expressing GFP, as well as the intensity of the fluorescence.

If antibodies were used to assay for the presence of specific cell surface antigens, following the two initial washes, additional steps are as follows. (First, a solution of IgG was used to block nonspecific binding using 40µl / $1x10^6$ cells for 15 minutes.) Immediately following this, 10µl of the desired specific phycoerythrin conjugated antibody was incubated with an aliquot of the cells. Following two more 0.05% PBS/BSA solution washes, a 20 minute incubation was performed.

The flow cytometer was set to record 10,000 events, at the same settings for all tests; FSC Voltage: E-1, FSC Amp Gain: 868, SSC Voltage: 304, SSC Amp Gain: 1.0, FL1 Voltage: 412, FL1 Amp Gain: 1.0, FL2 Voltage: 427, FL2 Amp Gain 1.0. Compensation was adjusted accordingly for each sample, yet FL2 compensation consistently ranged from 4.0% FL1 to 12% FL1 for optimal data acquirement.

Analysis of the data included setting the isotype or negative control at no more than a 3.0% error, in order to compensate for any autofluorescence or particulate matter that may have been contaminating the sample. No gates were used to isolate any specific populations in the analysis of the data, thus all recorded events have been included in the histograms.

## Clonal Expansion of OS521Oct-4/GFP Cells

Cells from an OS521Oct-4/GFP tumor that were heterogeneous for GFP expression were fractionated into enriched and depleted populations using FACS. The resulting populations were then counted, serially diluted, and plated in separate 96 well dishes at a density of one cell per well. Two hours post plating, allowing the cells enough time to adhere to the dish, the wells were visually inspected to confirm the presence of only a single cell per well. Wells with multiple cells or those without cells were discarded at that time. The cells were then visually checked every 24 hours to monitor proliferation. Additionally, if cells began to form colonies in two different areas in the well, they would be discarded, ensuring that clones used for experimentation proliferated from a single cell. The cells were incubated in standard culture media and allowed to expand for two weeks. As the populations reached confluence, they were expanded to larger wells, until they could be maintained in $25cm^2$ flasks.

## *In vitro* Osteogenic Differentiation

OS521Oct-4/GFP clones from monolayer were seeded at a density of $4x10^4$ cells/$cm^2$ in 24 well plates- in either standard culture media or in StemXVivo complete osteogenic base media (R&D Systems). The active ingredients in this media includes β-glycerol-phosphate, L-ascorbic acid, and dexomethasone. Samples were then cultured for 21-28 days, during which time the cells were passaged and analyzed for GFP expression by flow cytometry every 7 days.

## Treatment with Bone Morphogenic Proteins

Two different methods were used to examine the effect of BMPs on Oct-4/GFP expression and tumorigenicity of osteosarcoma tumor cells: adenovirus (Ad.) mediated transduction of BMP 2, 4, or 7, or culture in conditioned media derived from transduced cells. *Adenovirus-mediated transduction of BMP 2, 4, or 7 into* osteosarcoma *tumor cells:* For adenovirus infection, cells were isolated by enzymatic dissociation of freshly harvested tumors

and cultured overnight. The following day cells were trypsinized and reseeded at a density of $10^4$ cells/$cm^2$ in 24 well dishes in standard culture media and cultured for an additional 12-16 hours. The media were then aspirated, and the cells were washed 3 times with PBS. Cultures were then transduced in Optimem for 4 hours with Ad.BMP 2, 4, or 7 at an estimated multiplicity of infection of 10 infectious particles per cell. In control experiments using Ad.GFP this dose was found to result in transduction of 90-100% of the cells as determined by flow cytometry. Samples were then cultured for an additional five days. Cell media was collected from each sample on days 2 and 5 to determine approximate BMP concentrations using commercially available ELISAs according to the manufacturer's protocol (Quantikine, R&D Systems). The respective BMP conditioned media were then stored at -$20^0$C for later use (see below). On day 7 post-transduction samples were trypsinized and the cells were counted. $1x10^5$ cells were transplanted into NOD/SCID mice and monitored as previously indicated for tumorigenicity experiments. Aliquots of the samples were analyzed by flow cytometry for Oct-4/GFP expression.

*Treatment of* osteosarcoma *tumor cells with BMP 2, 4, or 7 conditioned media:* Additional aliquots of cells isolated from xenotransplanted tumors were initially seeded as described above, but instead of transduction with adenovirus, they were incubated in the BMP conditioned media from adenovirus infected tumor cell cultures, in a 1:1 ratio with standard culture media, for 7-10 days. BMP treated and untreated samples were then analyzed by flow cytometry for Oct-4/GFP expression and transplanted into NOD/SCID mice ($1X10^5$ cells/mouse) and monitored for tumor formation as before.

# CHAPTER 3
# LITERATURE REVIEW

## The Biology of Cancer

Cancer is typified by a culmination of genotypic changes in otherwise normal functioning cells that lead to malignant behavior. These genetic mutations can be characterized as either loss or gain of function which then lead to observed cancer phenotypes. Loss of function occurs in tumor suppressor genes, which normally function to repress proliferation, repair DNA damage, or promote apoptosis. Gene silencing of tumor suppressors such as p53 is seen in a majority of human cancers (Meyers et al. 2005) and often arises from epigenetic changes such as hypermethylation of the CpG islands in the proximal promoter regions of the gene (Jones and Baylin 2002). Proto-oncogenes are genes whose aberrant activity causes normal cells to become cancerous either because they are mutated or expressed at the wrong time in development. The gene products can cause increased cellular proliferation independent of extrinsic growth signals as well as resistance to apoptotic mechanisms; these in turn drive cancer progression (Hanahan and Weinberg 2000). Tumorigenesis generally does not occur after just a single mutation. It has been suggested that several mutations, typically between four and seven rate-limiting stochastic events, must occur in normal human cells for them to acquire tumorigenic properties (Gibbs et al. 2002). According to Hananan and Weinberg (Hanahan and Weinberg 2000), the evolution from a normal cell to a cancer cell is the culmination of at least six essential alterations in cell physiology that collectively result in the capacity for malignant growth. These alterations include: self sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis.

These are generalizations about changes that have been observed in all types of cancer. Most cancer types have hallmark genes that have been mutated, and each of these changes results in alterations in essential biological mechanisms. Understanding these mutations can change cancer treatments into a rational science with practitioners aiming treatments a specific molecular targets, thus improving patient comfort during treatment but more importantly increasing patient survival rates (Hanahan and Weinberg 2000).

## The Biology of Osteosarcoma

Osteosarcoma is a highly malignant mesenchymal tumor of bone in which the malignant cells produce osteoid. It can arise in any bone, but occurs primarily in the juxta-epiphyseal regions of rapidly growing long bones (Figure 5-1). The histopathologic appearance of high-grade intramedullary osteosarcoma is one of malignant spindle cells producing osteoid and immature bone. The bone structure is disorganized and can appear as a fine lacey trabecular pattern or as irregular clumps of osteoid, distinctly unlike normal bone formation. Classic osteosarcoma may also appear to be predominantly fibrous or chondroid with only small areas of osteoid formation (Gibbs et al. 2002). Grossly, osteosarcoma begins as a process destructive of medullary bone which progresses to destroy cortical bone, often with a large associated soft tissue component. The natural history of osteosarcoma is one of relentless local progression with loss of the function of the affected extremity and distant metastasis, most often to the lung(Chi et al. 2004; Meyers et al. 2005).A small percentage of patients develop bone metastases which are almost always fatal(Wuisman and Enneking 1990). Despite advances in the understanding of cancer biology, the characterization of the events that lead to the development of conventional osteosarcoma have yet to be defined. Difficulties impeding the molecular study of osteosarcoma include obtaining living samples that can be decalcified, as well as samples that are viable after

aggressive chemotherapies. Thus far, unlike many other cancers, there have not been specific translocations or chromosomal rearrangement events that can typify this malignancy.

Two genetic lesions that are often seen in osteosarcoma inactivate the p53 and retinoblastoma (Rb) tumor suppressor genes; however, because of the variety of genetic mutations involved in osteosarcoma, the relative contributions of these genes have yet to be determined (Sandberg and Bridge 2003). Alterations of the RB1 gene have been shown in up to 70% of reported cases, and loss of heterozygosity for RB1 has been shown to be a marker of poor prognosis (Li et al. 2005). Overexpression of the oncogene MDM2 has also been implicated as an important genetic alteration (Miller et al. 1996). Although this information has provided insight into aspects of the molecular dysregulation of osteosarcoma and its heterogeneous nature, to date these types of studies have been of limited value in establishing the molecular determinants of tumorigenesis or in the development of effective therapies(Ragland et al. 2002).

Additionally, drug resistance, primarily to methotrexate, by osteosarcoma cells, only increases the difficulty of treating patients. Two proposed reasons for drug resistance point to a defect in the reduced folate carrier, or alterations and/ or amplifications of the dihydrofolate reductase target. Likewise methotrexate resistance can stem from the functional loss of the RB protein. Resistance to other chemo therapeutic drugs can also be attributed to overexpression of p-glycoprotein, an ATP dependent transmembrane protein (Sandberg and Bridge 2003)

## Stem Cells

Stem cells are considered to be a rare group of cells with the capacity to self-renew and generate a developmental hierarchy of differentiating progeny. Pluripotent stem cells derived from the inner cell mass (ICM) of the blastocyst expand and differentiate to ultimately generate all the cells that comprise the adult individual. ES cells are derived from the ICM and retain the widest developmental capacity. They are pluripotent, indicative of few epigenetic changes due to

methylation, allowing them to differentiate into ectodermal, mesodermal, and endodermal lineages. Additionally, they maintain a stable karyotype and grow indefinitely in culture (Varga and Wrana 2005).

At the time of embryonic differentiation, alleged permanent silencing of embryonic genes is thought to occur via epigenetic modifications of their respective promoter regions. Histone modifications and DNA methylation serve to block the reactivation of these genes and thus prevent dedifferentiation of somatic cells (Hattori et al. 2004; Feldman et al. 2006).

Interestingly, several recent studies have challenged the apparent permanency of embryonic gene silencing by demonstrating the ability of differentiated mouse and human skin fibroblasts to be reprogrammed back to a pluripotent undifferentiated state (Meissner et al. 2007; Okita et al. 2007; Wernig et al. 2007; Yu et al. 2007). In these studies, murine fibroblasts were retrovirally transduced to express four transcription factors associated with pluripotency. Ectopic expression of these proteins was sufficient to induce epigenetic reprogramming of the somatic genome into an embryonic pluripotent state. The induced pluripotent stem (iPS) cells were shown to form viable chimeras and generate live late-term embryos when injected into tetraploid blastocysts (Meissner et al. 2007; Wernig et al. 2007). More recently, similar results have been obtained using human dermal fibroblasts lentivirally transduced to express transcription factors associated with pluripotency (Yu et al. 2007). Each of the generated iPS cell clones displayed human ES cell morphology, normal karyotype, and expressed cell surface markers and genes characteristic of human ES cells. All of the clones analyzed exhibited a demethylation pattern similar to that of human ES cells. These cells also demonstrated the capacity to differentiate into advanced cell types of each of the three primary germ layers (Okita et al. 2007; Yu et al. 2007).

