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Cancer stem cells in Osteosarcoma

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Abstract:

Osteosarcoma is the most common primary bone tumour in children and adolescents and advanced osteosarcoma patients with evidence of metastasis share a poor prognosis. Osteosarcoma frequently gains resistance to standard therapies highlighting the need for improved treatment regimens and identification of novel therapeutic targets. Cancer stem cells (CSC) represent a sub-type of tumour cells attributed to critical steps in cancer including tumour propagation, therapy resistance, recurrence and in some cases metastasis. Recent published work demonstrates evidence of cancer stem cell phenotypes in osteosarcoma with links to drug resistance and tumorigenesis. In this review we will discuss the commonly used isolation techniques for cancer stem cells in osteosarcoma as well as the identified biochemical and molecular markers.

Keywords: osteosarcoma, bone cancer, cancer stem cell, tumour heterogeneity

Cancer stem cells and tumour heterogeneity: what do we know about osteosarcoma?

Osteosarcoma predominantly initiates in the metaphysis of the long bones with a high prevalence in children and young adults. The origin of a tumour is potentially a single cell located in the bone marrow, which will eventually give rise to a polyclonal, heterogeneous tumour mass. Analysis of tumour heterogeneity allows us to decipher the steps that were taken from the initiating cell to the development of a heterogeneous tumour mass comprised of an array of distinguishable sub-clones. Indeed, osteosarcoma initiates as a monoclonal disease, which quickly develops into a polyclonal disease and is considered one of the most complex cancers in terms of molecular aberration. Deeper insight into this diversity therefore holds great promise to identify markers associated with the most aggressive tumour cells within a tumour mass. The vast heterogeneity found in osteosarcoma is shown in an exome sequencing study in which multiple pathways (14 driver genes) were identified (1). The authors suggest that no single driver gene can be pinpointed to be the cause of the majority of investigated tumours and that several oncogenic pathways cause genetic instability in osteosarcoma development. Importantly, this high level of heterogeneity adds increased complexity for effective treatment strategies, which is clinically reflected in refractory and recurrent disease.

The increasing knowledge of the cancer genome through in depth analysis using for example deep sequencing has significantly added to the understanding of intra-tumour heterogeneity and an evolutionary pattern of a subset of clones within a tumour has been reported (2). New technologies now allow us to view heterogeneity also on a single cell level. This has clearly increased the tumour complexity over performing analysis on bulk tissue showing even deeper levels of intra-tumour heterogeneity in many cancer types (3-6). Single

cell analysis on CSCs in osteosarcoma has, to our knowledge, currently not been reported but could significantly help to understand the diversity of these cells.

Cells-of-origin in osteosarcoma and properties of cancer stem cells

In osteosarcoma several cell-of-origin models have been proposed including transformation of undifferentiated mesenchymal stem cells (MSCs) as well as more committed osteogenic progenitor cells (7) (Figure 1). Osteosarcoma is a bone forming tumour invading frequently the surrounding soft tissues as revealed by conventional imaging associating X-ray and Magnetic Resonance Imaging (MRI) (Figures 1A-C). Osteosarcoma is a vascularized tumour characterized by typical osteoid matrix formed by cancer cells (Figures 1D,E). Evidence comes predominantly from i*n vivo* studies using MSCs and/or osteoprogenitors in which for example mutations in genes such as *P53* and *RB* and/or aberrant Hedgehog and NOTCH signalling were shown to induce osteosarcoma (7-9). The terminology of 'cancer stem cells' is still under debate with the association to stem cells remaining controversial. Indeed, cancer stem cells and cancer initiating cells are often used interchangeably although they may indeed exhibit different properties. In a cancer stem cell model, tumours are thought to be hierarchically organised with a subpopulation of self-renewing cells at the basis of tumour progression (10). These cancer stem cells (CSCs) are proposed to be unique subsets of clones within a tumour mass attributed with tumour propagation, resistance to therapy and have in some studies been attributed to initiate metastases. Evidence exists in osteosarcoma that patients may present with distant metastases decades after completion of their first treatment (11) potentially further highlighting the tumorigenic characteristics of CSCs although this currently remains speculative. Similar to the origin of osteosarcoma, the

