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# Evidence for the osteosarcoma stem cell

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## Abstract

Osteosarcoma is a highly malignant bone tumor of children and young adults. Cytotoxic chemotherapy combined with aggressive surgery only has a 60% survival rate. Historically, chemotherapy has been developed assuming that all cells within a particular cancer are clonal and near identical. Appreciating the now apparent functional heterogeneity of osteosarcoma cells within and between individual tumors will likely be critical in developing much needed novel effective therapies. The foundation for this heterogeneity may lie in the so called “cancer stem cell” or tumorigenic cell of origin. In this brief review, we will examine the evidence for the existence of this cell and its potential importance for future therapies.

## OSTEOSARCOMA AND ITS CLINICAL MANAGEMENT

Osteosarcoma is a highly malignant mesenchymal tumor of bone in which the malignant cells produce osteoid. It is one of the most common primary, non-hematologic bone malignancies in children.[1] Osteosarcoma occurs most frequently in patients between the ages of 10 and 25 years.[1] Before the advent of multi-agent chemotherapy, amputation provided a long-term survival rate of about 20%. The use of multi-agent chemotherapy combined with aggressive surgery has improved the long-term survival in these patients to approximately 60%.[2] Interestingly, survival of patients treated with chemotherapy alone is only 20%,[3] suggesting that populations of tumor cells in a large percentage of osteosarcomas are highly resistant to chemotherapy. Despite intensive efforts in both surgical and medical management, the survival rate has not improved over the last 30 years and fully 40% of osteosarcoma patients die of their disease.

Osteosarcoma can arise in any bone, but occurs primarily in the juxta-epiphyseal regions of rapidly growing long bones. Osteosarcoma often begins as a process destructive of medullary bone that progresses to destroy cortical bone, usually with an associated soft-tissue component. The histopathologic appearance of high-grade intramedullary osteosarcoma is one of malignant spindle cells producing osteoid and immature bone. The natural history of osteosarcoma is one of relentless local progression with loss of the function of the affected extremity, leading to distant metastasis, most often to the lung.[2,4]

Cytogenetic evaluation has revealed numerous complex chromosomal abnormalities that vary both within and between individual tumors in osteosarcoma. Different from other sarcomas, such as Ewing’s sarcoma, synovial sarcoma and alveolar rhabdomyosarcoma, osteosarcoma has not been associated with specific recurrent chromosomal rearrangements.[5] Molecular analyses have revealed a variety of genetic alterations in osteosarcoma, including inactivation of p53 and retinoblastoma tumor suppressor genes and overexpression of oncogenes such as MDM2.[6] For example, alterations of the retinoblastoma gene (RB1) have been shown in up to 70% of reported cases, and loss of heterozygosity for RB1 has been

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shown to be a marker of poor prognosis.[7] Major signaling pathway alterations also have been implicated. The Wnt pathway, specifically its downstream mediator β-catenin and co-receptor LRP5 have been associated with metastasis and a poor prognosis.[8*,9*] Abnormal expression of growth factors, such as TGF-b3, also has been associated with poor outcome. 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 improved therapies.[10]

## TUMORAL HETEROGENEITY

Cancer treatment, in general, often combines systemic cytotoxic chemotherapy with radiation, surgery or both. While this paradigm, overall, has been effective; not all patients respond equally, and relapses are common. The explanation for this disparity may lie in the assumption that a tumor, being clonally derived, is composed of a homogeneous population of cells;[11] therefore, all the cells within the tumor are expected to respond uniformly to a specific chemotherapeutic agent. Much to the contrary, it has long been recognized that the cells within individual tumors are not biologically equivalent and that tumors are comprised of cells of differing morphology, matrix production, proliferation rate, and tumorigenic/metastatic potential.[12] One theoretical paradigm that proposes to explain intra-tumoral heterogeneity is the cancer stem cell model.

## CANCER STEM CELLS

The cancer stem cell (CSC) theory holds that there is a small sub-population of cells within a tumor which, like normal stem cells, has the ability to self-renew. These CSCs are thought to 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 considered to be responsible for initiating and maintaining the growth of the tumor and if not completely eradicated by surgical extirpation or chemotherapy initiates local and distant recurrence.[13]

Reya *et al.*,[14] drawing parallels between CSCs and normal stem cells, has suggested that tumorigenesis can be viewed as aberrant organogenesis.[14] Both stem-cell types self-renew; however, normal stem cells do so in a tightly regulated fashion, and CSCs in a dysregulated manner. Both cell types exhibit extensive proliferative potential and give rise to new tissue, but normal stem cells generate tissue that is well differentiated, organized and functional, while CSCs generate disorganized, invasive, abnormally differentiated tissue. Both tissue types are heterogeneous, being composed of cells with differing phenotypes and proliferative capacity. As cancer is thought to be of clonal origin, the CSC must be capable of generating diverse progeny of both indefinite and limited proliferative potential.[15] Implicating the involvement of adult stem cells committed precursors in tumorigenesis, many tumors reflect functional and phenotypic aspects of the tissue from which they purportedly arose. Osteosarcoma is a typical example in that it generates a matrix that is clearly recognizable as bone by histologic as well as radiographic examination. It is also abundantly clear that this bone is aberrant in both form and function.