These experiments indicate that the silencing of embryonic genes in adult cells may not be permanent.

Adult stem cells generate mature, differentiated cells that form specific tissues (Eckfeldt et al. 2005). Adult stem cells are characterized by their ability to self renew as well as the ability to differentiate into mature cells of a particular lineage (Reya et al. 2001). These characteristics allow these cells to maintain their population, while also allowing a more specialized response to enable repair of injured tissues.

*In vivo*, stem cells reside in a "niche," which is a specific location in a tissue where the cells can reside for an indefinite period of time and produce progeny cells while self-renewing (Ohlstein et al. 2004). Cellular division within the niche can take two different directions and must be carefully regulated. Too little proliferation may lead to a depletion of the necessary stem cell population; yet, it is possible for unchecked proliferation to result in tumorigenesis. Normally, self renewal or symmetric division occurs when a stem cell divides into two identical daughter cells. These cells remain in the niche and serve as functioning stem cells, maintaining pluripotency and sustaining the population. Asymmetric division occurs where two daughter cells are formed, but one cell stays within the niche to act as a stem cell, and the other cell leaves the niche in order to differentiate and proliferate into additional progeny. Due to different physiologic conditions, both symmetric and asymmetric division can occur within two different cells residing in the same niche (Yin and Li 2006).

Stem cells are often identified through *in vitro* studies, where cell surface/cytoplasmic proteins, transcription factors, and proliferative behaviors are often the defining features observed. In these studies, the natural *in vivo* environments are mimicked as closely as possible through exposure to important growth factors and morphogenetic molecules. Yet, these attempts

at reproducing the *in vivo* environment fail to include many important events and interactions. Cellular communication through cytokines, nonphysiological amounts of growth factors, and morphogens can have potent effects on stem cell behavior not typically observed *in vivo* (Steindler 2007).

## Cancer Stem Cell Theory

The cancer stem cell theory holds that there is a sub-population of cells within a tumor which, like normal stem cells, has the ability to self-renew. These cells can divide asymmetrically, producing an identical daughter stem-like cell and a more differentiated cell which upon subsequent divisions generates the vast majority of the tumor bulk, which is essentially benign. This stem-like cell is responsible for initiating and maintaining the growth of the tumor and if not completely eradicated by surgical extirpation or chemotherapy is responsible for local and distant recurrence (Figure 5-2) (Pardal et al. 2003). Along these lines, Weismann, drawing parallels between cancer stem cells and normal stem cells, has suggested that tumorigenesis can be viewed as aberrant organogenesis (Reya et al. 2001)

The first definitive work describing a cancer stem cell was performed by John Dick and colleagues in studies of acute myeloid leukemia (AML) (Bonnet and Dick 1997). They identified a rare population of human SCID leukemia initiating cells that were able to propagate AML in a xenograft transplant system. The leukemic grafts generated were representative of the patients' original disease phenotype. They demonstrated that the human AML stem cells purified from patient samples were $CD34^{+}CD38^{-}$, resembling the normal hematopoietic stem cell phenotype. Cells from the CD34+CD38+ fraction could not transfer the disease despite having a leukemic blast phenotype. This suggested that the normal hematopoietic stem cell was the target of leukemic transformation. Others have subsequently implicated stem-like cells in the

pathogenesis of brain and breast malignancies suggesting a broader involvement of stem cells in carcinogenesis (Ignatova et al. 2002; Al-Hajj et al. 2003; Hemmati et al. 2003; Galli et al. 2004).

It has been suggested that cancer is a disease of unregulated self-renewal in which abnormal stem cells utilize the machinery of self-renewal to drive neoplastic proliferation (Pardal et al. 2003). That cancer could arise from a primitive stem-like cell or other precursor seems reasonable as it would require far fewer genetic or epigenetic alterations to effect a malignant change in a cell already equipped with the capacity for self-renewal. Several of the genes shown to play a role in the regulation of normal stem cell self renewal (WNT, Sonic Hedgehog, Notch) have been found to be active in cancer (Jhappan et al. 1992; van de Wetering et al. 2002; Lessard and Sauvageau 2003; Pasca di Magliano and Hebrok 2003; Qiang et al. 2003).

**Use of Agents That Induce Differentiation for the Treatment of Cancer**

Several lines of evidence suggest that aberrant expression of the key regulatory proteins of ES cell pluripotency can directly contribute to tumorigenesis in several cell types. Therefore, agents that serve to inhibit the activity, or alternatively block the expression of these proteins could be beneficial in the treatment of cancer. Along these lines alterations in the differentiation programs of cancer cells often result in changes to their phenotype related to survival, rate of growth, loss of differentiation and the ability to proliferate and invade surrounding tissue (Hanahan and Weinberg 2000)Anaplasia, the loss of differentiation, is associated with aggressive clinical behavior, suggesting differentiation confers a restraint on tumorigenesis (Thomas and Kansara 2006). In fact, apart from the presence of metastatic disease, the degree of differentiation of a sarcoma is the most powerful negative prognostic indicator. Interestingly, differentiation therapy is already in clinical use. The most striking example may be the use of all-trans-retinoic acid (ATRA) in acute promyelocytic leukemia, felt my most to be a stem cell

malignancy. 90% of patients can expect remission of their disease with combination therapy consisting of both conventional chemotherapy and ATRA (Ohnishi 2007).

The role of BMP signaling in cellular biology is far reaching and diverse. One of these roles lies in determining cell fate choices during differentiation. Data from work with BMP's in mouse ES cells shows that their role sharply contrasts with that of analogous human cells, where neural differentiation is blocked by BMPs in mice, yet induced in humans (Varga and Wrana 2005).

BMPs are secreted proteins and can direct mesenchymal stem cells (MSCs) to chondrogenic and osteogenic cell lineages, and in the presence of fibroblast growth factors, they can direct ES cells to differentiate into the trophoblast lineage in humans. BMPs are part of the TGF-β superfamily, and transmit signals through a defined pathway. The BMP binds tightly to BMPI/II receptor heterodimer upon which the type II receptor phosphorylates the type I receptor. In the cytoplasm, the downstream effect of BMP binding causes phosphorylation of BMP R-Smads, specifically 1, 5, and 8, which complex with Smad4. The heteromeric complexes then translocate to the nucleus where they can regulate transcription either directly or in concert with other transcription factors (Varga and Wrana 2005).

BMPs may have an important role in controlling the biology of stem cell cancers as they play a crucial role in early stem cell development, as well as self-renewal; losing the ability to regulate these functions may lead to tumorigenesis (Varga and Wrana 2005). In support of this are studies showing that mutation of the BMP I receptor is a huge risk factor in developing gastrointestinal cancers. Expanding the potential of differentiation therapy beyond leukemias, a recent report by Piccirillo et al. (Piccirillo and Vescovi 2006)showed that stem-like, tumor-initiating cells isolated from human glioblastomas when incubated with certain BMPs increased

the expression of markers of neural differentiation, and showed decreased proliferation and tumor formation when transplanted into experimental animals. Their work implies that certain populations of tumor stem cells retain an ability to respond to normal signals of maturation induction, and efforts to devise therapies to differentiate cancer cells might be fruitful.

Histone deacetylases (HDAC) play a major role in the epigenetic changes which regulate gene expression within a cell. HDACs catalyze the removal of acetyl groups, and thus stimulate chromatin condensation and promote transcriptional repression. Transcriptional repression can translate to the loss expression of tumor suppressor genes, contributing to the formation of cancer. HDAC over-expression has been observed in colon, breast, prostate, and other cancers. Due to their wide-spread involvement in cancer, HDACs have become a novel target for therapy. HDAC inhibitors, which include a variety of therapeutic agents, can lead to the reversal of epigenetic silencing. HDAC inhibitors come in the form of short chain fatty acids, hydroxamic acids, benzamides, and cyclic tetrapeptides. The actions of these drugs affect cellular processes such as inducing cell cycle arrest, stimulating tumor cell death, and promoting differentiation. HDAC inhibitors induce cellular differentiation and promote cell cycle arrest at the $G_1$/S checkpoint. Clinical trials have shown that HDAC inhibitors have anti cancer activity and are being tested as either monotherapies or in combination with chemotherapy (Carew et al. 2008).

## Preliminary Results

### Cells Isolated from Bone Sarcoma Cultures Exhibit Stem-Like Attributes

We initiated a preliminary series of experiments to explore the existence of stem-like cells in bone sarcomas. The culture system originally used by Reynolds and Weiss (Reynolds and Weiss 1992) to generate neurosphere clones from adult mammalian brain, has since been found to isolate cells possessing attributes of stem and progenitor cells. The stressful growth conditions of this system were found to positively select for primitive cells by eliminating the

differentiated cells, which are unable to survive. Similarly, suspending dissociated cancerous tissue in semi-solid media without serum selects primitive clonogenic cells that can be expanded and give rise to different classes of cells. This system enables isolation of stem-like cells from malignant human brain tumors by exploiting anchorage independence, serum starvation and necessary pleiotropic growth factors(Ignatova et al. 2002). Similar sphere culture systems have been used to identify tumor stem cells from both brain and breast malignancies that are capable of self-renewal in mouse models (Al-Hajj et al. 2003; Hemmati et al. 2003; Singh et al. 2003; Singh et al. 2004).

We found that all bone sarcoma cultures formed spherical colonies ("sarcospheres") at a frequency of $10^{-2} – 10^{-3}$ , similar to that reported by others for brain and breast malignancies(Ignatova et al. 2002; Al-Hajj et al. 2003; Hemmati et al. 2003). Sarcospheres were also generated at a similar frequency from fresh tumor dissociates produced at the time of biopsy. Furthermore, all cultures tested demonstrated the capacity for self-renewal by the formation of secondary spheres at a similar or increased frequency of approximately $10^{-2}$.

We also examined these cultures for expression of Oct-4 and Nanog transcription factors found to be indispensible for the maintenance of pluripotency and self-renewal in ES cells. Using semi-quantitative RT-PCR we found detectable transcripts in each culture type for both transcription factors (Figure 5-3).

Immunohistochemical staining of sarcospheres from paraffin sections enabled detection of both Nanog and Oct-4 in similar patterns. Interestingly, the staining of the smaller spheres showed a high proportion of cells that were positive for both transcription factors. As the spheres increased in size and cell number, the cells showed increasingly greater heterogeneity of immunostaining with a lesser percentage expressing these ES cell markers. These observations

suggested a situation whereby during early sphere formation relatively primitive cells undergo a transition from symmetric to asymmetric division and thereupon produce daughter cells of various states of differentiation.

