Published in final edited form as:

Cancer Lett. 2013 September 10; 338(1): 158–167. doi:10.1016/j.canlet.2012.05.028.

PERSPECTIVES ON CANCER STEM CELLS IN OSTEOSARCOMA

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Abstract

Osteosarcoma is an aggressive pediatric tumor of growing bones that, despite surgery and chemotherapy, is prone to relapse. These mesenchymal tumors are derived from progenitor cells in the osteoblast lineage that have accumulated mutations to escape cell cycle checkpoints leading to excessive proliferation and defects in their ability to differentiate appropriately into mature bone-forming osteoblasts. Like other malignant tumors, osteosarcoma is often heterogeneous, consisting of phenotypically distinct cells with features of different stages of differentiation. The cancer stem cell hypothesis posits that tumors are maintained by stem cells and it is the incomplete eradication of a refractory population of tumor-initiating stem cells that accounts for drug resistance and tumor relapse. In this review we present our current knowledge about the biology of osteosarcoma stem cells from mouse and human tumors, highlighting new insights and unresolved issues in the identification of this elusive population. We focus on factors and pathways that are implicated in maintaining such cells, and differences from paradigms of epithelial cancers. Targeting of the cancer stem cells in osteosarcoma is a promising avenue to explore to develop new therapies for this devastating childhood cancer.

Keywords

osteosarcoma; cancer stem cell; tumor initiating cell; mesenchymal tumors; bone cancer; Sox2; FGF; Wnt signaling

1. Osteosarcoma- The Clinical Disease

Osteosarcoma is the most common primary malignant bone tumor in children and adolescents, comprising almost 60% of all bone sarcomas [1; 2]. It is an osteoid producing solid tumor that occurs more often in larger bones close to epiphyseal areas of rapid growth. The incidence of osteosarcoma is approximately 4–5 children per year per million in the US. It shows a bimodal age distribution with a peak incidence of osteosarcoma in young adults. A second peak of incidence was identified in elderly adults where it is associated with defective bone remodeling [3; 4] With the advent of chemotherapy, the long-term cure rate after surgery for non-metastatic osteosarcoma has risen from 25% to 60% [5]. However, despite advances in chemotherapy and surgery, the survival rate for osteosarcoma has

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Conflict of interest: The authors declare no conflict of interest

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reached a plateau and 40% of osteosarcoma patients eventually succumb to the disease. The majority of osteosarcoma are high grade and are often metastatic at presentation [6]. They are associated with poor prognosis and the overall survival rate for patients with advanced disease remains low at 20% [7; 8]. A high proportion of patients will have a relapse due to metastasis to the lung, the primary site of osteosarcoma metastasis. Up to 20% of osteosarcoma patients present with detectable lung metastasis at initial diagnosis, whereas 80% of patients with only primary tumors develop metastasis in the lung following surgical resection [6]. Metastases are often resistant to conventional chemotherapy and current aggressive treatments pose a significant challenge and do not guarantee long-term survival [9]. A better understanding of osteosarcoma biology and pathogenesis is needed to advance the development of targeted therapies for both primary and metastatic osteosarcoma.

2. Genetics of Osteosarcoma

Molecular and cytogenetic analyses have detected a variety of alterations in osteosarcoma that include several complex chromosomal rearrangements often specific to each tumor [10]. Unlike Ewing's sarcoma, osteosarcoma is not associated with a specific oncogene or chromosomal rearrangement [11]. Comparative genomic hybridization analyses have identified several areas of DNA gain or loss, and oncogenes such as *MYC*, *FOS* and *MDM2*, as well as *RECQ* helicase mutations have been associated with a small proportion of osteosarcoma. Some recurrent alterations may even have prognostic value [12]. The mutations associated with osteosarcoma have been extensively reviewed by Tang et al [13]. Despite extensive cytogenetic analysis, the common underlying genetic alterations responsible for disease development remain elusive [14]. The strongest genetic association for sporadic and hereditary osteosarcoma is with the retinoblastoma (Rb) and p53 tumor suppressor genes. Li Fraumeni patients who carry mutations in p53 are predisposed to osteosarcoma and patients with Rb mutations have a 500 fold increased incidence of osteosarcoma compared to the general population.

Compelling evidence for the role of Rb and p53 in osteosarcomagenesis comes from genetically engineered mouse models that can recapitulate the human disease ([15; 16]. Conditional knockout using Cre-lox technology to inactivate Rb and p53 in the osteoblast lineage leads to short-latency spontaneous metastatic osteosarcoma similar to human tumors in which the cells are arrested in their differentiation [17; 18]. These studies demonstrate the essential role of p53 inactivation and the cooperating effect of Rb disruption in the development of osteosarcoma. While it is evident that several other alterations must accrue on the path to an cancer development, the loss of Rb and p53 are critical events in this process. Interestingly, the barrier that the p53 pathway poses to tumorigenesis, is also becoming evident from studies in induced pluripotent cells, where p53 blocks reprogramming of somatic cells to pluripotent cells [19; 20]. One could speculate then that inactivation of these tumor suppressors in a tumor-initiating cell could permit aberrant dedifferentiation to a more primitive state of the progenitor cell that originates the osteosarcoma. Rb and p53 DNA are also sentinels of the DNA damage checkpoint pathway targeted by radiation which is another inducer of osteosarcoma [21]. Thus, osteosarcoma is associated with the accrual of multiple genetic alterations, of which only a few predisposing mutations have been identified.