The first definitive work describing a CSC was performed by Bonnet and Dick[16] in studies of acute myeloid leukemia (AML). They identified a rare population of human severe combined immunodeficiency (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, breast, colon, pancreas and prostate malignancies, among many others, suggesting a broader involvement of stem-like cells in carcinogenesis.[17–22]

That cancer could arise from a primitive stem-like cell or other precursor seems reasonable because 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 in general.[23–37] Bmi-1, a gene involved in somatic stem cell self-renewal has been shown to be critical for cancer stem cell proliferation in hematologic malignancies.[26] Interestingly, it is also overexpressed in osteosarcoma but knockdown experiments do not reveal a functional role in its malignancy.[28]

## TUMOR INITIATING CELLS IN OSTEOSARCOMA

In considering the types of cancer likely to arise from the participation of stem/progenitor cells, osteosarcoma emerges as a particularly likely candidate. Bone is a rich reservoir of growth factors and adult stem/progenitor cells. It also is one of the few organs in the human with the capacity for regeneration. Indeed, children are capable of regenerating large segments of bone lost to trauma or surgery, and to a lesser extent this regenerative ability is retained even into adulthood. Osteosarcoma arises most commonly near the growth plates of adolescents, the period and location of the most rapid skeletal growth, when bone progenitor cells are active in expansion, proliferation and differentiation. This period of "organogenesis" would seem to offer multiple opportunities for perturbation in proliferation and differentiation programming, supporting the development of the malignant phenotype.

We initially examined osteosarcomas for the existence of sub-populations of stem-like cells by attempting to grow cells isolated from primary biopsies in a sphere culture system. The stringent growth conditions of anchorage independence and serum starvation, have been shown by others to select for cells with stem-like properties. Consistent with this, we found that ~1 in 100–1000 osteosarcoma cells were in fact capable of growing as spherical clones under these conditions[29]. Cells within these "sarcospheres" appeared to self-renew and could be driven to differentiate along multiple lineages. Moreover, relative to monolayer culture, sarcospheres exhibited enhanced expression of the embryonic stem-cell associated transcription factors, Oct-4 and Nanog. This suggested that embryonic transcription factors may play a role in facilitating an osteosarcoma stem cell phenotype and could provide potential targets for the development of novel therapies.

Other groups have since confirmed these initial findings in both human and canine osteosarcoma.[30,31] Interestingly, and in line with the thinking that CSC might be responsible for tumor recurrence after therapy, Fuji *et al*.[32] more recently showed that sarcosphere cultures demonstrated increased resistance to two standard osteosarcoma chemotherapeutic agents, doxorubicin and cisplatin. Although the sphere culture assay supports a potential role for CSC in osteosarcoma, the relevance of this system with regard to tumorigenesis still remains in question, as it has yet to be demonstrated that cells capable of forming sarcospheres possess increased tumorigenic capacity *in vivo*. Since O'Brien *et al.* and others[20–22,33] first identified leukemic stem cells based on the expression of specific surface antigens, numerous investigators have employed a similar approach in search of CSC in other malignancies. Surface antigens, often referred to as markers, are typically glycoproteins that exist on the external surface of a cell and can be seen indirectly by

fluorescently labeled antibodies targeted against them. Cells so labeled may be counted and separated from other populations by fluorescent activated cell sorting (FACS). The function of these markers in reference to cancer biology is usually unknown and their identification, fortuitous. Tirino *et al*. and others[34–37] demonstrated in three commercially available osteosarcoma cell lines a very small subpopulation of cells expressing CD133, a surface marker purported to identify CSCs in melanoma, brain and colon cancer. The CD133+ cells were able to produce sarcospheres and express the ABCG2 transporter, an active efflux pump associated with drug resistance and stem cells discussed later in this review. Of note, these investigators did not find that other surface antigens commonly associated with CSC, such as CD44, CD29 and CD90, were differentially expressed. Adhikari *et al*.[38] evaluated murine and human osteosarcoma cell lines for the expression of CD117 and Stro-1 (markers often found on mesenchymal stem cells). They identified a population of cells positive for these markers that demonstrated ABC transporter genes, enhanced tumorigenic capacity and drug resistance.

Because the function of most purported CSC surface markers is unknown, and therefore may very well not be involved in the pathophysiology of these cells, others have sought functional assays to identify and isolate stem-like cells in cancer. One such assay involves the detection of aldehyde dehydrogenase (ALDH1) activity. ALDH1 is an enzyme thought to be involved in vitamin A metabolism and drug resistance. Its activity has been used to identify CSC in several malignancies, including breast cancer and leukemia.[39] However, its role in osteosarcoma has only been implicated in one report.[40] In this study, the authors noted that expression of ALDH1 correlated with increased tumorigenic potential in an established osteosarcoma cell line. Curiously, ALDH1 expression was linked with tumorigenic potential only in those cells growing as xenografts and not those grown in culture. This suggests that its expression may be a marker of the tumorigenic phenotype but does not confer tumorigenic activity.