Following these observations, we addressed whether Oct-4 and Nanog are expressed in actual tumor tissue. For this, paraffin sections from eight bone sarcoma patients were evaluated using immunohistochemistry (Figure 5-4). Nanog and Oct-4 nuclear staining was observed in seven of the eight tumors studied. In each case, as determined by histologic criteria, the stained nuclei were of malignant cells, and not from infiltrating normal cells. Among different tumor specimens, the number of Oct-4 and Nanog positive cells varied considerably. Positive Oct-4 staining ranged from a few percent of the cells in some tumors to up to 25% in others. Nanog staining was also quite variable ranging from ~1% to nearly 50% in certain samples. These results showed that subpopulations of cells in bone sarcomas express regulatory proteins typically restricted specifically to embryonal cells.

We reasoned that if bone sarcomas express some of the molecular machinery of ES cells, they might, in addition to mesodermal genes, express genes from endodermal and ectodermal lineages. RT-PCR analyses of mRNA from adherent and sarcosphere cultures revealed expression of Gata-4, Gata-6 and alpha fetoprotein (AFP), indicative of endodermal differentiation (Figure 5-5a). Expression of β-III tubulin RNA was seen as well, which is believed to be a marker of neural-ectoderm, but has also been demonstrated in some poorly differentiated malignancies (Katsetos et al. 2003). Using Western blot analyses, expression of AFP and β-III tubulin was demonstrated at the protein level in each of seven cultures tested (Figure 5-5b). β-III tubulin was also detected in paraffin sections of tumors using immunohistochemistry and in cell culture samples by immunocytochemistry (Figure 5-5c-f). At

this point, we do not know the position that a putative bone-sarcoma stem cell might occupy in the stem cell hierarchy. It is possible that the expression of genes such as Gata-4 and Gata-6 is simply a consequence of a larger global pattern of dysregulated gene expression in these tumor cells. An alternative explanation is that these genes are indicative of aberrant pluripotent differentiation of cancer stem cells. Regardless, the detection of expression of ectodermal and endodermal genes implies that the tumor cells are not effectively lineage restricted and suggest epigenetic reorganization of the genome in bone sarcomas.

Although the bone sarcomas we have studied appear to express transcription factors associated with pluripotent ES cells as well as genes of endodermal and ectodermal lineages, the histologic phenotype of these tumors is by definition one of arrested mesenchymal differentiation. Thus, it seems reasonable to expect that a cancer stem cell in bone sarcoma would arise from a mutagenic lesion in a mesenchymal progenitor. Therefore, we examined the respective cultures for the presence of cells with characteristics of mesenchymal stem cells. Although the precise cell-surface phenotype of an MSC has not been determined, MSCs have been found to variously express the cell surface proteins Stro-1, CD90, CD29, CD73 CD44 and CD105(Simmons et al. 1994; Stewart et al. 1999; Li et al. 2005). Using immunocytochemistry and flow cytometry, we screened our tumor cell cultures and found that all the cultures expressed these antigens, but to different degrees. Although differences in the intensity of immunoreactivity of specific surface antigens were seen between cell cultures, within any particular culture the cells appeared largely homogenous for expression of these antigens. Unfortunately the apparent uniformity of the cultures with regard to expression of surface antigens precluded fractionation of the cells based on surface antigen, and thus could not be used strategically as a potential method to explore markers of tumorigenic cells. To determine if cells

within the cultures were indeed multipotent, we attempted to induce adherent cultures of bone sarcoma cells to differentiate along two distinct mesenchymal lineages by culture in osteogenic and adipogenic media. Within each adherent culture tested, we observed discrete foci of mineralization in cells grown in osteogenic medium, and fields of lipid laden cells in those grown in adipogenic medium (Figure 5-6).

Cumulatively, the data from the previous sections suggested that at least subpopulations of cells in bone sarcomas were capable of self-renewal, expressed transcription factors of ES cells as well as expression of ecto- and endodermal genes, and showed the capacity for differentiation along multiple mesenchymal lineages. Altogether these data support the involvement of stem like cells in bone sarcomas.

**Development of an *In vivo* Model to Examine the Role of Stem-Like cells in the Pathogenesis of Osteosarcoma**

To explore in more detail the functional relationship between "stemness" and tumorigenicity of osteosarcoma, we focused our studies on the OS521 line, derived from a high grade, poorly differentiated human osteosarcoma. This cell line was found to cause robust tumor formation following subcutaneous xenograft into the backs of NOD/SCID mice. In initial experiments we found that delivery of as few as $3 \times 10^4$ cells in saline suspension reproducibly produced tumors of >1 cm diameter in 4-6 weeks following injection. Similar to that observed from the biopsy of the original patient, tumors arising from the xenograft showed clear evidence of osteoid, recapitulating the characteristic phenotype of an osteosarcoma. Characterization of the OS521 culture for several cell surface markers showed that the cells in monolayer were comprised of a largely homogenous population without striking differences with regard to cell surface antigens. The cells were MHC class I+, CD90+, and NCAM+. Interestingly they were uniformly strongly positive for expression of CD44, a marker of breast cancer stem cells, and

negative for the presence of CD133 (Figure 5-8b) a marker of stem cells in colon, brain and prostate cancer.

**Expression of an Oct-4 Promoter/GFP Reporter Construct That Selectively Identifies Cancer Stem Cells in Osteosarcoma**

In an effort to selectively visualize and track living cells in culture that express ES cell-specific genes and determine their relative participation in tumorigenesis, we transfected the OS521 cells in monolayer with the plasmid construct phOct-4/GFP, (a generous gift from Dr. Wei Cui of the Rosalin Institute, UK) containing the human Oct-4 promoter sequence (spanning -3917 to +55, relative to the transcription start site) linked to the coding sequence for enhanced green fluorescent protein (Figure 5-7). This plasmid also contains an independent SV40-promoter driven neomycin resistance cassette, which allows positive selection of cells that have acquired the plasmid construct, irrespective of their capacity to transcribe the Oct-4 promoter. Thus in medium supplemented with G418 all surviving cells will contain the plasmid and express the neomycin resistance gene; however, only the subset of cells that are capable of activating the Oct-4 promoter sequence will fluoresce green. Gerrard et al.(Gerrard et al. 2005)showed that in human ES cells stably transfected with this construct, GFP expression driven by the Oct-4 promoter faithfully represented expression of Oct-4 in undifferentiated ES cells and during their differentiation. In these studies, GFP expression co-localized with endogenous Oct-4 protein as well as surface antigens SSEA-4 and Tra-1-60. Neural differentiation of the cells, as well as targeted knockdown of endogenous Oct-4 expression by RNAi, down-regulated GFP and correlated closely with the reduction in endogenous Oct-4 protein.

Following transfection of the OS521 cells with phOct-4/GFP and positive selection of transfectants in media containing G418 we characterized Oct-4 driven-GFP expression of cells in

monolayer using fluorescence microscopy and flow cytometry. Somewhat surprisingly, despite the apparent homogeneity of the cells in culture with regard to cell surface proteins, we found that following stable transfection of the OS521 line only about 24% of the G418 resistant cells were GFP+ (Figure 5-8a). To begin to determine the relative participation of the GFP+ cells (those that transcribe the Oct-4 promoter) in tumor initiation, we injected 3 x $10^4$ cells from the total neo resistant cell population (all cells both GFP positive and negative) subcutaneously into the backs of 6 NOD/SCID mice; the same dose of untransfected OS521 cells were injected into a separate group of 6 animals. At 3-5 weeks post-injection, tumors >0.5 cm diameter had formed in 5/6 and 4/6 animals of the two respective groups, which indicated that transfection of the phOct-4/GFP plasmid did not adversely influence the tumor-initiating potential of the OS521 cells. Tumors that were ≥ 1.0 cm diameter were harvested. Tumors from animals receiving the phOct-4/GFP transfected cells were brightly fluorescent under UV light, while tumors from animals receiving untransfected OS521 cells showed no evidence of GFP expression (not shown). Histologic section showed large clusters or foci of GFP+ cells distributed throughout the tumor mass (Figure 5-8a). Following harvest, the tumors were dissociated, and the cells recovered were characterized for GFP expression by flow cytometry. The proportion of GFP+ cells isolated from the tumors had increased to ~67%, nearly 3-fold over that observed in monolayer culture. There was no apparent change, however, in the expression of the various surface antigens with respect to the GFP positive and negative cell populations (Figure 5-8b). Altogether, these results suggested a selective amplification of cells that express the Oct-4 promoter during tumorigenesis.

To determine the relative tumor initiating capacity of the cells that expressed the Oct-4 promoter construct versus those that did not (i.e. GFP+ cells vs. GFP- cells, respectively), the

cells recovered from the harvested tumors were pooled and fractionated by FACS into GFP-enriched and GFP-depleted populations, as shown in Figure 5-9a-c. Subsequent flow cytometry analysis of a portion of the respective fractions showed that in the enriched fraction ~92% of the cells were GFP+; in the GFP-depleted fraction the number of GFP+ cells was reduced to about 3%. From a starting dose of 3 x $10^4$ cells, we injected ten-fold dilutions of the respective fractions, as well as equivalent numbers of unfractionated, phOct-4/GFP transfected cells, into individual groups of NOD/SCID mice at 8 animals/group and examined the rate of tumor formation.

The GFP-enriched fraction proved to be significantly more tumorigenic (>100-fold) than the GFP-depleted fraction (Figure 5-9d). At the 3 x$10^4$ cell dose it produced tumors in all mice with a mean time to onset of 22 days, while the GFP-depleted fraction only produced tumors in ~60% of the mice with a mean time to onset of over 6 weeks. At 3 x $10^3$ cells, again, all 8 of the animals receiving cells from the GFP-enriched fraction developed tumors, with a mean time to onset of 34 days. For the GFP-depleted group, only 1 of 8 mice developed a tumor over the 90-day time course. At the 3 x $10^2$ cell dose none of the mice from the GFP-depleted group developed tumors, while all of the animals receiving the GFP-enriched cells developed tumors, with a mean time to onset of 42 days. At this dose only 3 of 8 animals receiving unsorted/phOct-4/GFP transfected cells formed tumors, with a mean time to onset of ~60 days. Visualization of the freshly excised tumors using inverted fluorescence microscopy showed that all tumors formed in all groups were highly GFP+. Following dissociation, flow cytometric analysis of the recovered cells showed that tumors formed from the GFP-enriched fractions were comprised of ~70-80% GFP+ cells, while those from the GFP-depleted fractions were ~50-55% GFP+ (not shown).

Interestingly, we passaged the GFP+ cells through at least 3 rounds in mice, whereby the cells were injected, harvested from tumors, enriched and reinjected. At the 300 cell dose (~the lowest dose that we can reasonably expect to deliver) we found that the tumors appeared to increase in virulence with passage, producing tumors with shorter time to onset and more rapid growth rate. We also noted the formation of multiple local tumor nodules following a single injection. Analysis of the lungs of these mice using inverted fluorescence microscopy showed clear evidence of metastases, with clusters of GFP+ cells readily identified throughout. These data demonstrated that the cells that expressed the Oct-4 promoter construct displayed self-renewal *in vivo* and further supported the participation of a cancer stem cell in tumorigenesis of osteosarcoma.