3. Osteosarcoma and the Cancer Stem Cell (CSC) Hypothesis

Our view of cancer formation has increased in complexity over the past decade. Tumors are no longer viewed as homogenous masses of proliferating cells, each with identical genetic alterations, but more as a heterogeneous tissue that contains a hierarchy of cells, perhaps originating from a single cancer stem cell. Maintenance of the tumor is also dependent on a

stromal cell component from the tumor microenvironment or niche that nurtures the growth of cancer cells [22].

There is accumulating evidence suggesting that tumors are partially refractory to standard radiation and chemotherapeutic regimens because of their heterogeneous composition. Most anti-cancer agents work on the premise that the high cell division rate of tumor cells makes them more susceptible to anti-proliferative treatments. However, this traditionally held view that arose from initial studies on the clonal origin of leukemias and of oncogenic viruses, and postulated that all cancer cells are fast-dividing and possess tumorigenic potential, has been challenged. This view, called the clonal evolution or stochastic model, posits that all cells within a tumor can repopulate a tumor. A subset of these cells may sequentially acquire additional genetic alterations that promote their survival, aggressiveness and metastatic ability [23; 24; 25]. In contrast, the cancer stem cell (CSC) hypothesis postulates that a small subpopulation of cancer cells drives tumor growth and metastasis. This subpopulation, also referred to as tumor-initiating cells (TIC), is thought to be resistant to treatment and can repopulate a tumor after cessation of chemotherapy. Over the last decade, evidence for the cancer stem cells hypothesis has accumulated alongside the parallel development of the concept that normal tissues are maintained by somatic stem cells. This has led to the sometimes uneasy alliance between stem cell and cancer biology. Though the name “cancer stem cell” is still considered controversial by stem cell orthodoxy which requires not only self renewal but also a stringent demonstration of multipotency in order to identify cells as stem cells, cancer biologists refer to such cells as the “stem cell fraction” of a tumor since these cells possess many canonical stem cell properties of self renewal and differentiation to more lineage restricted cell types through symmetric and asymmetric division. Besides the biological properties that CSCs share with normal stem cells, CSCs also express higher levels of drug transporters and possess higher DNA repair capacities making them especially resilient to standard anti-cancer regimens. Thus, knowledge of the biology of these cells- the mutations and epigenetic changes that cause them to originate, and the niches that enable their maintenance depends on the unequivocal identification and isolation of such cells.

Although the concept of cancer stem cells was first put forth in the 1950's, the strongest evidence of a cancer stem cell emerged in 1994 in seminal studies by Dick and colleagues who showed that rare CD34⁺/CD38⁻ cells (frequency 10⁻⁵–10⁻⁶) derived from a leukemia patient were sufficient to cause acute myeloid leukemia when transplanted in severe combined immunodeficiency (SCID) mice [26]. These experiments established the proof-of-principle experiment for a canonical cancer stem cell – the ability to regenerate a tumor from a single cell *in vivo*. Since then, there has been a concerted search for small population of CSCs in other cancers. The stemness properties of such cells are usually assessed based on their ability to grow clonally as spheres under serum-free conditions, to undergo symmetric and asymmetric division and be able to generate tumors in immunocompromised mice. Such cells have now also been identified in solid tumors of epithelial and neuro-ectodermal origin and more recently, in tumors of mesenchymal origin.

CSCs isolated from these different tumor types share some common characteristics such as drug resistance, ability to repopulate tumors and asymmetric division. These characteristics have been exploited to identify CSCs but given the differences in the tissue of origin, it is becoming evident that CSCs isolated from epithelial, neuro-ectodermal and mesenchymal tumors might have differential requirements for their maintenance and growth, exhibit diverse cell surface glycoproteins and differentially exploit signal transduction pathways.

The existence of CSCs in osteosarcoma was first demonstrated by Gibbs and colleagues who showed that human osteosarcoma samples and cell lines contain a sub-population (10⁻²–10⁻³) of cells that are capable of growing in spherical, clonal clusters in suspension under

serum-free conditions and have the properties of self renewal and multipotency [27]. These cell clusters, referred to as sarcospheres or osteospheres, can be dissociated and replated to form secondary spheres, and have the ability to undergo osteogenic and adipogenic differentiation. Further studies demonstrated that sphere-forming cells from osteosarcoma lines can initiate tumors, and are more drug-resistant to chemotherapeutic agents [28]. Cells with stem-like properties can also be separated by fluorescent or magnetic cell sorting based on the high expression of cell surface glycoprotein markers preferentially expressed on mesenchymal stem cells such as CD133 (prominin), CD 117(c-kit) and Stro-1 [29; 30]. Subsequent studies have revealed that a small CD133-positive population (10^{-4}) from human osteosarcoma correlates with the sphere-forming ability and in vivo tumor-forming capacity in xenograft assays [30; 31].

Correlations with stem cell markers have been recently corroborated in mouse models of osteosarcoma. In mice with conditional deletion of the Rb and p53 genes in the osteoblast lineage, osteosarcoma originates with almost 100% penetrance. Cells isolated from these murine tumors are multipotent, possess a significant sphere-forming fraction with high expression of the mouse stem cell surface antigen, Sca-1. In these tumor cells, Sca-1 expression correlates with sphere and tumor-forming ability as well as multipotency [17; 32]. Besides the sphere-forming assay, the CSC content of osteosarcoma has also been assessed by assays that harness other properties of stem cells such as the ability to retain or efflux dyes or to exhibit high aldehyde dehydrogenase (ALDH1) activity [33; 34; 35; 36; 37]. These assays tend to identify a higher proportion of the cells which likely contain the CSC fraction and perhaps other progenitor cells that maintain these properties.

