Osteosarcoma: mouse models, cell of origin and cancer stem cell.

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Abstract

Osteosarcoma (OS) is the most common non-hematologic primary tumor of bone in children and adults. High-dose cytotoxic chemotherapy and surgical resection have improved prognosis, with long-term survival for non-metastatic disease approaching 70%. However, most OS tumors are high grade and tend to rapidly develop pulmonary metastases. Despite clinical advances, patients with metastatic disease or relapse have a poor prognosis. Here the cell biology of OS is reviewed with a special emphasis on mouse models as well as the roles of the cell of origin and cancer stem cells. A better understanding of the molecular pathogenesis of human OS is essential for the development of improved prognostic and diagnostic markers as well as targeted therapies for both primary and metastatic OS.

Introduction

OS is a highly malignant form of bone cancer that onsets during periods of skeletal growth, and thus primarily affects children [1,2,3,4]. The incidence of OS is approximately 750 to 900 cases each year in the United States, of which 400 occur in children and adolescents younger than 20 years of age [5,6]. OS progresses quickly and is characterized by a local invasion of bone, loss of the function of the affected extremity and distant metastasis, most often to the lung. Due to the high virulence of OS, current treatment is aggressive and involves a combination of 1) multidrug cytotoxic chemotherapy (doxorubicin, cisplatin, ifosfamide, etoposide and methotrexate [7,8]) which can slow tumor growth, but often induces cardiac myopathy, hearing loss and risk of secondary malignancy [4,8]; and 2) radical surgery, i.e. amputation or resection of the involved bone and soft tissues, and subsequent skeletal reconstruction with massive metallic endo-prostheses or cadaveric bone. Despite intensive efforts to improve both chemotherapeutics and surgical management, 40% of OS patients succumb to the disease. Additionally, the clinical outcome for metastatic OS remains poor; fewer than 30% of patients that present metastases survive 5 years after initial diagnosis. Therefore there is an urgent need for the development of novel therapeutics for OS-agents with increased capacity to eliminate systemic tumor burden as well as reduced toxicity in healthy tissues.

OS genetics

OS is characterized by a lack of recurrent translocations and a complex karyotype. Genetic approaches have identified several genes of potential importance in the development and progression of the disease [9,10,11]. However the widespread chromosomal alterations of the OS genome, has limited the interpretation of these findings. Genetic alterations of OS are usually sporadic; however genetic predisposition has been documented in patients with Li-Fraumeni and Retinoblastoma syndrome. Somatic *P53* deletions and point mutations occur in approximately 50% of human OS [12,13,14,15] and half of those mutations are associated with loss of the remaining allele [13]. Additionally almost 70% of OS have at least one *RB* allele alteration [16,17]. Homozygous deletions of *RB*

are seen in 23% of tumors, while point mutations appear in 6% [17,18]. In addition, numerous alterations that disrupt the *RB* pathway have also been reported; for example, the loss of function at the INK4a/ARF locus and the amplification of CDK4 have been found to occur (one or the other) in 22% of OS [19,20,21]. The prevalence of these alterations would suggest that the deregulation of the G1/S checkpoint in the cell cycle is a common event in OS.

Animal models in OS

Currently there is not an ideal animal model of OS that fully represents its biological and clinical features. Nevertheless, many aspects of the biology of the disease have been learnt from a variety of approaches: 1) the development of secondary OS as a consequence of animals receiving radiation, 2) human and murine OS cell lines, 3) xenotransplantation studies and 4) conditional mouse OS models. Animal models potentially hold significant promise in increasing our understanding of the genetic basis of OS and more importantly, in advancing pre-clinical studies aimed to the rational development of new therapeutic approaches as well as their validation prior to clinical trials. The more commonly used systems are briefly described below:

1. *Secondary OS after radiation*. The development of rodent models started with the exposure of rats and mice to chemical and radioactive carcinogens [22,23,24]. These models yielded tumors that histologically resembled human OS and derived cell lines that complement human OS studies [25]. Despite the high penetrance of the models, their relevance remains unclear since the majority of OS in humans is sporadic, while the carcinogen-induced murine model is more representative of a therapy induced disease.

2. *Xenotransplantation studies*. There is a significant body of literature related to the development and use of xenograft and allograft models of human and murine OS cells injected into immunocompromised mice. Injected cells form a solid tumor locally grown within days or weeks after implantation [25,26]. The use of this system has become a prominent tool in current oncological research due to the quick onset of the tumors, its cheap availability and ease of handling and maintenance. In addition, OS donor-derived cells often metastasize to the lungs, providing an opportunity to investigate primary and secondary tumor growth. The principal limitation is that the approach uses fully developed OS cells and therefore does not provide information about the initiation of the tumor and its etiology. In certain circumstances the injected cell line may not be metastatic in the rodent context, making it impossible to study the dissemination of the disease. Despite these limitations many groups have successfully used this model to identify factors involved in OS migration [27,28] and more importantly for screening drugs with tumoricidal potential [29].