# CHAPTER 4
# RESULTS

## Rationale for Aim 1

Based on previous data, we found that our exogenous Oct-4/GFP reporter could identify a subpopulation of cells with enhanced tumor forming capacity. These cells were capable of activating the Oct-4 promoter whose gene product is responsible for maintaining a pluri-potency in ES cells, hinting to the existence of a stem like tumor cell in human osteosarcoma. These cells were 100 fold more tumorigenic than those not expressing the Oct-4 promoter, and the resulting tumors were heterogeneous in nature for GFP expression. We questioned whether this heterogeneity was due to existing differences in the proliferation rates of the GFP+/- subpopulations, since fractions from FACS contained up to 8.0% cross contamination (Figures 5-9b, 5-9c), or if the differences represented changes in the ability of certain cells to express the Oct4 promoter. We aimed to investigate this by creating clonal populations of the respective phenotypes and repeating the tumorigenicity assays. Clonal expansion would create a population of cells that were homogeneous in nature, allowing any changes in Oct4/GFP expression to be attributed to changes in the biology of the progeny cells and not to selective amplification of GFP+ and GFP- subpopulations.

We reasoned that *in vivo*, tumor initiation occurs from GFP+ cells, which proliferate and differentiate to form cells that have lost Oct-4 expression as well as their tumorigenic potential. These GFP+ cells might be thought of as moving away from the stem like phenotype and becoming more specialized, as the transform from GFP+ to GFP-. Along these lines, we would not expect to see cells lacking the expression of the Oct-4 promoter to spontaneously gain the ability to express the gene either *in vivo* or *in vitro*, because this would signify an act of dedifferentiation towards a more stem like state. Due to epigenetic silencing, this is usually an

irreversible phenomenon. Using cells of a pure clonal population would allow us to observe any shifts in Oct4/GFP expression and to determine if the GFP- cell populations arise from GFP+ populations and not vice versa. Additionally, it would show that the appearance of a GFP- population was a direct result of biological changes in the tumorigenic cell and not a shift in the dynamics of the population due to proliferative differences.

**Rationale for Aim 2**

If in fact, cancer stem cells in osteosarcoma rely on the molecular machinery of ES cells that function to maintain pluripotency, then it would be rational to expect that the forced induction of cellular differentiation in these cells should either reduce the expression of these proteins, or alternatively modulate their effects, and in so doing reduce tumorigenicity. Along these lines, Feinberg and others have suggested that cancer stem cells from certain types of tumors retain the ability to respond to extrinsic differentiation signals (Jones and Baylin 2002).

In exploring the effects of differentiation agents on osteosarcoma stem cells, we examined if exposure of OS521Oct-4/GFP clones in monolayer to osteogenic differentiation media would stimulate cellular differentiation and change the expression of osteogenic-associated genes in these cells. If we could associate an increase in osteogenic associated gene transcription with a lack of expression of Oct-4, then we could assume that loss the stem like phenotype has occurred along with the differentiation event. This would be important in linking the lack of Oct-4 expression with a cell that has experienced differentiation.

We also chose to use BMPs 2, 4 and 7, which are well known inducers of osteogenic differentiation in human mesenchymal stem cells, to perform tumorigenicity assays with. If treatment with these agents induces a loss of Oct-4 expression via differentiation, resulting in a loss of tumorigenic capacity, we could assume that the tumor initiating potential of osteosarcoma depends on the retention of a stem like phenotype.

If we find that any of these differentiating agents decrease tumorigenicity, these data could form the platform for the development of novel therapies for this devastating disease.

**Clonal Expansion of OS521Oct-4/GFP cells**

In order to investigate our hypothesis that heterogeneity can be derived from a clonal population, we had to first create clones from our patient derived OS521 cell line. After completing this task, we observed the following characteristics of the different clones.

For the GFP+ fractions, we observed that 90% of the 96 wells held viable cells following serial dilutions and allowing cellular adherence. Any wells that were observed to contain more or less than one cell were immediately discarded. Over the course of two weeks, all of the GFP+ cells began to divide, yet three of the clones displayed more aggressive proliferation and had reached confluency. These clones, named A1, S1, and T1, were allowed to expand for experimental purposes. At six weeks the S1 and T1 clones had divided enough to populate a $25cm^2$ flask. The A1 clone inexplicably stopped dividing and expired four weeks into the expansion process. Fluorescence microscopy and flow cytometry were used to confirm the homogeneous expression of GFP in both clones. Figure 5-10a shows that the S1 clonal population in monolayer was 98% positive for GFP expression, which was also comparable to that seen in the T1population in culture.

Likewise, 90% of the GFP- population survived initial plating, yet after two weeks the cells had failed to achieve proficient division rates. Additional time was allotted for the expansion of the GFP- clones, but none of the cells succeeded and eventually died by 8 weeks. Fluorescent microscopy confirmed the absence of GFP in any of the cells, but there were not enough cells in any of the wells to perform a flow cytometry assay.

**Flow Cytometric Analysis of Clones and Clonally Derived Tumors**

We wanted to determine if the tumors formed from our largely homogeneous clonal populations would recapitulate the heterogeneity observed from the parental OS521 lines. This would allude to the ability of the cells to lose the expression of Oct-4 and proceed towards a more differentiated phenotype.

Following delivery of 3 x $10^4$ cells of the respective clones into NOD/SCID mice, tumors readily formed in animals within 2-3 weeks. Analysis by flow cytometry showed that the cells recovered from the tumors were markedly heterogeneous with respect to GFP expression, as shown in Figure 5-10b, with a clear reduction in the mean intensity of fluorescence. On first passage (Figure 5-10b), tumors exhibited fluorescence over a range of three logarithmic orders. The histogram shows a clear shift to the left in fluorescence intensity compared to that of the parental *in vitro* population, though there is no clear distinction between the two populations in the histogram. Interestingly, serial passage of unsorted cells from freshly dissociated tumors derived from these clones resulted in the production of discrete GFP+ and GFP- populations. They were composed of approximately 60% GFP+ and 40%GFP- by the third passage (Figure 5-10c). The GFP+ positive fraction more closely resembled that of the parent clonal population, with the majority of the cells having a fluorescence intensity greater than$10^4$.

We questioned whether these more discrete GFP+ and GFP- populations might also be distinct with regards to tumorigenic capacity. To this end we harvested passage three tumors, which are composed of the most segregated populations, from one of the clones (S1) and fractionated the cells by FACS into GFP enriched and depleted populations. We then transplanted them as previously described for tumorigenicity experiments. We observed results similar to those shown for our parental cultures/tumors using these clonally derived populations regarding the frequency and kinetics of tumor formation by the two fractions at the indicated

doses (compare Figure 5-9d and 5-11b). Analysis of the resulting tumor cell populations for Oct-4/GFP expression by flow cytometry showed that GFP enriched fractions formed tumors composed of 85% GFP+ cells, and GFP depleted fractions formed tumors composed 55% GFP+ cells (Figure 5-11c).

The data to this point show that Oct-4/GFP+ and Oct-4/GFP- cells generate heterogeneous tumors upon transplant, and are capable of extensive self-renewal *in vivo*: all of which are traits associated with cancer stem cells. However, in this instance the cancer stem cell is not a rare, slowly dividing stem-like cell but a highly proliferative cell whose active cell division directly supports the growth of the tumor.

**Culture of OS521Oct-4/GFP Clones in Osteogenic Media**

To attempt to link differentiation to the loss of the expression of Oct-4, we took OS521Oct-4/GFP clones from monolayer and attempted to stimulate differentiation with commercially available osteogenic media for a period of 21 days. Culturing of human ESCs and MSCs in these conditions has been shown to result in the activation of genes associated with osteogenic differentiation and matrix deposition (Gronthos and Simmons 1995). We believe that a loss of Oct-4 expression during the incubation would show that Oct-4/GFP- cells are indeed more differentiated than their fluorescent counterparts.

In order to prevent "rolling up" of the confluent monolayers, the cultures were trypsinized and reseeded every 7 days to remove dead cells and for analysis by flow cytometry. We were unable to detect significant matrix deposition or changes in the expression of genes associated with osteogenesis in samples cultured in osteogenic media by staining of the confluent monolayers with Alizarin Red S and RT-PCR, respectively (not shown). As shown in Figure 5-12, OS521Oct-4/GFP clones cultured in osteogenic media for 14 and 21 days were composed of both GFP+ and GFP- cells. On day 14 there were 57% GFP+ and 43% GFP- cells, and by day 21

the population was composed of 64% GFP+ and 46% GFP- cells. Controls grown under standard culture conditions remained unchanged for GFP expression (95%). These cells were not used in tumorigenicity assays.

**Culture of OS521Oct-4/GFP Clonally Derived Tumor Cells in BMP Conditioned Media**

We hypothesized that inducing differentiation through various agents, namely BMPs, would adversely affect the tumorigenic capacity of the OS521 clonally derived tumors. We attempted to stimulate differentiation using either adenovirus transduction or exposure of the cells to BMP treated media. Subsequent injection of the treated cells yielded the following.

BMP conditioned media was generated by infection of cells from tumors derived from clone S1, with adenovirus encoding the cDNAs for either BMP 4, or 7 as described. BMP 2 appeared to be toxic to the cells and they did not survive the transduction; as a result this portion was omitted from the experiment. Analysis by ELISA for BMP concentrations of cell free supernatants from both infections showed high concentrations of BMP protein (200>ng/ml). Approximately 3 days following the start of induction we first noted by visual observation that BMP-treated cultures adopted a flatter cellular morphology and took longer to grow to confluence than untreated controls. Unsorted tumor cell populations cultured in BMP 4 or -7 conditioned media for 7-10 days also resulted in alterations in the proportion of the GFP+ and GFP- fractions, with a clear decrease in mean fluorescence intensity (compare 13A and B). Interestingly this effect was more pronounced in the BMP 4 treated samples.

Tumors formed by transplantation of these cells ($1.0X10^5$ cells/mouse) also showed differences in the relative proportions of GFP+ and GFP- cell fractions (Figure 5-13c). We observed that for the BMP 4 treated sample, 10% of the cells were GFP+ following transplantation. This result is in stark contrast to the BMP 7 treated sample, where 70% of the cells were GFP+. This result contradicted what we had consistently observed for untreated

samples following transplantation; the GFP+ cell fraction usually expands or remains constant (compare Figures 5-13c and 5-11c). Time to tumor onset, penetrance and kinetics were all comparable to tumors formed by untreated controls.