4 Issues in Isolation and Functional Characterization of CSCs from Osteosarcoma

Most techniques for the isolation of CSCs from osteosarcoma rely on methods that exploit the differences between CSCs and bulk of the tumor population. A major concern of such methods is the assumption that such differences are irreversible and that there exists primarily one type of CSC population. Detailed reviews of the different methods used for isolation and identification of osteosarcoma stem cells have been provided [13; 38]. Studies utilizing different methods for osteosarcoma CSC enrichment are summarized in Table 1. We focus here on highlighting some of the issues and caveats with each of these methods.

4.1. Isolation based on expression of surface markers

Such isolation strategies rely on the differential expression of surface markers on the CSC fraction of the tumor cells. In tumors of mesenchymal origin, markers that are commonly expressed on mesenchymal stem cells have been employed to isolate osteosarcoma CSCs. Of these, only a few have been useful in isolating a small fraction of cells with CSC characteristics. Unfortunately, normal stem cells in mesenchymal tissues do not have a well-defined hierarchical organization of cells as in the hair follicle, intestine or hematopoietic systems, and there is no clear consensus on the best mesenchymal stem cell markers to use [39; 40]. As evident from the studies in Table 1, multiple surface markers, singly and in combination, have been used to identify and isolate CSCs from both established cell lines, and disaggregated human tumor samples. However, these results need to be interpreted with caution since marker expression is highly dependent on the cell line used (freshly isolated tumor sample, primary cell line, established cell line), and on the extent of passaging (passaged *in vitro* or *in vivo*). Additionally, marker expression differs between species and is variable based on the method of isolation (enzymatic versus mechanical disaggregation) since proteolytic cleavage of surface proteins can alter marker-dependent isolation. This is made clear from studies that demonstrate differing fractions of CD133-positive CSCs based

on culture conditions [41]. As seen in Table 1, different markers used in these studies identify a variable content of putative CSCs. Passage in culture is also likely to be responsible for expanding this population. Therefore, given the lack of definitive markers for mesenchymal-derived CSCs, and various factors affecting their expression, CSCs determination based on surface marker expression cannot be interpreted in isolation but needs to be correlated with functional aspects of CSCs. Furthermore, different subpopulations of cells are likely being identified with different surface markers which could indicate that more than one type of stem cell exists in the bulk population. Although there is no available functional distinction between these, their existence is feasible. The emerging situation in normal tissues indicates that a hierarchy of stem cells coexist with quiescent and active stem cells making up the stem cell population [42], and recent data have uncovered a more fluid situation in which stem cell populations actually have the potential to interconvert [43].

4.2. Isolation based on intrinsic cellular properties

Dye retention—A commonly held assumption is that CSCs are relatively quiescent with slow proliferation rates and undergo asymmetric cell division. PKH26 and PKH67 are lipophilic dyes that uniformly label the cell membrane and partition equally into the two daughter cells after a labeled cell has undergone cell division. Thus, the rate of dye retention is inversely proportional to the rate of cell division. While a slow dividing cell effectively retains the dye, faster dividing cells rapidly dilute the dye from their membrane. Since CSCs are capable of asymmetric division, it is assumed that the quiescent CSC retains the membrane label for longer periods as compared to the more differentiated daughter cells that divide rapidly and form the bulk of the tumor population. This technique was used to isolate PKH26+ CSCs from established human osteosarcoma cells [33]. Gene expression profiling reveals that the PKH26+ represents more immature cells as compared to the overall adherent cell population. However this PKH26+ fraction is much larger than the CD133+ fraction, again pointing to the identification of different subpopulations of cells with different identification methods.

Dye exclusion and Side Population—Several reports have demonstrated that CSCs are drug-resistant due to higher expression of drug transporters such as the ABC multidrug efflux transporters [44; 45]. This property has been utilized to isolate osteosarcoma CSCs from both established cell lines [34] and biopsied human osteosarcoma samples [35], based on their ability to exclude fluorescent DNA-binding dyes such as Hoechst33342. The dye-excluding population is often referred to as the side population (SP). The SP from established bone sarcomas contained the sphere-forming population, and could also rapidly initiate tumors at a higher frequency. Additionally, the SP was able to undergo asymmetric division since they gave rise to both a SP fraction and a non-SP-fraction [34; 35].

Aldehyde Dehydrogenase (ALDH1) activity—The search for a universal marker for normal and cancer stem cells has yielded the drug detoxification enzyme, aldehyde dehydrogenase (ALDH1) as a possible candidate [46; 47]. The identification method (Aldefluor assay) relies on ALDH1 enzymatic activity to convert a non-fluorescent substrate to a fluorescent one, which allows isolation of ALDH1+ cells by fluorescent activated cell sorting. ALDH1+ osteosarcoma cells were found to be enriched in the sphere-forming fraction [36], and were associated with a more aggressive, tumor-forming population [37]. The ALDH1 assay has not been extensively used in osteosarcoma and can depend on the membrane of the cell type in some tissues where ALDH1+ cells are slower proliferating and have decreased ability to migrate.

A caveat of all these assays is that they depend on two main assumptions that may not be universally applicable to CSCs from different tumors – a) that CSCs proliferate more slowly than the rest of the population b) that CSCs retain or exclude dyes differentially than the rest of the population.

4.3. Functional Characterization of Osteosarcoma Stem Cells

Functional characterization of CSCs are typically stringent assays that utilize two main aspects of such stem cells: a) the ability to give rise to a tumor that is similar to the original tumor in an immunocompromised host and b) the ability to give rise to cells of different lineages that compose the tissue of origin. Such functional characterization is usually a sequel to isolation techniques and is an absolute requisite to establish the identity of CSCs. In the studies described in the previous section, the marker used to isolate osteosarcoma CSCs was usually correlated with *in vivo* tumorigenicity (Table 1). The ability to form sarcospheres/osteospheres has been demonstrated to be well-correlated with the ability to form tumors in xenograft assays [33].