existing hypotheses for the origin of CSCs in general include the transformation of undifferentiated stem cells or more committed cells to gain aberrant self-renewal properties (12). Remarkably, a stem cell transcription factor (Sox2) has been shown to maintain osteosarcoma CSCs (13) through inhibition of the Hippo pathway (14). The complex self-renewal process of normal stem cells (15) is partly regulated by external signals from the surrounding microenvironment, the stem cell niche. Such a specialised microenvironment has also been proposed for CSCs, influencing CSC function and survival and in some cancers these niches (e.g. vascular, immune, bone) may even overlap with the normal stem cell niche (16). In addition, the bone niche and the bone microenvironment that osteosarcoma cells and the putative CSCs are surrounded by apply continuous pressure thus further influencing genomic instability, dormancy and potential new resistant mechanisms (17).

Methods to identify cancer stem cells

Several methods have been developed to identify and isolate CSCs based on their self-renewal properties and have been discussed in detail elsewhere (10, 18, 19). Functional *in vitro* assays are frequently initially applied to enrich for CSCs. This is often followed by more descriptive assays and *in vivo* verification; however, the order and selection of the tests appears to be interchangeable depending on the study. The functional *in vitro* test of formation of tumour spheres under non-adherent and serum-free conditions often serves as the initial step to enrich for CSC-like cell populations (Figure 1F). The standard method to verify CSC candidates *in vivo* is the serial transplantation of isolated putative CSCs into immunocompromised mice to assess tumorigenic capacity at low cell numbers. Both the *in vivo* and *in vitro* functional assays have the disadvantage that truly quiescent CSCs will

possibly not be identified. The functional methods are frequently used in connection with descriptive assays like the measurement of gene expression levels of stem-like factors and identification based on cell surface markers. In order to assess cell populations for proposed drug resistance properties side population analysis (dye exclusion assay and ALDH (aldehyde dehydrogenase) activity are frequently applied to enrich for CSCs with these characteristics. Importantly, all methods require careful consideration because under none of these experimental conditions a pure CSC population or detection of all CSC sub-populations can be assumed. The methods are more likely to enrich the sample for (specific) CSCs through experimentally-induced selection and in some cases environmental pressure. The use of a number of methods including functional experiments and careful interpretation should therefore be applied to CSC research.

Identification of cancer stem cells in osteosarcoma

In the following section the main identified markers and evidence for the existence of CSCs in osteosarcoma will be discussed. The studies were grouped based on their initial enrichment method and an overview can be found in **table 1**.

Side population (dye exclusion assays)

One main feature of CSCs is their potential to evade treatment and it is suggested that this is achieved through an increase in ATP-binding cassette (ABC) multidrug efflux transporters such as MDR1/ABCB1 and BRCP1/ABCG2 and ABCB5 expression. This trait has been used for CSC identification by measuring the ability of cells to exclude DNA-binding dyes (Hoechst

33342 or Rhodamine 123) by fluorescence-activated cell sorting (18, 20-22). Cells expressing high ABC transporters exclude the dyes and are visible as a 'side population' during analysis. Murase *et al* (23) studied the side population fraction in seven osteosarcoma cell lines but only one line (NY) was reported to have a side population fraction while side population cells were hardly detected in the rest of the cell lines. They also studied one bone human malignant fibrous histiocytoma cell line (MFH2003) which was shown to have the largest side population fraction and to exhibit increased ABCG5 expression as well as cancer-initiating characteristics detected by increased sphere formation and tumour formation *in vivo* when compared to non-side population cells.

A higher detection rate of side population cells in osteosarcoma was shown in human primary samples (24). Downstream analysis of the side population cells showed upregulated gene expression (ABC transporters, Oct4, Nanog), increased sphere formation and higher multidrug resistance to doxorubicin, methotrexate and cisplatin compared to non- side population cells. When injected into immunocompromised mice, the proposed CSCs showed higher tumorigenic potential although non-side population cells at higher numbers could also form tumours. Similar experiments carried out with U2OS cells did not show a side population fraction.