# CHAPTER 5
# DISCUSSION

Our group has identified a putative CSC population in osteosarcoma using an Oct-4 promoter driven GFP reporter. We observed that the GFP enriched fraction of xenotransplanted tumor cells was roughly 100-fold more tumorigenic than the GFP depleted fraction when transplanted subcutaneously into NOD/SCID mice. We believe that silencing of the Oct-4/GFP transgene and concomitant loss of tumorigenic ability is the result of differentiation/proliferation of these cells during tumor initiation and growth.

In the present study we have focused on two distinct aims. We first showed that individual Oct-4/GFP+ clones were capable of generating heterogeneous tumors composed of both GFP+ and GFP- cells. This confirms that this cell retains a stem cell like phenotype and possesses key stem cell attributes, such as the ability to self renew and recapitulate heterogeneity of the initial population. Additionally it supports our hypothesis that changes observed in our experiments were due to biological effects and not the heterogeneous nature of the collective population. Secondly, we cultured cells from these tumors in osteogenic media or in the presence of BMP 4 or 7 and found that we were able to induce silencing of the Oct-4 promoter. We then took these treated cells and performed tumorigenicity assays. At a dose of $10^5$ unsorted cells tumors formed within 4 weeks however there were distinct differences between tumors formed by BMP 4 treated samples compared to samples exposed to BMP 7 regarding days to tumor onset and the proportions of GFP+ and GFP- cells fractions.

In order to obtain a truly homogeneous population to which variation could be attributed to biological changes, we clonally expanded the cells from OS521 Oct-4/GFP cell line. Successful expansion of the population expressing the Oct-4 gene product can be attributed to the fact that these cells maintain their pluripotency *in vitro* and thus their stem like qualities. The

ability of stem cell like cancer cells to divide both symmetrically and asymmetrically can explain the expansion and restoration of the cell population. Symmetric division, which is what we saw in our clones *in vitro*, contributed to the homogeneous nature of the clonal population. This makes sense since the cells still expressing Oct-4 maintain an ES cell like phenotype. We did not observe any asymmetric division *in vitro*, leading to more differentiated cells, until the cells were exposed to the *in vivo* environment in our tumorigenicity assays. The *in vitro* environment apparently lacks the necessary cues to induce change or gene silencing in the population.

The lack of Oct-4 expression in the GFP- cells allude to the reason why they cells were not able to clonally expand. Loss of Oct-4 expression points directly to a cell which no longer transcribes the Oct-4 gene, which is crucial in maintaining pluripotency in ES cells. Along these lines, a cell that is no longer behaving as a stem cell would be unable to divide in the manner required to reinstate a stable self maintaining population.

Phenotypic characteristics of the clones that were established *in vitro* showed that they were 98% positive for GFP expression. Upon multiple passages of these cells *in vitro*, the relative homogeneity remained at the highly uniform levels. Loss of Oct-4 promoter activity was not observed in our cultures, which we attribute to the absence of the proper cues that a cell would normally be exposed to in an *in vivo* environment. Basic media and cell/cell contacts do not provide stimulation for the cells to differentiate or initiate any of the associated epigenetic changes, and they symmetrically divide to maintain their population and existence.

Changes in GFP expression can be induced through serial passage *in vivo* in the NOD/SCID mouse model. Tumor progression is often viewed as driven by epigenetic plasticity and genomic instability. Epigenetic heterogeneity within stem and progenitor populations could in part account for tumor cell heterogeneity. From these observations we postulate that one

possible explanation for our results is that Oct-4/GFP activity identifies tumor cells with a stem-like epigenetic signature with the capacity for infinite self-renewal. Loss of Oct-4/GFP expression may be the result of differentiation of the cancer stem cell population during tumor growth and then an adoption of a more differentiated epigenetic signature. This leads to a loss of stem-associated activities and the ability to initiate and sustain tumorigenesis *in vivo*.

As the number of serial passages increased, we observed that the gap between GFP+ and GFP- populations increased as well (Our results clearly show that from initial injection and a nearly pure GFP+ cell population, we can form tumors that are comprised of a substantial number of GFP- cells.) We believe that this phenomenon occurs when the tumor initiating GFP+ cells are exposed to the *in vivo* micro-environment providing contact with the extracellular matrix, cellular cues of proliferation, and growth and proliferation factors. These cells are now stimulated to divide asymmetrically, where changes induce a loss of Oct-4/GFP expression, and thus increase the number of GFP- cells. These differentiated cells accumulate and contribute to the bulk of the tumor mass, while the less differentiated GFP+ cells maintain their primitive stem like qualities and their tumorigenic potential. Additionally, these changes cause the GFP- cells to lose their tumor initiating potential. With passage *in vivo*, the clonally expanded Oct-4/GFP+ cells diverge, eventually adopting a profile of GFP expression similar to the parental line (Figure 5-10). They display a population composed of both GFP+ and GFP- cells. This observation supports two concepts consistent with a cancer stem cell. First, this shows that tumors formed from the clonally expanded line are capable of recapitulating the heterogeneity of the Oct-4 expression of the original population. More importantly though, this suggests that GFP- cells are derived from GFP+ cells, suggesting that any GFP- cells arise as a result of cellular differentiation.

FACS of clonally derived tumor cells into GFP enriched and GFP depleted fractions, followed by xenotransplantation over a range of cell doses, showed that cells that continued to express the Oct-4 promoter sequence were significantly more tumorigenic than cells that had lost that capacity (Figure 5-11). Consistent with results from the non-clonally derived cell population, the GFP+ cell fraction produced tumors in all animals at all doses. Administration of as few as 300 cells was sufficient to cause tumor formation in all animals tested. The GFP- fraction in contrast formed tumors in only 50% of the mice at 30,000 cells and 25% at 3,000 cells. Analysis of the resultant tumors from each cell fraction showed that first passage tumors from the GFP+ fraction were comprised of 85% fluorescent cells. Tumors from the GFP- fraction were also highly GFP+ with about 55% of the cells showing fluorescence above background.

As before, we believe the tumors arising from the GFP- population in all probability are the result of low level contamination of GFP+ cells present in the depleted fraction. Foremost, the majority of the cells in tumors generated from the GFP depleted fraction are GFP+. In our exprerience, we have never observed cells reacquire the capacity to express the Oct-4 reporter once lost. Furthermore, as seen in Figure 5-11b a dose of only 300 GFP+ cells is sufficient to form tumors in all mice. Since typically between 3-8% of the cells in the respective fractions are contaminants of the opposing fraction, we would expect between 900-2400 GFP+ cells in the 30,000 cell dose and 90-240 in the 3,000 dose, numbers at both dilutions that approach or exceed the minimum established tumorigenic dose.

Cells from the GFP- fraction only formed tumors in doses of $3x10^3$ or greater, and in 50% or less of the mice. Tumor formation in this condition is most likely due to cross contamination of GFP+ cells in the GFP depleted fraction. Figure 5-9a-c shows that the level of cross contamination can reach 8.0% in the fractionated samples. Along these lines, a dose of $3x10^3$

GFP- cells contains enough GFP+ cells to initiate tumor formation, and doses of 300 cells or less do not contain enough cells with tumor forming capacity. Additionally, we see that the tumor is composed of 55% GFP+ and 45% GFP- cells (Figure 5-11c). This heterogeneity is also most likely due to the cross contamination of GFP+, putative tumor initiating cells that were injected. This small subpopulation would be required to divide more times to form a tumor, when compared to tumors formed from a GFP enriched fraction. As this small number of GFP+ cells divide, they will lose the ability to express the Oct-4 promoter, resulting in a larger population of GFP- cells in the tumor.

As the population of the serial passed clones regressed back to resemble the Oct-4/GFP expression of the parental line, we found that we could diverge the two populations further by exposure of the cells to osteogenic differentiation media as well as BMP 4 and BMP 7.

Exposure to osteogenic media resulted in an overall reduction in GFP expression from the Oct-4 promoter producing a heterogeneous population of approximately 60% GFP+ and 40% GFP- cells. Despite these changes in transcription of the Oct-4 promoter were unable to detect significant matrix deposition by staining of the confluent monolayers with Alizarin Red S, or changes in the expression of genes associated with osteogenesis using RT-PCR. This may have occurred for several reasons. First, OS521 is classified histologically as a highly dedifferentiated osteosarcoma and thus may be relatively resistant to certain differentiation cues. Along these lines, it is possible that this cell line may require a more extended incubation to stimulate the cells to begin secreting matrix. It is also possible that deposited extracellular matrix components were lost during passage of the confluent cultures during the extended incubation. Additionally, changes in the expression of genes associated with osteogenesis may not have been detectable

because of the heterogeneity of the treated cell population. Fractionating the cells into their respective GFP+ and GFP- populations, before RT-PCR analysis, may have resolved this issue.

.Differentiation from BMP stimulation occurs when the protein causes the transmembrane BMPI receptor to phosphorylate the BMPII receptor and form a heteromeric complex. Intracellularly, the BMPII receptor phosphorylates the SMAD1/5 proteins, which are cytoplasmic. These, in turn, induce the upregulation of the transcription factors RUNX2 and Osterix, which are critical to osteoinduction. BMPs are also known to activate MAPKs which upregulates RUNX2. We postulate that by activating these pathways, we have induced osteogenic differentiation which causes a loss of the expression of our transgenic reporter gene. This in turn would allow a population of more differentiated but less tumorigenic cells to increase in number, consistent with the increased GFP- population.

Upon xenografting the cells at a dose of 100k cells/mouse, we observed tumorigenesis in 2/3 of the mice inoculated in both BMP conditions. We attribute this to the large number of cells injected. It should be noted that in previous work we established that in GFP enriched populations, tumors readily formed at doses of three hundred cells. Along these lines it is highly probable that at least three hundred Oct-4/GFP+ cells were delivered that maintained their tumorigenic potential. Even if a smaller dose was administered ($\leq$ 30k cells/mouse), we believe we would most likely observe tumor formation because we would still be injecting a sufficient dose of tumorigenic cells. Though a large portion of the cells from the sample were indeed induced to differentiate (loss of GFP expression), and thus had lost their tumorigenic potential, the underlying fact that a small dose of cells retain their tumorigenic capacity does not change. The remaining GFP+ population, still retaining their tumorigenic potential, had perhaps not been given enough time to respond to the BMP signaling. It may also be the case that under the

conditions used; they did not receive a sufficient amount of stimulation from the BMPs. As a treatment option, differentiation therapy using agents such as BMP4 or BMP7 exposure might do little to halt tumor formation due to the very small number of cells required to initiate a tumor. Even though these molecules do induce differentiation, the relatively small subpopulation of cells that can form a tumor persists. If this were to be used as an effective therapy, stimulatory effects must induce virtually every cell in the tumor to differentiate.

What is the nature of the relationship between Oct-4/GFP expression, tumorigenesis, and differentiation at the molecular level? This is a difficult question to address since tumorigenic ability is a complex phenotype involving multiple factors and pathways and BMPs have pleiotropic effects. It is likely that Oct-4/GFP activity is a product of the activity of a pathway or group of factors critical to the tumorigenic phenotype.