Limiting Dilution Assay (LDA)—This is the gold standard for establishing the identity of a tumor cell as a CSC. This assay is dependent on the ability of CSCs to form tumors in a dose-dependent manner that exhibits a linear relationship. In other words, a tumor cell is designated a CSC if it can initiate tumors at a much higher frequency and at lower cell numbers, as compared to the bulk tumor fraction or other cells. The validity of a limiting dilution assay is highly dependent on the linearity of the cell number-tumor frequency relationship, strain of the murine model, lack of an immune response that is an important regulator of tumor growth *in vivo* and presence of species-specific signals, and thus should be interpreted with caution [48; 49; 50]. Furthermore, given the intimate association of the tumor with its microenvironment, the site of injection also contributes to the efficiency of tumor initiation. Thus, orthotopic models in which cells are delivered directly to a bony niche may be a preferred site for assessing osteosarcoma CSCs [50]. The LDA assay can be applied to assess sphere formation *in vitro*. [51]. Although the mechanism by which stem cells preferentially form clonal spheres is mostly unknown, the assay is based on assessing the self-renewing populations of tumor cells that are able to grow in suspension under serum-free conditions. However, the sphere-formation assay has come under criticism for the followings reasons: 1) inability to distinguish it from aggregation at higher cell densities 2) inability to detect quiescent stem cells [52]. Establishment of clonality can be difficult, especially at higher cell densities, where clumping is observed. However, this can be averted by incorporating a LDA approach to establish that a single cancer stem cell can give rise to primary and then secondary spheres. We have effectively incorporated LDA along with a sphere-formation assay to demonstrate that Sca-1+ correlates with the sphere-forming ability of mouse osteosarcoma CSCs and with the expression of the stem cell transcription factor, Sox2. [32].

Multipotency—Unlike the bulk tumor fraction, CSCs undergo symmetric and asymmetric division to differentiate into several cell types that can be found in the original tissue and are therefore considered multipotent. While the definition of a canonical CSC does not include multipotency, it is an essential feature of the stem cell dogma [53]. Like MSCs, osteosarcoma CSCs also can differentiate into osteoblasts, adipocytes and chondrocytes under appropriate stimuli. Osteosarcoma CSC are relatively easy to characterize via multipotency assays due to the simplicity of differentiation protocols and identification of the differentiated cell types. These *in vitro* assays are generally conducted after marker-based separation to characterize the stem- and non-stem fraction.

Thus the assays for multipotency in combination with LDA analysis provides the best available assessment for CSCs. Whether the better control cells against which these CSC-delineating parameters are assessed are the total adherent population, or marker negative cells, would depend on the proportion of CSCs within the population.

5 The Case for Pluripotency Factors: Correlation and Causation

It is a generally accepted axiom that cancer cells are in a relatively undifferentiated and more stem-like state. Several groups have reported that mRNAs for pluripotency-associated embryonic stem cell factors such as Oct4, Sox2 and Nanog are preferentially expressed in the subset of sphere-forming, stem-like cells in human osteosarcoma and osteosarcoma cell lines [28; 30; 54], as well as in canine osteosarcoma [55]. These findings added weight to the notion of “stemness” in the sphere-forming population. Levings et al found that an Oct4-promoter-GFP construct was highly expressed in xenografts and in rapidly dividing cells in 40–60% of the tumor mass [56]. Clonal isolates of GFP⁺ cells were highly tumorigenic compared to the rest of the population. While these studies suggest a correlation between expression of these pluripotency genes and stemness, a serious caveat in the OCT4-GFP experiments is the inability to detect authentic endogenous OCT4 expression [57].

The first evidence that expression of an embryonic stem cell pluripotency transcription factor played an important role in osteosarcoma stem cells comes from osteosarcoma in mice with the conditional deletion of p53 and Rb. We found that tumor cells positive for the mouse MSC marker Sca-1, have enhanced sphere-forming capacity, and express the pluripotency transcription factor, Sox2. Sphere forming cells are enriched for Sca1 and Sox2 expression but exhibit decreased expression of Runx2 and OSX markers of osteoblastic differentiation. We found no evidence of Oct4 expression. Knockdown of Sox2 expression by shRNA led to loss of the sphere-forming ability and to loss of tumorigenic potential of the cells. Knockdown of Sox2 also caused decreased Sca-1 expression and an increase in cell cycle inhibitors p21 and p27, thus reestablishing the osteogenic differentiation program. Sox2 was also expressed in human osteosarcoma and osteosarcoma cell lines as well as in cells from freshly biopsied patient samples. Sphere forming ability in these human cells was also dependent on Sox2 expression [32]. These findings strongly support the idea that osteosarcoma contains a de-differentiated cell population of primitive cells that has lost its osteoblastic properties. Importantly, this is the first demonstration that Sox2, a pluripotency maintaining factor, is required for self renewal and maintenance of cancer stem cells in osteosarcoma. [32].

Sox2 expression closely correlates with that of Sca-1. Since Sox2 is a nuclear protein that cannot be used to sort live cells we sorted cells into Sca-1^{HI} and Sca-1^{LO} fractions by Sca-1-based magnetic separation, and FACS sorting, (Sca-1^{HI} Sox2^{HI} and Sca-1^{LO} Sox2^{LO}). The Sca-1^{HI} Sox2^{HI} fraction formed spheres more efficiently than Sca-1^{LO} Sox2^{LO} fraction, expressed very low levels of the osteoblast markers RUNX2 and osterix (OSX) and was essentially blocked in osteogenic differentiation. Interestingly, they had greater adipogenic potential than the Sca-1^{LO} Sox2^{LO} cells. In contrast, the Sca-1^{LO}, Sox2^{LO} fraction expressed significant levels of RUNX2 and OSX RNA and was capable of osteogenic differentiation. Upon passaging, Sca-1^{HI} Sox2^{HI} cells could give rise to Sca-1^{LO}, Sox2^{LO} cells, but we did not find evidence of the reverse. This result indicates that Sox2/Sca-1 expression mark a population of stem-like cells but have maintained the ability to undergo symmetric and asymmetric division but that are defective in osteoblastic differentiation and preferentially enter the adipocytic fate [32].