3. An alternative to injecting cell suspensions into recipient animals is to *transplant* into them pieces of tumor directly harvested from the patient. The advantage is that the human tumor can grow in its native stroma, which in some reports has been shown to enhance tumor growth and metastasis. With the use of cell suspension and transplants mouse cells can infiltrate the tumor, possibly influencing the activities of the tumor cells and in some cases, mouse cells can overgrow the human cell population [30]. Alternatively, *intratibial implantation* of OS cells has been shown to induce OS orthotopic formation in local and metastatic sites (proximal tibia and lung) [26,31,32,33]. This model allows the study of primary tumor formation within its native context as well as the early stages of metastatic progression of OS, thereby reconstituting the entire metastatic process. However, its use is limited by the lack of reproducibility due in part to the technical skill required to perform the implantation.

4. *Transgenic mice*. The ability to alter the expression of specific genes in OS became available in the mouse with the evolution of gene targeting technologies [39,40]. Many murine OS models have been developed to recapitulate the *P53* and *RB* mutations in hereditary and sporadic human OS. *Germ-line* deletion of *p53* resulted in an OS incidence of 4% in homozygous *p53* null mice [41] and 25% in heterozygous *p53* mice [42], underlying the importance of altered *P53* in driving OS. However the rapid development, the higher penetrance of other tumors (mostly lymphomas) and the long latency of OS [43] necessitate to sacrifice the mice before disease onset, hampering in many cases, the utility of these models. The role of *P53* was further highlighted by tumor analysis of *p53* knock-in mice containing a mutant copy of *p53R172H* (corresponding to the *R175H* hot-spot mutation in humans) that not only develop primary tumors but also metastasize to the lungs as well as other organs [44,45]. Conversely, mice with germ-line deletions of *Rb* did not develop OS: homologous deletion of *Rb* is embryonic lethal and the heterozygous are not predisposed to OS [46,47].

The application of conditional gene regulation and the availability of tissue specific *Cre expressing mouse lines* [48] has greatly enhanced our ability to generate specific models of mesenchymal osteogenic lineage that more faithfully resemble human OS [40,49] (table 1). Using *Cre* recombinase activated by the gene promoter of Paired related homebox 1 (*Prx1-cre)* [50] that deletes LoxP flanked alleles in the early limb mesenchyme, 22% of mice with *p53* heterozigosity developed OS. Not surprisingly, homozygous deletion of *p53* had a three-fold increase in OS incidence over the heterozygous animals. In contrast, the deletion of *Rb* in the mesenchymal *Prx* expressing progenitors did not produce any OS tumors [34,35]. Interestingly, the highest incidence (92%) of OS occurred with the combined deletion of one allele of *Rb* with homozygous *p53* deletion [35]. Nonetheless homozygous deletion of both genes yielded only 18% of OS tumors with a strong preference for poorly differentiated soft tissue sarcomas (PD-STS) [34,35].

Cell	Cre	Gene	OS penetrance	Metastatic disease
MSC/skeletal Progenitors	*Prx-1*	$p53^{fl/+}$	22% [34]	
		$p53^{fl/fl}$	61% [34]; 62%[35]	Yes (24%)
		$p53^{fl/fl}$-$Rb^{fl/+}$	92% [35]	
		$p53^{fl/fl}$-$Rb^{fl/fl}$	18% [34]; 29% [35]	
Pre-osteoblasts	*Osx*	$Rb^{fl/fl}$	0% [36]; 0% [37]	
		$p53^{fl/fl}$	100% [36]; 100% [37]	Yes (40%); Yes (32%)
		$p53^{fl/fl}$-$Rb^{fl/+}$	53% [36]; 100% [37]	
		$p53^{fl/fl}$-$Rb^{fl/fl}$	72% [36]; 100% [37]	Yes (37%)
	Col1α1-3.6	$p53^{fl/fl}$	60% [38]	
Osteoblasts	*Col1α1-2.3*	$p53^{fl/fl}$	85% [34]	

Table 1. Summary of OS murine genotypes and incidence rates

Development of OS with a penetrance of 100% [36,37] has been observed following osteoblast specific deletion of *p53* using Osterix-mediated Cre expression (*Osx-Cre*) [51]. As with mesenchymal progenitors *Rb* deletions have no effect and combined deletion of *Rb* and *p53* in osteoblasts once again generated OS with high penetrance (100%) [36,37]. Of potential clinical importance was the existence of short-latency spontaneous metastatic OS similar to human tumors in which cells are arrested in their differentiation [36,37].