Aldehyde dehydrogenase (ALDH)

A second, frequently investigated approach through which CSCs apply their chemoresistance is the expression and activity of the drug-detoxifying enzyme ALDH. A subpopulation of $ALDH1^{high}$ MG63 cells was detected by Honoki *et al* (25) who further reported $ALDH1^{high}$ cells to have increased expression of stem-like genes (Nanog, Oct3/4, Stat3, Sox2), higher

resistance to doxorubicin and cisplatin and increased self-renewal ability as shown by sphere formation. Importantly, $ALDH^{high}$ cells were also detected in human osteosarcoma samples and increased ALDH activity was further correlated to metastatic potential (26). It was also shown that inhibition of ALDH activity using disulfiram resulted in reduced cell proliferation suggesting direct targeting of CSC phenotype cells. A second study reported large ALDH-bright cell population in the OS99-1 osteosarcoma cell line compared to lower percentages in Hu09, Saos-2 and MG63 (27). Interestingly, injection of the OS99-1 cells into mice followed by ALDH activity measurement showed a lower ALDH-bright fraction compared to the parental line cultured *in vitro*. The ALDH-bright cells were further characterised to be more tumorigenic *in vivo* and exhibit higher stem like gene expression (Nanog, Sox-2, OCT3/4) compared to ALDH-low cells.

Cell surface markers

Cell surface markers are arguably the most attractive and sought after identification method of CSCs. A specific cell surface marker exclusively to CSCs and not normal cells, including stem cells, would drastically simplify the isolation and treatment potential for these cells.

CD133 (prominin)

Tirino *et al* (28) showed that CD133, a membrane glycoprotein, may be a marker of CSCs in osteosarcoma. $CD133^{+}$ cells were identified in three osteosarcoma cell lines (Saos2, MG63, U2OS). The $CD133^{+}$ cells were further characterised to be more proliferative, overexpress OCT3/4 and ABCG2, have a small side population fraction and formed spheres in serum-free

conditions while $CD133^-$ cells did not. Interestingly, the authors detected intracellular CD133 staining in $CD133^+$and $CD133^-$ cells and reported that mRNA levels of CD133 were identical between the two populations. The group was unable to grow $CD133^+$ or $CD133^-$ tumours *in vivo*. In a follow up study, $CD133^+$ cells were shown to be present in two human primary osteosarcomas and exhibiting stem-like gene expression (e.g. OCT3/4, Nanog), sphere formation and side population fractions (29). In addition, cells isolated from $CD133^+$ derived spheres were shown to form large tumours *in vivo* compared to the adherent cells. Interestingly, in both studies it was shown that $CD133^+$ cells could give rise to $CD133^-$ cells in culture suggesting a phenotypic switch.

The expression of CD133 on FFPE samples of human osteosarcoma was shown by He *et al* (30) who detected the marker on 46 out of 70 analysed samples and positively correlated CD133 expression with lung metastases and decreased overall survival. In addition, characterisation of MG63 cells showed that $CD133^+$ subsets were more migratory, invasive and overexpressed Oct4, Nanog and CXCR4. $CD133^+$ cells in patient samples of osteosarcoma and a $CD133^+$ sub-population in Saos2 cells were also reported by Li *et al* (31). A comprehensive assessment of CD133 expression and associated CSC phenotypes on cell lines (Saos2, U2OS, MG63, HOS, MNNG/HOS, 143B) and primary tumours was followed by in depth analysis of miRNA expression profiling which identified miR-133a in connection with CD133 expression (32). The micro RNA was further shown to regulate tumorigenic potential since silencing in combination with chemotherapeutic treatment significantly reduced the aggressive tumour type and lung metastases *in vivo*. Interestingly, CD133 expression could be induced by chemotherapy treatment and was associated with enhanced miR-133a expression suggesting a CSC induction through therapeutic challenge. Overall, CD133 appears as potential therapeutic target in osteosarcoma and pharmacologic induction of a

switch from the CD133+ to CD133- phenotype through siRNA could be a new therapeutic approach.

CD117 (c-kit), Stro-1

A study assessing the feasibility of using MSC surface markers to enrich for CSCs in osteosarcoma was done by Adhikari *et al* (33), who convincingly showed that $CD117^+$/$Stro\text{-}1^+$ double-positive cells were present in mouse and human osteosarcoma cell lines as well as primary cultures. Intriguingly, the double-positive fraction showed higher sphere formation ability, increased resistance to doxorubicin treatment and overexpressed CXCR4 and ABCG2. Furthermore, double-positive cells more readily formed tumours at a lower cell number and it was shown that this CSC fraction had increased metastatic potential compared to double negative cells. This study was one of the first to comprehensively assess the molecular constitution of the identified CSCs and to furthermore show functional CSC characteristics in a number of experiments.