Thus mouse and human osteosarcoma contain at least two populations of cells, with high Sox2 expression marking cells with stem cell properties, and blocked in osteogenic

differentiation, while low Sox2 expressing cells as well as Sox2 depleted cells can differentiate into mature osteoblasts and show activation of the Wnt pathway. These findings support the hypothesis that Sox2 marks a population of osteosarcoma stem cells that, despite other mutations, maintain a requirement for Sox2 for tumor initiation. Agents that inactivate Sox2 could represent a new avenue for targeting osteosarcoma CSCs.

6. The Osteoblast Differentiation Program: Disruption in Osteosarcoma

Cancer stem cells share some properties with their normal counterparts and tumor formation has been likened to anomalous organogenesis [58]. The heterogeneous nature of osteosarcoma tissue indicates the presence of cells arrested at different stages of differentiation, and its' development coincides with periods when bone undergoes rapid growth or remodeling. MSCs are somatic stem cells that can differentiate into several lineages including adipocytes, osteoblasts or chondrocytes, which can further undergo proliferation, terminal differentiation or apoptosis [59; 60; 61]. During bone formation, immature cells of the osteoblast lineage differentiate into mature osteoblasts that are responsible for synthesis, deposition and mineralization of matrix to form bone [62]. Primitive osteoprogenitors arise from multipotent mesenchymal stem cells that commit to the osteoblast lineage and differentiate to preosteoblasts and mature osteoblasts. Few markers are known that can be used to distinguish differentiation stages along this lineage. The earliest known marker and a key regulator of osteoblast commitment is the transcription factor RUNX2/CBFA1, while osterix (OSX)/SP7, another crucial regulator of osteoblastogenesis, functions downstream of RUNX2. As preosteoblasts expressing RUNX2 and OSX differentiate further along the osteoblast lineage, they gradually stop proliferating, as they synthesize and deposit matrix proteins, notably Collagen1. Differentiation then proceeds to mature osteoblasts along with the expression of osteocalcin (OCN). Finally the matrix is mineralized to generate bone (Figure 1).

Although osteosarcomas are heterogeneous tumor masses interspersed with osteoid and bone, the disease state is generally characterized by increased proliferation and defective differentiation. Loss of differentiation is a prognostic feature of high grade osteosarcoma while low grade disease is associated with more differentiated morphology. Several studies including ours point to a clear link between tumorigenesis and defective differentiation in osteosarcoma. The two tumor suppressors, Rb and p53, that are commonly inactivated in osteosarcoma, are also required for controlled osteoblast differentiation. The Rb protein interacts with and increases the transcriptional activity of RUNX2, thereby increasing osteoblast differentiation [63; 64]. These findings have been corroborated by bone-specific Rb knockout mice that exhibit impaired skeletal development with increased numbers of defective osteoprogenitors that are unable to undergo terminal cell cycle exit [65; 66]. In fact, Rb deficient osteoblasts have an increased capacity to form multipotent spheres [66]. Similar trends have been reported in bone-specific p53 knockout mice that show defective bone differentiation along with a high propensity for the development of osteosarcoma [67; 68]. It is highly likely that Rb and p53 mutations disrupt the bone differentiations program, thereby selecting for immature progenitors with defective DNA damage checkpoints and a propensity to accumulate further mutations.

Molecular evidence for the inverse relationship between differentiation and tumorigenesis was described by Hinds and colleagues who showed that the RUNX2 and p27 differentiation pathway is disrupted in osteosarcoma to maintain the undifferentiated state [69]. New evidence from an unrelated pathway supporting this notion comes from Williams et al, who recently demonstrated that osteosarcomas preserve a deubiquitination mechanism in common with mesenchymal stem cells. They found that ID (inhibitors of DNA binding) proteins, that antagonize the Rb pathway, are stabilized by the deubiquitinating enzyme

USP1. High levels of ID proteins promoted the stem cell features and blocked osteoblastic differentiation in mesenchymal stem cells, and USP1 knockdown in osteosarcoma led to ID destabilization, growth arrest and differentiation [70]. Thus, although osteosarcoma stem cells must have some capacity to undergo asymmetric division and give rise to more differentiated daughter cells, osteosarcoma is characterized as a disease of defective differentiation [71].

6.1. Cell of Origin

Although most investigators assume that osteosarcoma originate from MSCs, the stage of the cell of origin remains difficult to pinpoint [13; 72]. Mouse models of spontaneous osteosarcoma in which Rb and p53 are inactivated in the osteoblast lineage, trace the cell of origin to osteoprogenitors. This is because the *OSX-Cre* transgene that mediates the excision of Rb and p53 floxed alleles, is driven by the osterix promoter that is expressed in osteoprogenitor cells. This suggests that osteosarcomas derive from a more restricted uni- or bipotent preosteoblast. However, it cannot be ruled out that the transgene may be expressed more broadly than the endogenous osterix promoter and may be expressed at more immature stages. Alternatively, the loss of Rb and p53 in a CSC may confer the ability to dedifferentiate to a more primitive state. Thus, whether CSC from osteosarcoma retain the ability to form multiple lineages, because they represent a de-differentiated fraction of osteoprogenitors that re-acquire MSC-like properties, or whether they retain multipotency because they derive from MSCs, remains to be elucidated (Figure 1).