A prominent cellular feature of conditional inactivation of *p53* in osteoblastic progenitors resulted in the hyperproliferation of osteoblasts prior to tumor formation, possibly providing insight into the initiating events of osteosarcoma [38]. *Rb* has been proposed to have a role influencing late osteoblast differentiation by interacting with *Runx2* [52]. However the removal of *Rb* alone is not sufficient to induce OS in a number of independent studies. The different experimental approaches strongly suggest that mutation on the *p53* pathway can serve as an initiating event in OS with a mutation in *Rb* pathway strongly accelerating tumor development.

Other genes such as C-FOS, TWIST, p14ARF, p16INK4a, and p21CIP have also been implicated in OS pathogenesis based on studies of human OS samples. Their mutation appears to complement the defects in the *p53* and *Rb* pathways and their involvement in osteosarcomagenesis is also demonstrated from genetically engineered mouse models.

Cell of origin

The cell of origin has been widely discussed in the literature and many times confused with the cancer stem cell (CSC). The *cell of origin* is defined as a normal cell that acquires the first cancer-promoting mutation (s) and it is not necessarily related to the CSC [53]. The cell of origin can also be referred as *tumor-initiating cell* (TIC), while the CSC is a *cancer-propagating cell*. Two major questions when considering the OS cell of origin are; 1) what is the differentiation stage of the tumor initiating cell and 2) is this cell phenotype common among all OS?.

OS is a diverse tumor type that is variable both histologically and in its clinical course. It is possible that its heterogeneity is a reflection of the diverse nature of cells of origin. However by definition, OS produces bone matrix so the TIC are likely to be from osteogenic lineage. Therefore we can speculate that OS initiating cells may arise from genetically unstable cells at all stages of osteogenic differentiation ranging from an early precursor, to a growth plate chondrocyte. However, from the existing data it is not possible to define.

Initially the OS cell of origin was considered to be a differentiated osteoblast, however in the recent years the focus has shifted towards progenitors [54,55]. It has been proposed to be a *mesenchymal progenitor cell* due to its potential to give rise to osteoblasts and the lack of terminally differentiated osteoblastic cells [56,57,58] in tumor tissue. RUNX2 expression has been used to identify mesenchymal progenitors proposed to be the source of OS initiating cells [54,55,59,60].

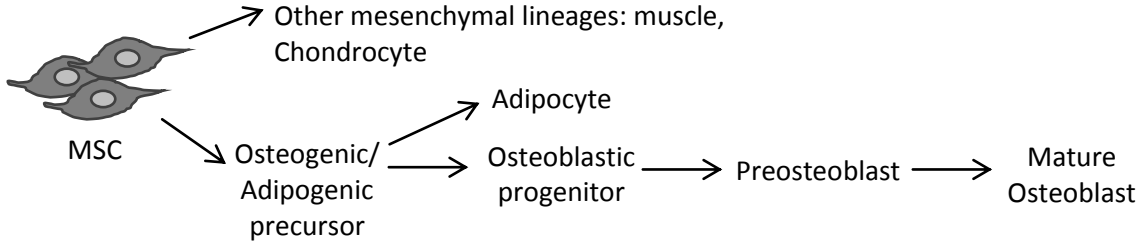

Figure 1: Differentiation potential of MSC. They can differentiate in a large variety of human tissues including osteogenic, chondrogenic, adipogenic and neuronal lineages

Data derived from a range of genetic approaches most strongly favors the OS cell of origin to be a progenitor cell committed to the *osteoblast lineage* [36,37,61] rather than an uncommitted, naïve stem cell. This is

highlighted by *p53* gene inactivation in mesenchymal progenitor cells using *Prx-1*, in which only 61% develops OS, while the remainder was PD-STS [34]. Comparatively, the deletion of *p53* in osteoblast progenitors using *Osx* resulted in 100% of OS penetrance [36,37] (Table 1). These observations enable distinction to be made regarding the influence of the cell developmental stage over tumor phenotype; with primitive multipotential cells favoring the development of PD-STS while committed osteoblast precursors solely give rise to OS. Nevertheless, a lack of consensus still remains over the precise phenotype of the cell of origin. This is in part due to an absence of cellular differentiation markers that enable sufficient discrimination between MSC and osteoblast progenitor cells.