Future work on this project will involve further characterization of the isolated cancer stem cells to identify differences at the molecular level between the GFP+ and GFP- populations. These studies should provide insight into the pathways and mechanisms responsible for conferring tumorigenicity and provide clues to the development of therapeutic strategies that target them.

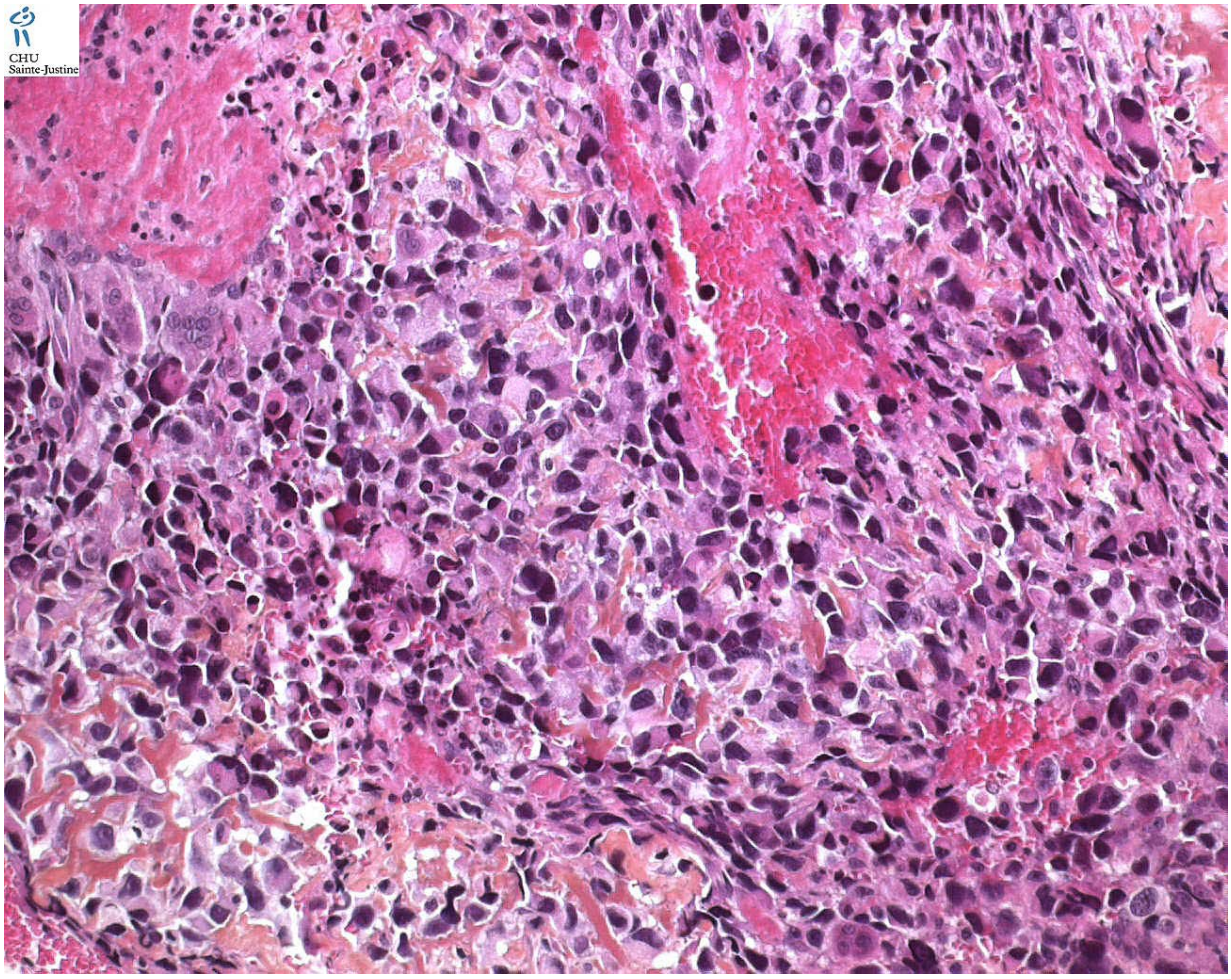



Figure 5-1. Osteosarcoma: H&E stain of a femoral osteosarcoma.

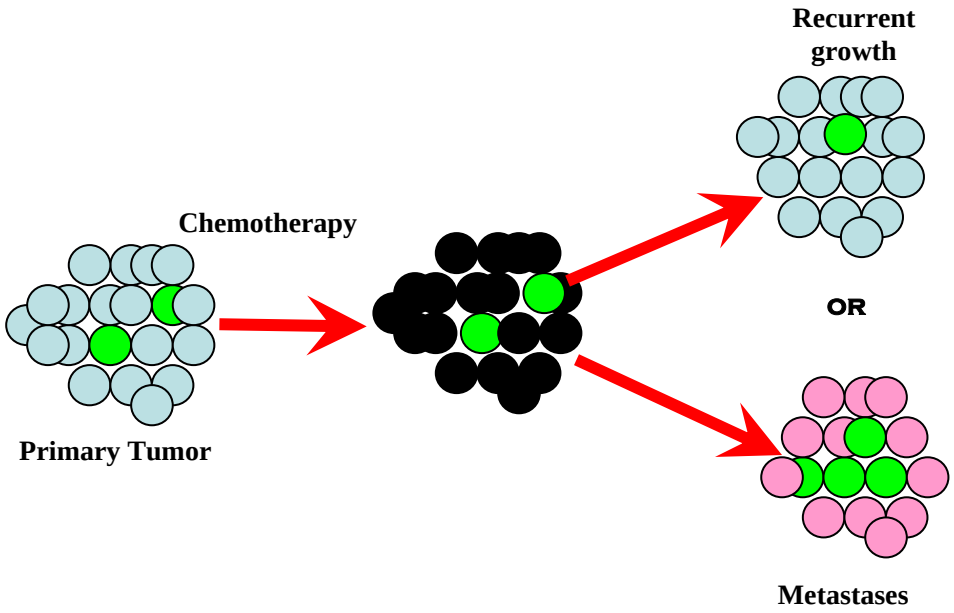



Figure 5-2. Cancer Stem Cells. Treatment of the primary tumor with chemotherapeutic agents kills the rapidly dividing cells of the tumor bulk (blue to black) but fails to eradicate the tumor stem cells (green) which can then cause re-growth of the primary tumor or disseminate to form distal metastases.

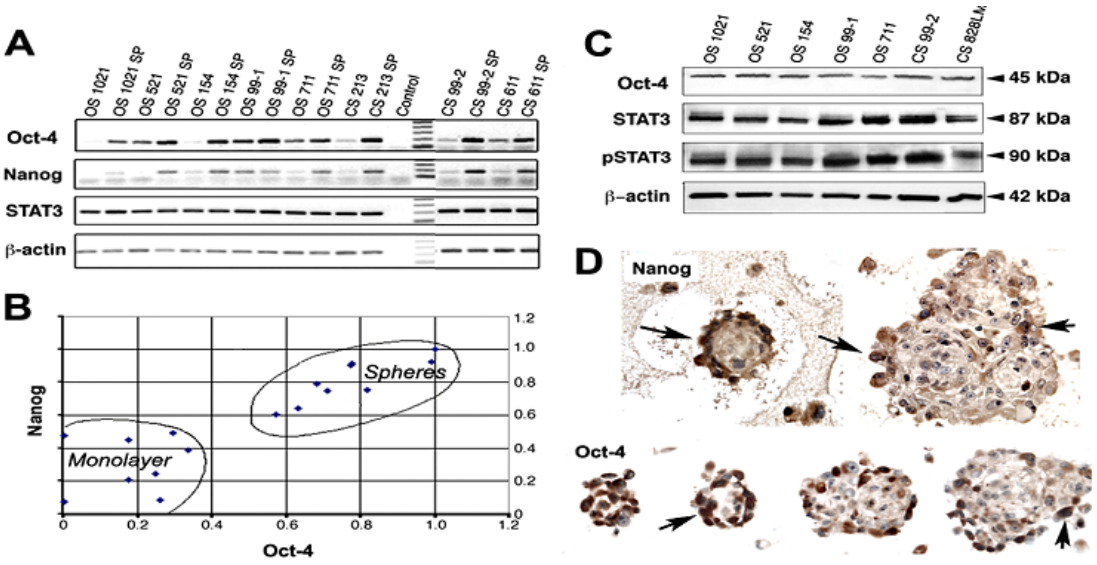



Figure 5-3. ESC-specific Genes in Sarcospheres. Genes specific to ESCs show increased expression in sarcosphere cultures derived from bone sarcomas. (A) Monolayer and sarco-sphere (SP) cultures from five osteosarcoma (OS) and three chondrosarcomas (CS) were analyzed for Oct-4, Nanog and STAT3 mRNA by RT-PCR; β-actin expression was used as a positive control. Sphere cultures demonstrate increased transcription of both Oct-4 and Nanog over adherent cultures; STAT3 expression was uniform between both culture types. (B) Relative band intensities for Oct-4 and Nanog for each culture from *(A)* were quantitated by densitometry, normalized relative to β-actin and plotted on the graph shown (Oct-4, x-axis; Nanog, y-axis). As indicated by the grouping, the sphere cultures of each sarcoma showed significantly greater expression of both Oct-4 and Nanog than adherent monolayer cultures ($p<0.05$, Pearson's correlation). (C) Western blot analysis of lysates from representative bone sarcoma cell cultures for protein expression of Oct-4, STAT3 and activated (phosphorylated, p) STAT3. β-actin was used as a positive control for loading, membrane transfer and immunoblotting. All cultures showed positive staining of protein bands of the appropriate sizes as indicated. (D) Small and large sarcospheres were embedded in fibrin and then paraffin section and stained using immunohistochemistry for Nanog and Oct-4 as indicated. Small spheres show intense staining in cells in the periphery. Large spheres show similar numbers of darkly staining cells with dramatically increased numbers of poorly staining cells in the interior of the sphere.