7. Signaling Factors that Influence Osteosarcoma CSCs

An important component of the osteoblast differentiation program is the stem cell niche of the bone marrow. Bone marrow is a source of hematopoietic and mesenchymal stem cells and these two populations can be differentiated based on phenotypic and functional assays [73]. MSCs could maintain osteosarcoma cells in an undifferentiated state through secretion of cytokines such as interleukin-6 [74]. Recent work by Brune et al suggest that mesenchymal stem cells (MSCs) isolated from human osteosarcoma samples are genetically distinct from tumor cells, suggesting that maintenance of a separate MSC niche might be crucial in maintaining osteosarcoma cells in an undifferentiated state [75].

Apart from mesenchymal stem cells, the bone marrow is a rich source of factors that influence the bone differentiation program [76; 77]. Several signaling pathways such as Hedgehog, Notch and MAP kinase play a role in maintaining stem cells in the osteoblast lineage and may play a role in maintaining osteosarcoma CSCs, while BMPs that normally have a pro-differentiation function, have been reported to decrease osteosarcoma CSCs [78]. Fibroblast growth factor (FGF) signaling have been implicated in the maintenance of the cancer stem cell niche [79], and serve as a potential mechanistic link to the development of osteosarcoma stem cells. The FGFs function by binding to their cognate fibroblast growth factor receptors, (FGFRs) [80]. The finding that activating mutations in FGFRs cause a number of human autosomal dominant skeletal disorders provided clear evidence for the importance of FGF signaling in bone-development [80; 81]. FGF stimulates proliferation and blocks differentiation in immature osteoblasts [82; 83], and induces the expression of Sox2. [84]. FGF signaling also induces proliferation of MSCs [85]. In There is emerging evidence that FGF signaling may play a role in maintaining osteosarcoma cells. FGF stimulation enhances proliferation of human osteosarcoma cells [86], and the FGFRs have been reported to be overexpressed in human osteosarcoma samples [87]. Treatment with inhibitors of FGF signaling leads to decreased Sox2 expression and decreased proliferation in mouse osteosarcoma cells [32]. A recent report points to a role for stromally-derived FGF in maintaining the immature state and aggressiveness of osteosarcoma in a mouse model [88].

Wnt signaling plays an important role in bone development and several studies have established that Wnt signaling promotes osteoblast differentiation and bone formation. [89; 90]. In the canonical Wnt pathway, the transcription of Wnt target genes is regulated by accumulation of nuclear β -catenin. The binding of the Wnts to their receptors blocks the phosphorylation and degradation of β -catenin which is translocated to the nucleus to regulate Wnt target genes [90; 91]. While Wnt activation has been linked to cancer development in cells of epithelial origin, such as colon cancer, its role in mesenchymal tumors is likely to be tumor-suppressive [92; 93]. The reports on Wnt signaling have generated some controversies in the literature about its activation or repression in osteosarcoma. Although the inactivation of a Wnt inhibitor, Wif-1, has been associated with radiation-induced osteosarcoma [94] implying deregulation of Wnt signaling, other groups have shown that Wnt signaling is down-regulated in osteosarcoma [95; 96]. In contrast to Matushansky et al who found that Wnt is inactive in a variety of human sarcomas, Vijay Kumar et al reported that Wnt signaling was active in fifty percent of human sarcomas and cell lines examined [97]. One reconciling explanation for these findings could be the heterogeneous nature of osteosarcoma and the differentiation stages of the cells examined. Wnt signaling would be expected to be active in more differentiated cells within the tumor.

In line with the findings of Matushansky et al [92], our studies clearly point to a tumor suppressive role for Wnt signaling in osteosarcoma. Using mouse and human osteosarcoma cells bearing a Wnt reporter gene, we found that Wnt signaling is inactive in sphere forming stem cells. Knockdown of Sox2 leads to induction of Wnt signaling along with the ability to undergo osteogenic differentiation [32].

Our data also suggest that FGF and Wnt signaling have opposing functions in osteosarcoma. FGF increases osteosarcoma sphere formation [32], and along with Sox2, FGFR2 expression is enriched in the sphere-forming fraction. Thus FGF could increase the stem population while more Wnt signaling is associated with differentiation which could maintain the osteosarcoma stem cell population. In line with these observations, we find that activation of Wnt signaling through the inhibition of the glycogen synthase kinase (GSK3 β) (with CHIR99201) in human epithelial (colon cancer HCT116) and a human osteosarcoma (NYU1-established in our laboratory) cell line provides contrasting results: while activation of Wnt signaling significantly increases the number of spheres formed by a colon tumor cells, osteosarcoma cells show a dramatic decrease in the number of spheres formed when Wnt is active (Figure 2). In several studies examining the effect of Wnt signaling on CSC derived from epithelial tumors, sphere formation is increased by Wnt activation and decreased by Wnt inhibition [98; 99; 100; 101] (Figure 2). Thus the effect of Wnt signaling is dependent on cellular context [93].

We have also found that Wnt signaling can decrease the amount of Sox2 protein in osteosarcoma further pointing to the antagonism between the pro-proliferation and tumorigenicity function of Sox2 and differentiation-inducing function of Wnt [32]. It remains to be determined whether the malignant state can be suppressed by activating Wnt signaling and suppressing Sox2 in osteosarcoma cancer stem cells.