CD271

CD271 is a neural crest low-affinity nerve growth factor receptor and a marker of bone marrow mesenchymal stem cells. A study investigating the stem like phenotype in $CD271^+$ cells was performed by Tian *et al* (34). $CD271^+$ cells were found on FFPE tissue of human osteosarcoma samples and the cell lines MNNG/HOS, U2OS and Saos2 also contained $CD271^+$ subpopulations. The authors went on to show that $CD271^+$ cells had upregulated stem cell gene expression, resistance to chemotherapy and were more readily detected in spheres. When $CD271^+$ and $CD271^-$ cells were separated the $CD271^+$ fraction showed higher

tumorigenic potential in the sphere formation assay and when injected into immunocompromised mice.

Sphere formation assays

Tissue stem cells have been identified through the ability of cells to form spheres and this method has also been used to assess CSC presence. Sarcospheres (spheres growing from sarcoma cells) have been grown under similar experimental set ups to those published for neural stem cells including non-adherent, serum-free conditions and the supplementation of media with growth factors (N2, epidermal growth factor etc.).

The first to describe osteosarcoma stem cells using sphere formation was Gibbs *et al* in 2005 (35). This was followed by several other groups, which further showed that sphere forming cells were tumorigenic in immunodeficient mice and that these cells were more drug resistant suggesting a CSC phenotype (36). A detailed study on the sphere forming fraction of MNNG/HOS cells was done by Martins-Neves *et al* (37) who showed that cells isolated from spheres had mesenchymal stem cell properties including upregulated gene expression of stemness genes Oct3/4, Nanog, and ABC transporters. They went on to show that sphere cells were more resistant to chemotherapy and radiation and that these cells exhibited higher cancer-initiating properties *in vivo* when compared to parental cells. It is noteworthy that the main CSC selection method was sphere formation suggesting that the experimental conditions of this assay induced CSC-like characteristics. Indeed, the same group later showed that the selection or enrichment method (i.e., sphere forming ability, side population analysis or ALDH activity) applied to each cell line of a larger panel was pre-selecting CSCs with specific and dissimilar characteristics in single cell lines (38). Importantly,

these data suggest that CSCs with specific molecular and functional characteristics are enriched depending on the methods and thus heterogeneous CSC sub-populations may exist side by side in one and the same cancer cell line. To our knowledge this study is one of very few applying several initial CSC enrichment methods on the same samples thus suggesting that heterogeneity may be present within the osteosarcoma CSC population.

The potential plasticity of the CSC phenotype in osteosarcoma was shown by Zhang *et al* (39). The authors presented that MNNG/HOS, Saos2 and MG63 cells readily formed spheres and that treatment with TGFβ1 or application of hypoxic conditions (both abundant in the bone microenvironment) significantly increased sphere formation suggesting an induction of CSCs through environmental factors. Sphere cells had increased stem like factor and ABC transporter expression levels, increased resistance to cisplatin and adriamycin and *in vivo* tumour formation. The role of TGF in general tumour progression was widely studied and its role in osteosarcoma suspected (40). Indeed, the relevance of TGFβ1 to propagate a stem like phenotype in osteosarcoma was shown when inhibition of the TGFβ1-receptor resulted in reduced sphere formation. *In vivo* experiments using CSCs from spheres, CSCs from TGFβ1-induced spheres and parental cells further confirmed the enrichment of aggressive CSCs in the TGFβ1-induced sphere population since this group was capable of forming the most tumours. The authors concluded that CSCs may develop de novo from differentiated cancer cells and that they can revert back into a more differentiated state underlining a potential of plasticity. In addition, the study elegantly highlights the importance of the microenvironment (or experimental conditions) for CSCs.

MicroRNAs (miRNA) regulate gene expression on a post-transcriptional level and have been proposed to be involved in cancer progression and initiation. The micro RNA miR-

26a was shown to be significantly lower in sarcospheres or the ALDH positive fraction of several osteosarcoma cell lines compared to the parental lines (41). Doxorubicin treatment also reduced miR-26a levels suggesting an increase in stem like cells after treatment and a correlation between low miR-26a levels and CSCs. This was confirmed since over-expression of miR-26a significantly reduced gene expression of stemness markers, resulted in fewer and smaller spheres, formed fewer tumours and increased the sensitivity to chemotherapeutic challenge compared to control. As a mechanism the group suggested that miR-26a reduces osteosarcoma malignancy via Jagged1 (notch pathway) suppression. Importantly, analysis of miR-26a levels in patient samples linked high levels to a better prognosis. Finally, a link between osteosarcoma CSCs and telomerase activity was reported by Yu *et al* after detection of cells with high telomerase activity under sphere formation conditions (42).