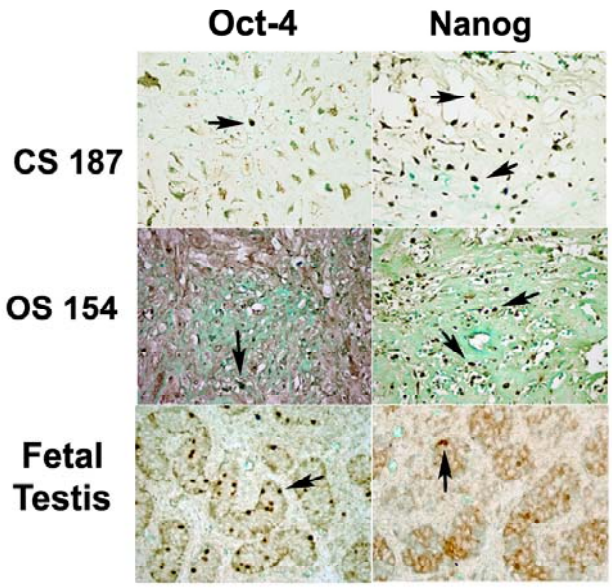



Figure 5-4. Immunohistochemical staining for Oct-4 and Nanog in sections from tumor biopsies of chondro- and osteosarcoma. One representative osteosarcoma (OS-154) and chondrosarcoma (CS-187) and a positive control, human fetal testis, are shown as indicated. CS-187 shows a single nuclei positive (brown) for Oct-4 and multiple nuclei positive (brown) for Nanog in a lung metastasis from a chondrosarcoma. OS 154 sections demonstrate scattered Oct-4 nuclear staining and near complete nuclear Nanog staining in a primary fibular osteosarcoma. Twenty-six week fetal testis with scattered Oct-4 and Nanog nuclear staining are shown as positive controls.

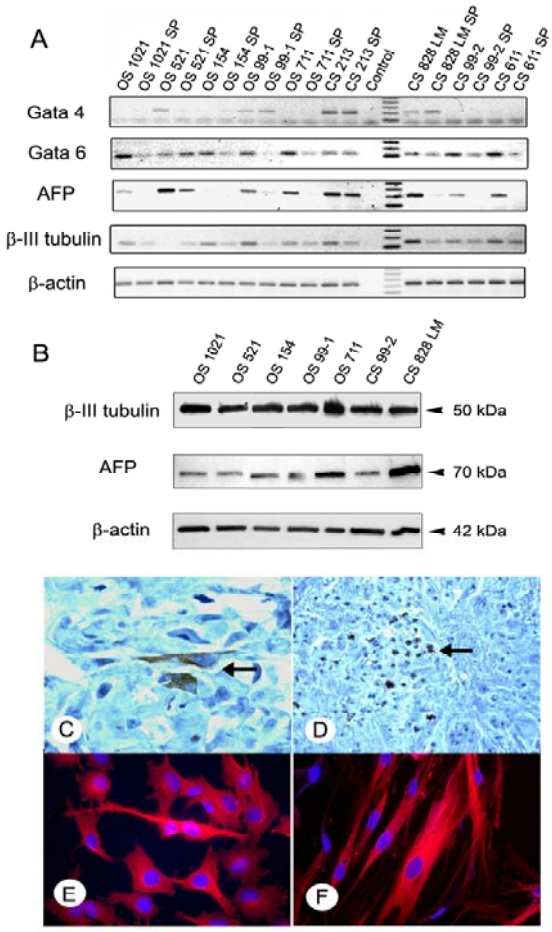



Figure 5-5. Analyses of bone sarcoma cultures for expression of genes of endo- and ectodermal lineages. (A) RT-PCR analyses of adherent and sarcosphere cultures for transcription of endoderm-associated genes (Gata-4, Gata-6, and alpha fetoprotein [AFP]) and the neuro-ectoderm marker, β-III tubulin. Primers for b-actin were used as positive reaction controls as indicated. Control lane represents parallel RT-PCR reactions performed without reverse transcriptase. (B) Western blot analyses for expression of b-III tubulin and AFP from lysates of adherent cultures shown in panel A, demonstrating protein expression of endoderm and neuro-ectoderm-associated genes. (C and D) Expression of b-III tubulin in tissue specimens from bone sarcomas as detected by immunohistochemistry, and in adherent cultures (E and F) demonstrated by immunocytochemistry. In panels *C and D*, arrows indicate regions of positive staining. In panels E and F areas of b-III tubulin staining are seen in red. Nuclei were counterstained blue using Hoechst's stain.

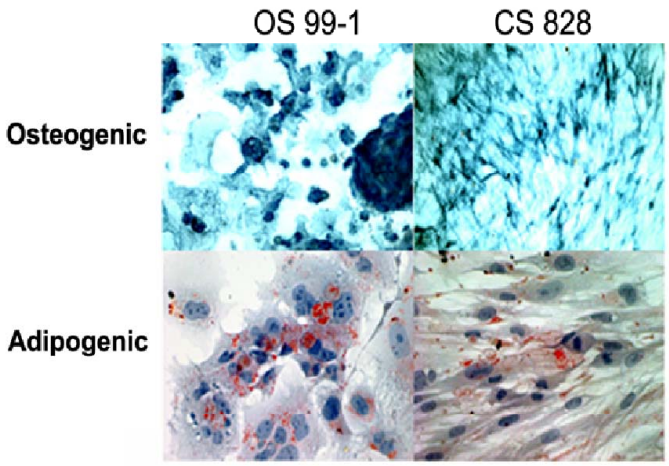



Figure 5-6. Multipotent cells in bone sarcoma**.** Following incubation in osteogenic or adipogenic media to induce differentiation along mesenchymal lineages, the respective cultures were analyzed for mineralization by Von Kossa staining or for lipid vacuoles by staining with Oil-red –O. As shown, both cultures showed focal staining for osteogenic and adipogenic differentiation.

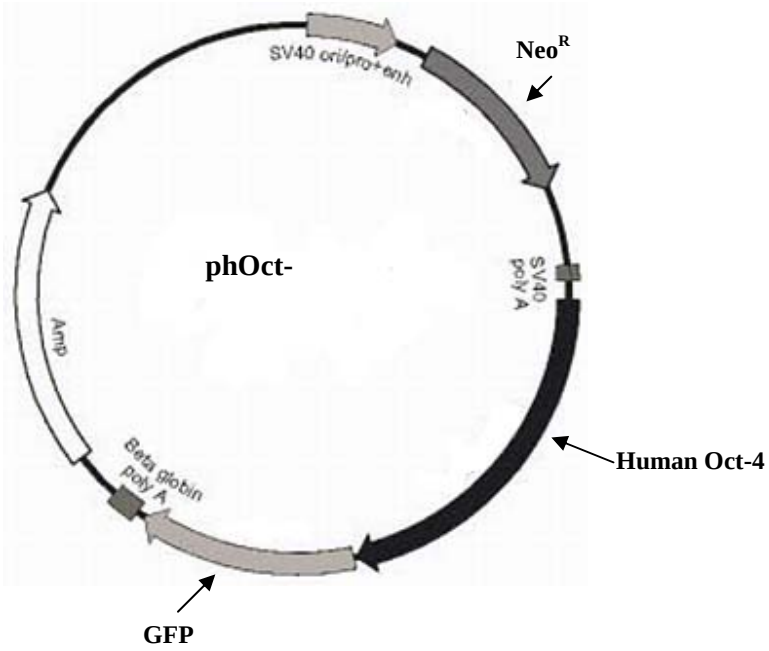



Figure 5-7. Schematic of phOct-4/GFP**.** The human Oct-4 promoter (~4kb) was cloned upstream of the gene for Green Fluorescent Protein (GFP). The plasmid also contains an SV40promoter-driven Neomycin resistance gene allowing for selection using G418 ($Neo^R$).

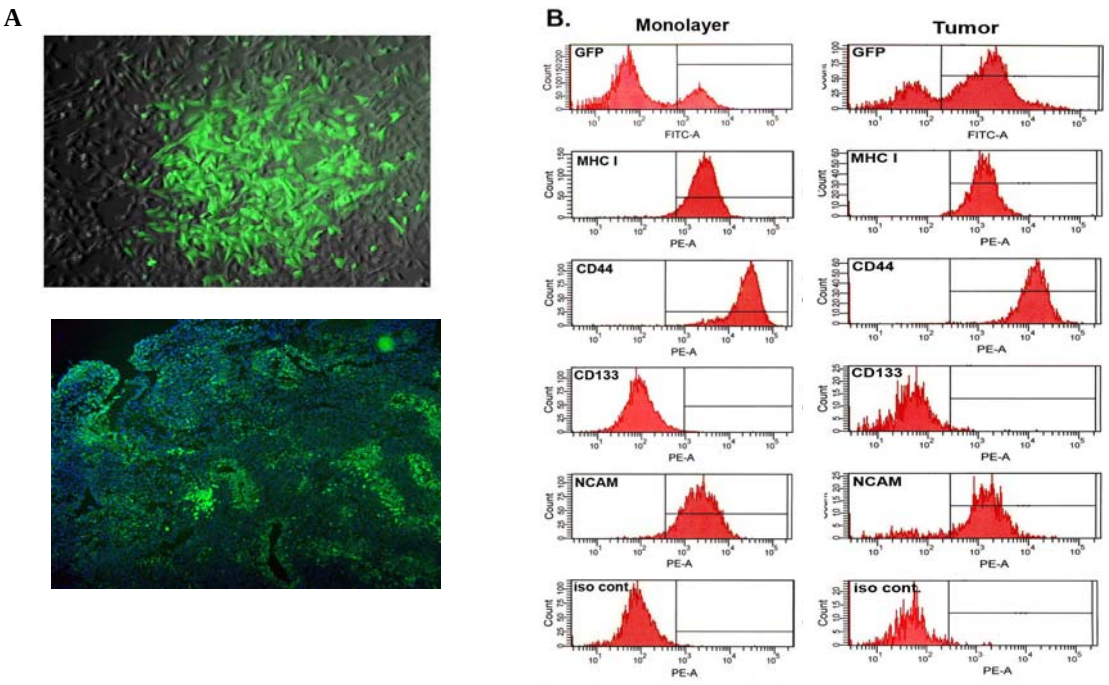
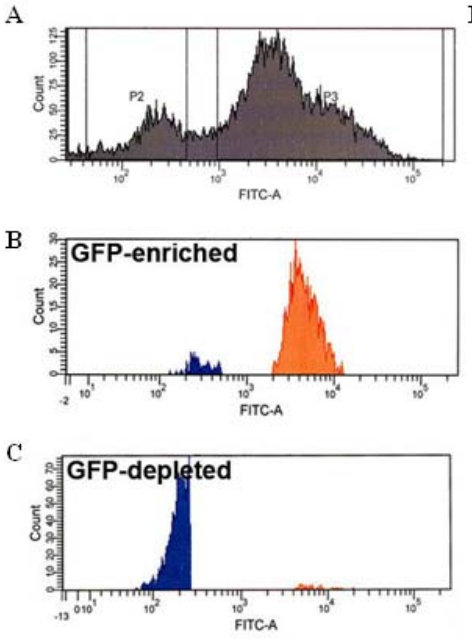



Figure 5-8. Expression of phOct-4/GFP and surface antigens in OS521 in vitro and in vivo. (A) Visualization Oct-4/GFP transfected monolayer cultures using inverted fluorescence microscopy (top panel) and in tumors by immunofluorescence (bottom panel). (B) Analysis of OCT-4/GFP and surface antigen expression by flow cytometry in monolayer culture and xenotranplanted tumors of OS521.