8. Conclusions and Future Perspectives

Since the discovery of oncogenes and tumor suppressor genes established the genetic nature of cancer, the cancer stem cell hypothesis is probably the only really novel notion that has inspired cancer research in the last decade. This hypothesis, imperfect as it is, has found considerable support from the studies of osteosarcoma, a highly aggressive tumor derived from the osteoblast lineage. Numerous laboratories have identified a subpopulation of human and murine osteosarcoma cells which express stem cell markers, are capable of

tumor formation, and can form spheres in suspension culture. Differently from adult stem cells, which have a finite life span, such cells are immortal, as they carry numerous mutations (most commonly affecting the p53 and Rb pathways) that make them capable of continuous self-renewal.

A property of CSC that has been consistently described for tumors of epithelial origin, that they are slow-growing, and relatively quiescent, has not generally been found in osteosarcoma CSC, that, with few exceptions, have been reported to be growing faster than the rest of the tumor population. It is possible that CSC of mesenchymal origin differ from those of epithelial cancers. An attractive hypothesis that could reconcile these observations has been suggested by work indicating that adult stem cells can exist in a quiescent as well as in an active state, where the cells cycle but are long lived, in a continuous equilibrium. The major role of quiescent cells would be to replace the “damaged” active population. Bias in the methods of isolation and identification of CSC could inadvertently focus on one or another type of cell. Alternatively, it is possible that, depending on the number and nature of the oncogenic mutations, the original stem cell clone could evolve into a population where the relatively quiescent or the active CSC predominate. In fact, the sphere formation assay itself, may not select for quiescent stem cells, but a faster-self renewing population [52]. A similar bias could be responsible for the very varied estimates of the frequency of CSCs in the tumor population. In breast cancer, a high frequency of CSCs characterizes the most aggressive and advanced cancers [102].

Another important question that has not often been addressed in osteosarcoma is whether the asymmetric division of CSC is unidirectional, i.e. whether more differentiated progenitors can dedifferentiate back to CSC. In principle, this should be highly possible, since the expression of only a few transcription factors is sufficient to convert fibroblasts into induced pluripotent cells. However, we found such dedifferentiation does not occur spontaneously in culture. It cannot be excluded however that appropriate stimuli could achieve this conversion. For example, it will be interesting to know, whether FGF signaling, which induces Sox2 expression, can promote dedifferentiation of osteosarcoma progenitors that express very low levels of Sox2.

Controversial reports have been made on the role and effect of Wnt signaling in osteosarcoma. The concept that Wnt signaling may be oncogenic in osteosarcoma is in line with what found in some epithelial cancers, but contrasts with the clear pro-differentiating effect of Wnt signaling in normal osteoblasts. Such an observation could derive from the examination of osteosarcoma where the non-CSC portion is predominant, as examination of Wnt signaling in CSC fraction shows that it is inactive, but it is indeed active in the non-CSC, more differentiated osteosarcoma cells. Alternatively, there may be different types of osteosarcoma, perhaps depending on the type of cell where the initial oncogenic event took place. Future experiments using Wnt inhibitors or activators may shed light on this controversy.

The issue of a stem cell signature is of great importance towards finding therapies targeted to CSC. Surprisingly, little has emerged this area, with the exception of a few reports. Salinomycin, a K ionophore, was reported to be a potential selective killer of osteosarcoma CSC [103], and immunotherapy with modified lymphocytes has been reported to eliminate osteosarcoma CSCs [104]. Importantly, many markers of osteosarcoma CSC identified so far are molecules that are probably not critical to maintain stem cell viability or self-renewal, but only indicative of their differentiation state. The discovery that Sox2, a transcription factor associated with stemness in many organs, is overexpressed in osteosarcomas and is required for their viability and tumorigenicity gives hope that other factors critical for CSC may be identified and targeted for therapy. Future therapies for improving current outcomes

for osteosarcoma patients will likely combine cytotoxic drugs, which eliminate the bulk of tumor cells, with CSC-targeting therapy. With more accurate identification of the CSC population and further elucidation of the molecular pathways they depend on for survival, targeted drug therapies for osteosarcoma should be on the horizon.

Acknowledgments

This work was partly supported by PHS Grants AR051358 from the NIAMS and DE013745 from the NIDCR. AM is a recipient of a St Baldrick's Research Award and UBR was supported by a grant from the Children's Cancer Research Fund.

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Figure 1. Schematic of the commitment of MSCs and progression of the osteoblast lineage
 Markers of osteoblast commitment and differentiation are in green = Runx2, osterix (osx), collagen1, alkaline phosphatase (ALP). Self-renewal of MSCs and osteoprogenitors (curved arrows). Sox2 decreases and Wnt signaling increases as differentiation progresses. Sox2 is expressed in MSC and immature osteoprogenitors. Osteosarcomas are thought to originate from multipotent MSCs, or progenitors in the osteoblast lineage that still retain bipotency. Osteosarcoma induction is promoted by Rb and p53 inactivation (*).

Figure 2. Opposite Wnt activity in epithelial and mesenchymal CSCs (sphere forming cells)
A. Spheres of osteosarcoma (OS-NYU1) [32] and colon cancer (HCT116) [105] cells expressing a Wnt-eGFP reporter that is also marked with RFP [106]. Wnt activity in the sphere is evident by expression of eGFP (green). dsRFP expression (red) measures transduction efficiency of the reporter. **B.** Composite data showing relative sphere formation in osteosarcoma and epithelial tumors (colon and prostate) before (control) and following canonical Wnt activation ("ON") or inhibition ("OFF"). Data for epithelial tumors have been adapted from references [98; 99; 100; 101]. Data for the osteosarcoma cell line was generated by treating the OS-NYU1 cell line with the Wnt activator (CHIR99201) and assaying sphere formation.