Induction of cancer stem cell phenotype through chemotherapeutic treatment

The presence of a therapy-resistant, tumorigenic sub-population of cancer cells in a tumour is visible in patients with refractory disease and significantly highlights the necessity to further understand the nature of these cells. This chemoresistant characteristic has been exploited in a few studies investigating whether chemotherapy treated or treatment-resistant cells could contain CSCs (43-46). Indeed, it was shown that methotrexate pre-treated or resistant cells not only expressed $CD117^{+}$/$Stro\text{-}1^{+}$ and had a side population but that these cells also formed more spheres *in vitro* and were more tumorigenic when injected subcutaneously into immunodeficient mice (44). The finding that cells with CSC characteristics could be enriched by treatment via activated Notch signalling pathways was shown in a recent study using cisplatin-resistant 143B and U2Os cells (46). Cisplatin-resistant

cells were enriched for $CD117^{+}$/$Stro\text{-}1^{+}$ double-positive cells, formed more spheres and were more tumorigenic *in vivo*. Importantly, the group also assessed relapsed tumours after cisplatin treatment *in vivo* and reported an increase in CSCs with elevated Oct4, Sox2, CD117, Stro-1 levels and sphere formation capability.

The exploitation of chemoresistant cancer cell traits was also used by Martins-Neves *et al* (43) who found that stem-like cells could be induced with chemotherapy treatment in a number of fibroblastic and osteoblastic osteosarcoma cell lines. The identified treatment-induced cells had increased $ALDH^{high}$ populations, upregulated ABC transporters and showed overexpression of stemness genes as well as Wnt/beta-catenin signalling pathways. The stem like features could be reduced when the Wnt/beta-catenin pathway was inhibited *in vitro* and it was shown that a combination of chemotherapy and Wnt-inhibitor was the most efficacious anti-tumour treatment *in vivo*. The findings of the induction of a stem-like phenotype through chemotherapy were strengthened with the correlation of 'poor response' to chemotherapy and stem-like gene expression in osteosarcoma patients. The selection of an aggressive CSC phenotype after cisplatin treatment could be suggested by the work of Tsuchida *et al* (45). Side population cells of the HOS cell line treated or not treated with cisplatin were isolated and while cisplatin-induced side population cells formed tumours *in vivo* the side population fraction isolated from untreated cells did not. Furthermore, cisplatin-induced activation of VEGF/Flt1 signalling was reported to accumulate the highly tumorigenic CSC-type cells.

Collectively the reports using chemotherapeutics show that a CSC phenotype can be induced through therapeutic challenge. It remains to be established whether different chemotherapeutic challenges will give rise to different CSC sub-populations.

Conclusion

The debate about a cancer stem cell definition is still ongoing and importantly it is not clear whether the stem cell phenotype of a cancer cell is intrinsic or plastic. Tumour cells can switch in and out of a stem cell phenotype further increasing the complexity of the cancer stem cell hypothesis and suggesting that environmental pressure (experimental or in patients) may be a trigger for heterogeneity and plasticity. For example increased environmental pressure through chemotherapy treatment has the potential to induce a switch from a differentiated towards a stem-like phenotype in osteosarcoma (43-46). This potential plasticity and the many different experimental enrichment methods may help to understand the variations of reported CSC markers between studies. Importantly it appears that the enriched CSC phenotype may depend on the applied isolation method(s), however, more studies are required to verify this. Moreover, it is currently unclear whether CSCs inherently exhibit metastasis-initiating properties.