D

| Cell Dose | GFP-enriched | Unsorted | GFP-depleted |
|---|---|---|---|
| 30,000 | 8/8 22 days | 6/8 26 days | 5/8 47 days |
| 3,000 | 8/8 34 days | 6/8 44 days | 1/8 51 days |
| 300 | 8/8 45 days | 3/8 60 days | 0/8 90 days |

Figure 5-9. The Oct-4/GFP enriched tumor fraction of OS 521 is highly tumorigenic following delivery into NOD/SCID mice. (A) Xenotranplanted tumors were harvested and fractionated by FACS into GFP-enriched and depleted populations for tumorigenicity experiments. The resulting populations were 92% and 97% pure as shown (B and C, respectively). (D) The frequency and rate of tumor formation of the two fractions.

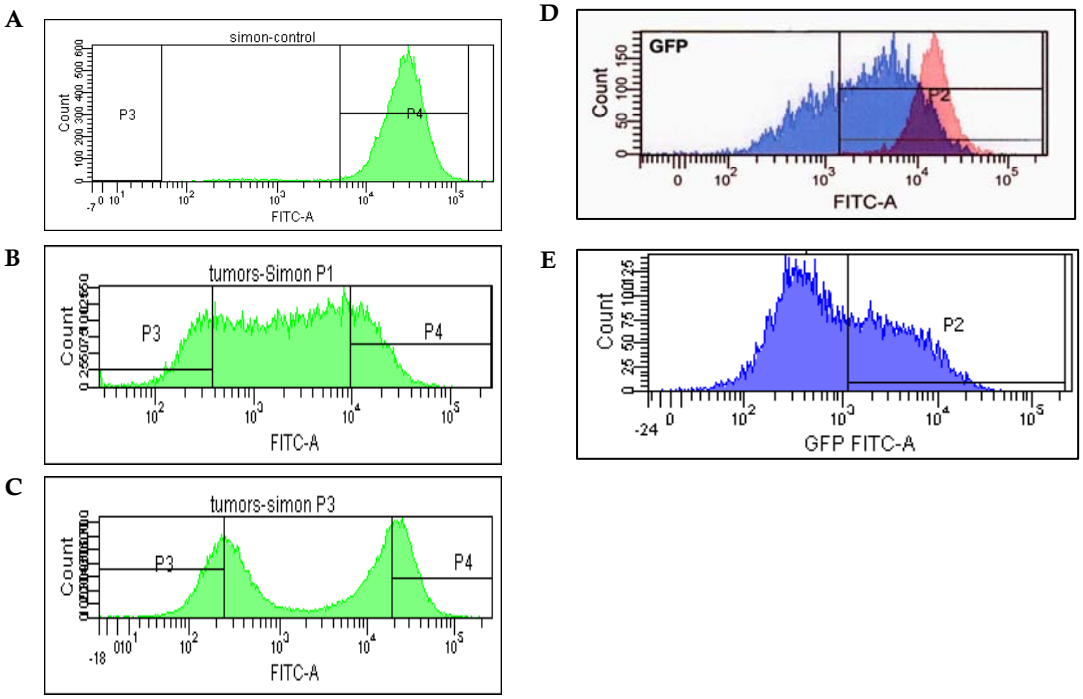
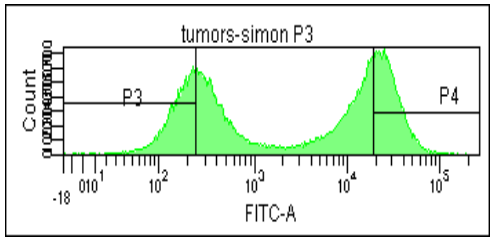



Figure 5-10. Serial transplant of Oct-4/GFP clones *in vivo***.** Flow cytometric analysis for Oct-4/GFP expression in clones isolated from xenotransplants showing the emergence of a GFP- population following serial passage *in vivo* (A) Clone S1 in vitro prior to xenotransplantation (B) following 1 passage in vivo (C) following 3 passages in vivo tumor (D and E) Clone T1 in vitro (red) and passage-1 tumor (blue) (E) third passage tumor.

A

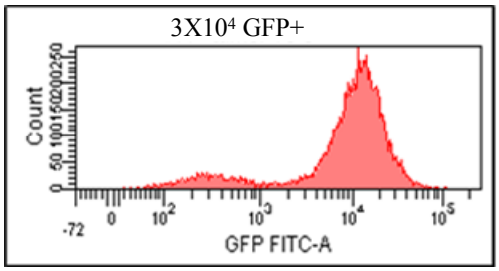


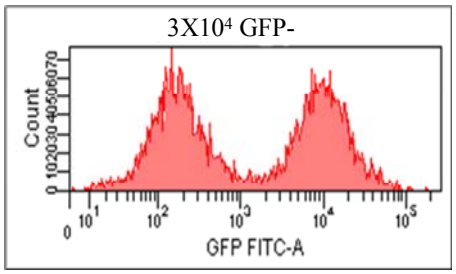

B

| Cell Dose | GFP + | GFP - |
|---|---|---|
| 30,000 | 4/4 23 days | 2/4 47 days |
| 3,000 | 4/4 36 days | 1/4 50 days |
| 300 | 4/4 44 days | 0/4 90 days |

C

85%GFP+




55%GFP+

Figure 5-11. The Oct-4/GFP-enriched fraction of clonally derived tumors is highly tumorigenic in vivo. (A) Transplanted tumrs were fractionated by FACS and the Oct-4/GFP enriched and depleted fractions were transplanted into NOD/SCID mice. (B) The clonal Oct-4/GFP-enriched fraction is more tumorigenic than the depleted fraction. (C) Analysis by flow cytometry of tumor transplants shows the tumors arising from the GFP+ and GFP- fractions are composed primarily of Oct-4/GFP+ cells.

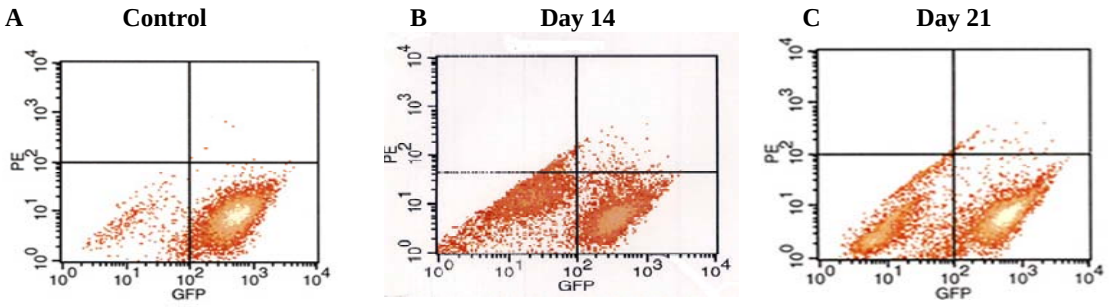



Figure 5-12. Culture in osteogenic media induces silencing of the Oct-4/GFP transgene. FACS analysis of cells isolated from transplanted tumors cultured in standard culture media for 21 days (A) or in osteogenic media for 14 or 21 days (B and C respectively).

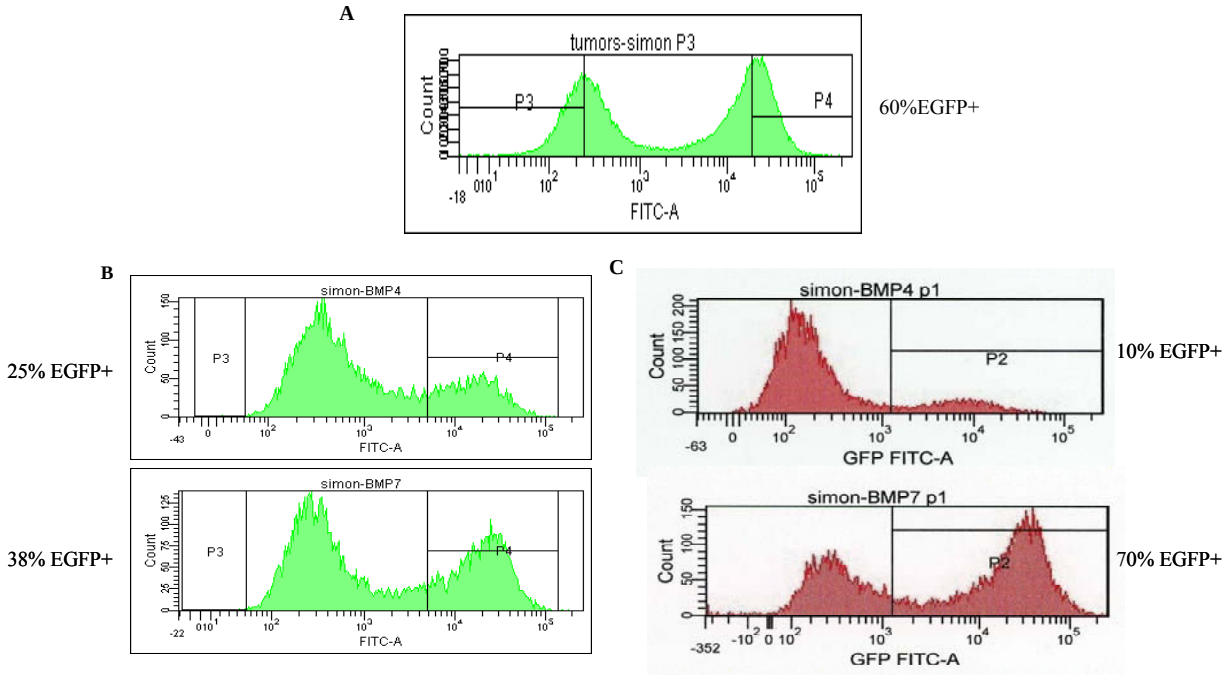



Figure 5-13. Treatment of OS tumor cells with BMPs induced silencing of Oct-4/GFP expression in vitro. (A) FACS analysis of tumor samples cultured in standard culture media. (B) Tumor samples cultured in BMP-4 or -7 conditioned media for 10 days. (C) Tumors generated by transplantation of 1X10 $^{5}$ treated tumor cells.

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# BIOGRAPHICAL SKETCH

Thomas Currie was born in 1980, in Cleveland, Ohio. Shortly thereafter, his family moved to Florida, where his father worked as an Anesthesiologist and his mother raised him and his three brothers and sister. After receiving his high school degree in the international baccalaureate program from Sebastian River High School, Thomas began his undergraduate career at the University of Florida in 1999 on a 100% Florida bright futures scholarship. In 2004 he received his bachelor degree in psychology and completed the pre-requisites needed for dental school. In 2006, before applying to dental school, Thomas decided to pursue a master of science degree from the University of Florida's College of Medicine. He focused his studies on molecular biology and aimed his research toward understanding human osteosarcoma. During this time, he was accepted into the University of Florida's College of Dentistry, for the 2008 entering class. In the future, Thomas wishes to work as a dentist in the state of Florida, while enjoying outdoor activities in his spare time.