Cancer stem cells in osteosarcoma

The cancer stem-cell model posits that some cancers are organized into a hierarchy of subpopulations of tumorigenic cancer stem cells and their non-tumorigenic progeny [62]. In these cases, cancer stem cells are thought to drive tumor growth and disease progression, perhaps through therapy resistance and metastasis. However, difficulty replicating CSC markers, patient-to-patient variability and a lack of consensus among different xenograft models has meant that it is unclear which tumors follow this model [63,64,65,66].

Tumorigenic cells are rare and phenotypically distinct in some cancers, but are common and phenotypically diverse, with no clear hierarchical organization in others. We do not know yet what portion of cancers follows the stem-cell model. Even in cancers that clearly contain a hierarchy of tumorigenic and non-tumorigenic cells, it is not necessarily clear which phenotypic and functional differences among cells arise from which sources of heterogeneity. Further it is not known to what extent metastasis, therapy resistance and disease progression reflect the intrinsic properties of CSC as opposed to genetic evolution or other sources of tumor heterogeneity.

The CSC model has mainly been tested using transplantation assays that analyze the potential of a cancer cell to form a tumor. These assays have demonstrated the existence of phenotypically distinct populations in different types of human cancers. The other criterion that must be satisfied according to this model is that tumorigenic cells give rise to a non-tumorigenic progeny. However this is based on the existence of membrane markers that can distinguish tumorigenic from non-tumorigenic cancer cells and in many cases there is a lack of knowledge of the proper marker to define those cells. Fortunately, new experimental approaches have been developed to circumvent this issue. Genetic approaches that map the contributions of cancer cells to tumor growth in mice have provided evidence to support the CSC model in some contexts, and evidence against the model in others [67,68,69,70,71]. Also deep sequencing of human tumors has also provided insight into genetic heterogeneity within tumors and the cells that are responsible for relapse after therapy [72].

A variety of methods have been employed to identify OS CSCs that are briefly described below (Table 2).

The existence of OS CSCs was first suggested by Gibbs and colleagues [73] who showed that human OS contains a subpopulation of cells (0.1-1%) capable of growing in spherical, clonal clusters in suspension under serum free conditions. The sarcospheres (or osteospheres) could be replated multiple times, and in some undergo osteogenic and adipogenic differentiation and showed at least by PCR expression of the embryonic stem cell markers OCT4 and NANOG compared to adherent cells. Several other groups also confirmed the ability of OS cells to form spheres [74,75,76,77]. Strikingly, spheres derived from the MG-63 cell line were less sensitive to the chemotherapeutic drugs doxorubicin and cisplatin than adherent cells and had an

increased expression of the DNA mismatch repair enzyme genes MLH1 and MSH2, suggesting that these cells might confer chemoresistance [75,77]. Further fractionation of OS subpopulations has been achieved based on activation of the *OCT4* promoter using *OCT4* driven GFP reporter [78]. These Oct4/GFP$^+$ cells from xenografted tumors expressed CD105 and ICAM-1 MSC markers were 100 fold more tumorigenic than GFP negative cells, and with the ability to metastasize to the lung. Basu-Roy *et al*. identified a subpopulation of CSC based on expression of SOX2 in SCA-1 positive cells with

Method	Reference
Spheres	[73,74,75,76,77]
Oct4	[78]
Sox2	[79]
CD133	[80,81]
CD117, STRO-1	[82]
ALDH	[83]
Side population	[84,85]

Table 2. Methods used to characterize CSC in OS.

conditional deletion for *Rb* and *p53* [79]. They could separate two populations within the tumor: one with high SOX2 expression (and SCA-1), conferring stem-like properties to the cells that were blocked in osteogenic differentiation, conversely low SOX2 expressing cells and SOX2 depleted cells could differentiate into mature osteoblasts and had enhanced Wnt pathway activation.

Tirino *et al.* [80,81] identified CSCs based on cell surface marker expression of CD133 (PROMININ-1) in human OS cell lines (SAOS2, MG63 and U2OS). The cells exhibited increased stem-like properties compared to CD133$^-$ cells. They were able to form spheres in culture that after extended passage showed increased expression of Oct4. In addition CD133$^+$ cells also expressed NESTIN (a marker for neural stem cells and brain CSCs) suggesting the use of both markers for the identification of OS CSCs [82]. Similarly, Adhikari *et al*. [86] investigated a CSC population positive for CD117 (c-KIT, receptor for stem cell factor, proto-oncogene and marker for ovarian CSCs) and STRO-1 (cell surface marker for osteogenic progenitor cells in the bone marrow) that was able to form spheres and were more tumorigenic than CD117$^-$/Stro1$^-$. Additionally, CD117, STRO-1 and ABCG2 were expressed at higher levels.