Table 1

Studies identifying stem like-cells in osteosarcoma

Isolation Strategy	Marker/ Substrate [reference]	Source	Functional Characterization	Comments
Markers	CD133 [30,31]	Established human cell lines (Saos-2, U-2OS, MG63) 3–5%	<ul style="list-style-type: none"> Higher anchorage-independent growth, proliferation Higher sphere forming ability 	<ul style="list-style-type: none"> Side population fraction a small sub-set (0.97%) of the CD133^{High} fraction. Co-enrichment of Oct3/4 and Cd133+ in sarcospheres.
		Human Tissue Samples (1.35–7.85% of total population in three osteosarcoma samples).	<ul style="list-style-type: none"> Tumor-initiating capacity of CD133+ sarcosphere fraction Colony-forming ability Multipotency Side population compared to the total adherent population. 	<ul style="list-style-type: none"> Both CD133^{High} and CD133^{Low} fractions positive for CD29, CD44 and CD90 antigens Side population fraction a small sub-set (0.97%) of the CD133^{High} fraction. Co-enrichment of Oct3/4 and CD133 in sarcospheres.
		Saos-2 human cell line	<ul style="list-style-type: none"> Sphere formation 	<ul style="list-style-type: none"> Sarcospheres enriched for Sox2, Oct3/4 and Nanog. SP was positive for CD133.
	Stro-1/CD117 double positive (DP) [29]	Murine DP cells (1% – 3%) of total monolayer cultures, and 6% – 14% in the sphere fractions, respectively in three murine cell lines) and human (KHOS – (1% DP cells) cell lines	<ul style="list-style-type: none"> Drug resistance (2-fold increase in IC₅₀) tumor initiating capacity (as low as 200 DP cells) Multipotency 	<ul style="list-style-type: none"> Stro-1/CD117 useful in both murine and human samples. Higher expression of CXCR4 (metastasis gene) and ABCG2 (drug transporter) in DP population
	Sca-1 [17,18,32]	Murine osteosarcoma cell lines (30 to 70%) in 4 cell lines	<ul style="list-style-type: none"> Sca-1 and Sox2 expression correlated with increased tumor formation, increased sphere-forming frequency in limiting dilution assay for sphere-forming ability, and multipotency 	<ul style="list-style-type: none"> Loss of Sox2 depletes the Sca-1 positive fraction.
		Murine osteosarcoma (primary tumors –1.2%) and cell lines established from tumors).	Sca-1 expression correlates with tumorigenicity	
Intrinsic properties	Hoechst 33342-excluding side population (SP) [34,35]	Established human cell lines (< 1% – 5.28%) in eight different cell lines.	<ul style="list-style-type: none"> Asymmetric division (SP giving rise to both SP and non-SP populations in culture) Sphere-forming assay (only seen in SP) 	<ul style="list-style-type: none"> Gene expression profiling by microarray analysis (higher expression of drug transporter ABCG2 in SP)

Isolation Strategy	Marker/ Substrate [reference]	Source	Functional Characterization	Comments
			<ul style="list-style-type: none"> limiting dilution analysis for tumor initiation 	
		Human tumor samples (3.7% – 10.2% in five human tumor samples)	<ul style="list-style-type: none"> Asymmetric division Limiting dilution analysis for tumor initiation Clonogenicity assay (SP forming colonies under adherent conditions at higher efficiency) Sphere-forming assay SP more drug-resistant with much higher IC50 to doxorubicin, cisplatin and methotrexate 	<ul style="list-style-type: none"> Higher expression of ABC transporter, Nanog and Oct4 in SP. Surface marker analysis reveals no difference between SP and non-SP in expression of CD44, CD117 and CD133
	aldehyde dehydrogenase (ALDH1) [36,37]	Human cell line (MG63-11%)	<ul style="list-style-type: none"> Sphere-forming ability, drug resistance 	<ul style="list-style-type: none"> ALDH1 activity higher in the sphere-forming fraction.
		Human cell lines (OS99-1 – 45.07%, Hu09 – 1.84%, Saos-2 – 1.56% and MG63 – 0.59%) OS99-1 xenografts (3.13%)	<ul style="list-style-type: none"> ALDH^{hi} cells from xenografts from OS99-1 cells showed higher tumor initiating frequency in limiting dilution assay, enhanced proliferation and colony-forming ability 	<ul style="list-style-type: none"> Increased expression of Oct3/4, Sox2 and Nanog in the ALDH^{hi} cells Authors use the ALDH^{hi} cells (3.13%) from xenografts they injected and not the original OS99-1 cells which showed a higher proportion of ALDH^{hi} cells (45.07%)
	Long-term dye retention (Use of PKH26 or PKH67) [33]	Human cell lines (8 – 25%)	<ul style="list-style-type: none"> sphere-formation limiting dilution analysis for tumor initiation (27-fold higher than PKH26^{Low} cells) 	<ul style="list-style-type: none"> Gene expression analysis of PKH26^{High} cells indicate upregulation of bone development/migration and downregulation of acetylation-related genes, No significant change found in Oct3/4 and Nanog expression between the two fractions.
	Expression of exogenous Oct4 promoter-driven GFP transgene [56]	Human cell line OS521 (24% of total adherent population)	<ul style="list-style-type: none"> Higher tumor initiation capacity and earlier age of onset in in vivo tumorigenesis assay seen in Oct4-GFP^{High} cells, 	<ul style="list-style-type: none"> Higher expression of mesenchymal stem cell markers (CD29, CD44, CD56, CD90, and CD105) and no correlation with canonical stem cell markers (CD133, EpCAM, or CD44) seen in the Oct4- GFP^{High} cells