The high level of heterogeneity found in osteosarcoma, including the effect of the tumour microenvironment may be more comprehensively assessed in PDX (patient derived xenograft) models and evidence exists that cells with CSC phenotype are present in patient derived osteosarcoma samples (24, 46). The use of patient derived material could potentially not only reflect the high heterogeneity of this rare cancer but would also reflect the influence of the human microenvironment in more detail compared to studies on cell lines. Despite these potential advantages the approach may be limited by the low availability of primary chemonaive tumours. The potential expansion of CSCs through the growth of PDX material *in vivo* could possibly help overcome this limitation and requires further in depth

investigation in both model development and CSC research in these models. Moreover technical refinements and research on novel techniques may allow the identification and isolation of pure subpopulations of CSCs from osteosarcoma. The major goal and biological interest of CSC research is the identification of specific treatment targets to effectively deplete CSCs. Several studies have linked CSCs directly to clinical events (24, 30, 41, 43) in osteosarcoma and treatment studies have reported inhibition of osteosarcoma CSCs or the CSC phenotype (reviewed in 7) suggesting that these elusive cells can indeed be targeted.

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Conflict of interest

The authors declare no conflict of interest.

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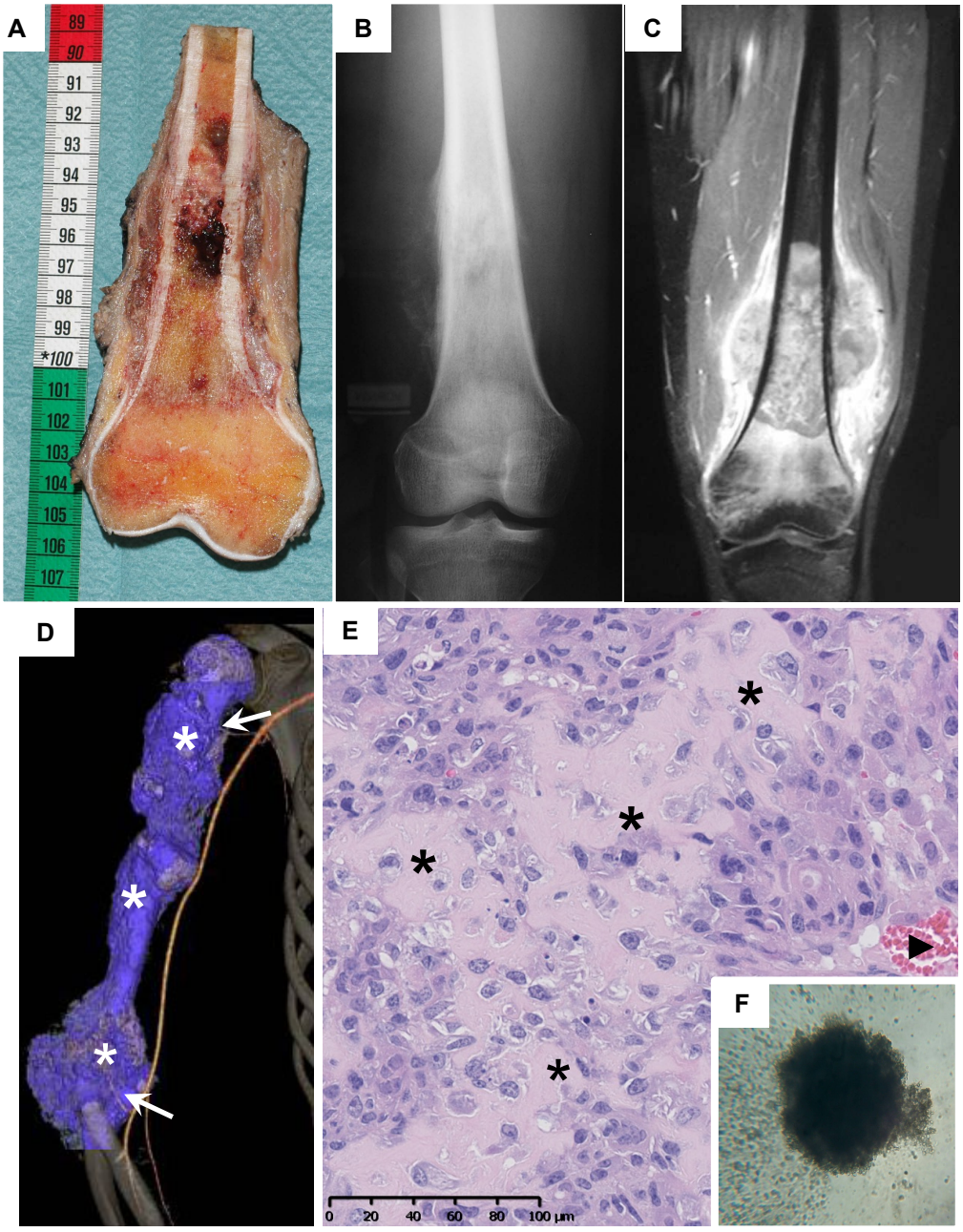
Figure 1: Representative imaging of osteoblastic osteosarcoma. (A) Macroscopic view of a resected osteosarcoma infiltrating surrounding soft tissue. Conventional X-ray **(B**) and (**C**) Magnetic Resonance Imaging (MRI) of osteosarcoma. (**D)** Computed tomography of an osteosarcoma in a 15-year old patient (adapted from "Bone Cancer" 1st Edition, Ed. Heymann D., Academic Press, 2009). Tumour tissue is composed by mineralized component detectable to X-Ray (*) and is strongly associated with the vasculature (arrows). (**E**) Typical histological view showing osteoid extracellular matrix produced by osteosarcoma cells (*), tumour tissue is vascularized (arrow head). (**F)** Sarcosphere generated from the human MNNG-HOS osteosarcoma cell line under non-adherent serum-free conditions.