Subpopulations of cells known as side populations also have been implicated by two groups as containing CSCs in sarcomas. Side population cells are identified by their capacity to actively efflux a toxic DNA binding dye, Hoechst 33342, when exposed to the dye *in vitro*. This observation is thought to be caused primarily by expression of ATP binding cassette (ABC) transporter proteins and may be responsible for resistance to certain chemotherapeutic agents. Wu *et al*.[41] evaluated multiple primary sarcoma cultures and found that in those tumors demonstrating a side population that these cells were more tumorigenic in immunocompromised mice than those cells that could not pump out the dye. Similarly, Murase *et al*.[42] showed a side population in several commercially available cell lines that also had increased tumorigenic potential and formed spherical colonies in anchorage independent growth conditions. Proponents of the side population method of identifying CSC suggest that as a functional assay based on transporter activity it may be superior to other methods. However, one criticism of the assay is that as Hoechst 33342 is toxic, those cells incapable of effluxing the dye may be “sick” and thus inhibited from forming tumors by the assay itself.

Recently, in a further attempt to identify tumor-initiating cells in osteosarcoma by way of a functional assay, we looked at transcriptional activity in an early passage primary human osteosarcoma cell culture. We found that tumor formation in a xenograft model was functionally linked to the capacity to activate an exogenous Oct-4 promoter-driven GFP reporter; cells in xenograft tumors capable of activating this reporter showed more than 100 times increased tumor-forming capacity. Additionally, we found that tumors derived from a single Oct-4/GFP+ cell could be serially passaged in mice, recapitulated the original parental phenotype and intra-tumoral heterogeneity with both GFP+ and GFP- cells. Thus, the GFP+ cell population exhibited the critical stem-like traits of self-renewal and generation of

heterogeneous populations. However, unlike the classic CSC model in which the tumor initiating cell is slow growing and rare, these cells divided rapidly and comprised 40–70% of the tumor mass.[43*]

Interestingly, despite numerous attempts, we have been unable to unequivocally link expression of the endogenous Oct-4 gene/protein with tumorigenic activity. We have found striking inconsistencies across the various types of assays we have employed, which we attribute to splice variants, pseudogenes and the questionable specificity of commercial reagents used to detect this protein. Indeed, the question of whether Oct-4 plays any role in somatic tumors remains an area of ongoing controversy in the literature. Regardless of our inability to establish a causative link between Oct-4 protein and tumorigenicity, activation of the Oct-4/GFP reporter remains a robust identifier of tumorigenic cells in our osteosarcoma xenografts. Along these lines, this reporter construct is routinely used as a marker of cellular reprogramming after transplant of somatic nuclei into ooplasms and during fusion of somatic and ES cells. In this regard, our data suggests that the Oct-4/GFP reporter is activated by the “reprogrammed” transcriptional state of the malignant cell. Loss of this signature (reflected by the loss of GFP expression) occurs in response to extrinsic differentiation signals that induce broad changes in global transcriptional patterns and the loss of tumorigenic capacity.

## TUMORIGENIC CELL OF ORIGIN

Although intra-tumoral heterogeneity and the functional characterization of cell populations of cells within osteosarcomas are relatively new areas of study, the data reported thus far are largely consistent with the CSC model. The limited reports in the literature indicate that it is possible to prospectively identify discrete subpopulations of cells within these tumors with enhanced tumorigenic activity. In this respect, the term “cancer stem cell” may be somewhat misleading and is certainly controversial. Proponents can be found who think the cell of origin is indeed a stem cell “gone bad” while others believe tumors arise from a more committed progenitor.[44,45] There are still others who believe that the initiating cell can arise from a terminally differentiated cell that “de-differentiates” to a more primitive and less regulated state. Osteosarcomas are a diverse tumor type, and are highly variable histologically and in clinical course. It is possible, if not likely, that this heterogeneity of function is a reflection of the diverse nature of cells of origin. The osteosarcoma initiating cells may arise from cells at all stages of osteogenic differentiation from early precursor to fully differentiated osteoblast, with numerous complex genetic and epigenetic lesions. Early work evaluating differentiation therapies that might target the more tumorigenic cells has shown some promise and supports an epigenetic component in the pathogenesis of this disease.[8,43,46]

## CONCLUSION

Regardless of the origin of the heterogeneity, it must be taken into account when designing new therapies against this aggressive disease. Standard drug development paradigms targeting presumably homogeneous cells in culture are likely inadequate. Further development will require an understanding of the molecular underpinnings of the differences between the tumorigenic and non-tumorigenic cells within an individual tumor.

Hopefully, as more tumors and cells are investigated functional themes can be identified that will allow categorization of these cells and lead to more effective diagnoses and improved treatments targeted at their functional differences.

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