Wang *et al.* discovered a subpopulation of stem cell-like cells with high ALDH in OS cell lines following xenograft transplantation. Aldehyde dehydrogenase is a detoxifying enzyme responsible for oxidation of intracellular aldehydes. High ALDH expression identifies CSCs in a number of cancers including breast, liver, colon and acute myelogenous leukemia [87,88,89,90] and has been linked to chemoresistance. It was shown that while adherent Hu09, Saos-2 and MG-63 cell lines possess small subpopulations of high ALDH activity (1.8%, 1.6% and 0.6%, respectively), OS99-1 contained 45%. Interestingly, the growth of those cells in tumor xenografts dramatically decreased the percentage of cells with high ALDH, however they showed characteristic cancer stem cells features of self-renewal, ability to produce differentiated progeny and increased expression of the stem cell genes OCT3/4, NANOG and SOX2. The use of ALDH as a marker of CSC is still controversial because it also serves as a marker of cell viability and detoxifying ability in normal cells, therefore its enhanced expression in CSCs may simply reflect a healthier cell population.

Murase and colleagues screened seven OS cell lines and a bone malignant fibrous histiocytoma (MFH) cell line for the presence of a side population (SP) [84]. SP cells are capable of effluxing the DNA-binding dye Hoechst 33342 using ATP-binding cassette transporters. This property has been used to identify CSCs in

gastrointestinal and ovarian cancers [91,92]. Of the OS cell lines one tumor population as well as MFH cells had an identifiable SP fraction. Moreover, only the SP cells derived from the MFH cell line were able to form tumors in immunocompromised mice. In contrast Yang *et al.* [85] isolated SP cells from primary OS cell lines and found that SP cells and non-SP cells were capable of tumor formation, thus questioning the validity of SP as a marker of CSCs. These results point out the importance of utilizing alternative assays and cell surface markers for the identification and isolation of putative sarcoma CSCs as SP-based isolation does not appear to be sufficient.

Evolution of the CSC theory incorporates interaction with the tumor microenvironment and the potential for directional interconversion of tumor cells between tumorigenic and non-tumorigenic phenotypes to explain the heterogeneity of the tumors [93,94]. This adds more difficulties in the identification of a CS population in osteosarcomas and will require the use of more sophisticated techniques for isolation and culture to preserve their niche for sustaining the true stem cell phenotype *in vitro*.

Conclusions

Our understanding of OS biology is hampered by its sudden onset, low prevalence, and absence of predisposing conditions or precursor lesions. With limited human tissue available for study, animal models provide a valuable tool to investigate the underlying mechanisms driving tumor initiation, progression, metastatic events and therapeutic interventions. While no model has yet faithfully recapitulated all aspects of OS, there is no doubt that the study of murine models has enabled some insight into the genetics of OS tumor initiation as well as the cellular and molecular profiles of tumor growth and metastasis. In particular, gene knockout studies have been instrumental in identifying genetic mutations that promote OS tumor initiation (*P53)*, as well as co-operative mutations that increase disease incidence (*RB*, *c-FOS*).

Integral to the study of the mechanisms of tumor initiation is identifying the cell of origin. While this area has received much focus the identity and role of the cell of origin is poorly understood. With the use of cell lineage specific markers it is now possible to introduce genetic mutations at defined developmental stages to investigate OS incidence and tumor pathology. With this strategy *Prx1* and *Osx* have been used to identify mesenchymal and osteoprogenitors cells, respectively, following conditional mutation of *p53.* It remains to be seen, however, whether these populations are truly distinct, as *Prx1* could be coexpressed with *Osx* in a certain subpopulation of cells. Another consideration particularly relevant in OS is tumor heterogeneity, which may infer that multiple cell types could act as cell of origin. Its identification may permit a more systematic analysis of the genetic lesions involved in OS initiation and progression, and could serve as a platform for the identification of early disease biomarkers. Cell of origin identification may also have important implications in the prevention of relapse and elucidate key molecular pathways and driver mutations that could lead to new therapeutic approaches to prevent the disease.

There is evidence to suggest that osteosarcomas contain tumor-propagating CSCs that can self-renew and induce tumor recurrence. Thus, cytotoxic therapies directed toward shrinking the tumor tissue must eliminate these cells. In order to develop CSC-targeted therapies for future studies, it will be necessary to establish unequivocal markers that identify and isolate these cells from non-

tumorigenic populations. To this end, certain candidates have been identified (OCT4, SOX2, CD133, etc.), and better characterization is needed to confirm the validity to establish molecular profiles of tumorigenicity as well as their potential for future drug development.

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