Table 1: Studies investigating CSCs in osteosarcoma

Initial CSC identification/ enrichment method	Osteosarcoma cell lines/patient samples	Characterisation of enriched CSC phenotype	Reference
Side population	OS2000, KIKU, NY, Huo9, HOS, U2OS and Saos2	Sphere formation, stem like gene expression, *in vivo* tumorigenicity	(23)
	Human primary, U2OS	Stem like genes, sphere formation, drug resistance, *in vivo* tumorigenicity	(24)
ALDH	MG63	Stem like gene expression, drug resistance, sphere formation	(25)
	OS99-1 Hu09, Saos-2, MG63	Stem like gene expression, *in vivo* tumorigenicity	(27)
CD133	Saos2, MG63, U2OS	Stem like gene expression, side population, sphere formation	(28)
	Human primary	Stem like genes, side population, sphere formation, *in vivo* tumorigenicity	(29)
	Human primary (FFPE), MG63	Stem like gene expression	(30)
	Human primary (FFPE), Saos2	Stem like gene expression	(31)
	Human primary, Saos2, U2OS, MG63, HOS, MNNG/HOS, 143B	Sphere formation, drug resistance, *in vivo* tumorigenicity, stem like gene expression	(32)
CD117/Stro-1	K7M2, KHOS/NP, MNNG/HOS, 318-1, P932, BCOS	Sphere formation, drug resistance, stem like gene expression, *in vivo* tumorigenicity and metastatic potential	(33)
CD271	Human primary (FFPE), MNNG/HOS, U2OS, Saos2	Stem like gene expression, sphere formation, drug resistance, *in vivo* tumorigenicity	(34)
Sphere formation	Human primary, MG63	Stem like gene expression	(35)
	MG63	Drug resistance, *in vivo* tumorigenicity	(36)
	MNNG/HOS	Stem like gene expression, drug resistance, in vivo tumorigenicity	(37)
Sphere formation, ALDH, side population	HOS, MG-63, MHM, MNNG-HOS, SJSA-1, L2531, L3312, OHS, U2OS	Stem like gene expression, *in vivo* tumorigenicity	(38)
Sphere formation	MNNG/HOS, Saos2, MG63 + TGFβ/hypoxia	*In vivo* tumorigenicity, drug resistance, stem like gene expression	(39)
Sphere formation, chemotherapeutic treatment, ALDH	Human primary, U2OS, MG63, Saos-2 and 143B, link to miR-26a	Stem like gene expression, *in vivo* tumorigenicity, drug resistance	(41)
Chemotherapeutic treatment	U2Os	$CD117^{+}$/Stro-1, side population, sphere formation, *in vivo* tumorigenicity	(44)
	Cisplatin-resistant 143B and U2Os	$CD117^{+}$/$Stro\text{-}1^{+}$, sphere formation, *in vivo* tumorigenicity, stem like gene expression	(42)
	HOS, MG-63, MHM, MNNG-HOS, OHS, U2OS	ALDH high, stem like gene expression	(43)
	HOS	Side population, *in vivo* tumorigenicity, stem like gene expression	(45)

Figure 1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