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The Heterogeneity of Osteosarcoma: The Role Played by Cancer Stem Cells.

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Abstract

Osteosarcoma is the most common bone sarcoma and is one of the cancer entities characterized by the highest level of heterogeneity in humans. This heterogeneity takes place not only at the macroscopic and microscopic levels, with heterogeneous micro-environmental components, but also at the genomic, transcriptomic and epigenetic levels. Recent investigations have revealed the existence in osteosarcoma of cancer cells with stemness properties. Cancer stem cells are characterized by their specific phenotype and low cycling capacity, and are linked to drug resistance, tumour growth and the metastatic process. In addition, cancer stem cells contribute to the enrichment of tumour heterogeneity. The present manuscript will describe the main characteristic features of cancer stem cells in osteosarcoma and will discuss their impact on maintaining tumour heterogeneity. Their clinical implications will also be briefly addressed.

Key words: osteosarcoma; cancer stem cell; tumour heterogeneity; drug resistance; cell dormancy

Introduction

Osteosarcoma is part of the family of malignant bone sarcomas which originate from a common mesenchymal precursor located in the bone marrow, and known as mesenchymal stem cells (1). Osteosarcoma is the main bone sarcoma in adolescents and young adults, with a peak of incidence at around 18 years old. Osteosarcomas are preferentially detected in the metaphysis of long bones and the tumour tissue is characterized by the presence of osteoid matrix produced by cancer cells (Figure 1). Microscopic heterogeneity is the first marker for osteosarcoma with the presence of highly vascularized, necrotic, proliferating and osteoid foci. Depending on the morphological features of the cancer cells, osteosarcomas can be classified as osteoblastic, chondroblastic, fibroblastic or telangiectatic. Current treatment combines neo-adjuvant chemotherapy, surgery and adjuvant chemotherapy including at least three cytotoxic agents such as doxorubicin, methotrexate and ifosfamide. Unfortunately, prognosis remains poor and overall survival has stagnated in the last four decades (10). Overall survival reaches 50-70% at 5 years depending on the series in the absence of detectable metastases, but drops to 30% when lung metastases are detected at the time of diagnosis.

Tumour heterogeneity can be directly related to both the natural history of the cancer cells and to their dialogue with the protagonists in the local micro-environment (2-6). The local micro-environment is composed of numerous cells types, including immune (e.g. tumour-infiltrating lymphocytes, tumour-associated macrophages) (7-9) and non-immune cells such as endothelial cells, fibroblasts and mesenchymal stem cells, which are spatially, temporally and functionally linked to cancer cells (1). Cancer cells can control the behaviour of their neighbours, which in turn play a part in fuelling tumour growth and the metastatic process. Cancer cells are composed of numerous cell clones competing together to preserve the overall survival of their congeners through selective advantage. Some of these clones drive tumour initiation and are called cancer stem cells (11). Even if the term “stem cell” is not perfectly appropriate, it describes a subpopulation of cells capable of reconstituting the characteristics of all cancer cells detectable in the tumour mass. Consequently, cancer stem cells can generate a tumour mass after inoculation into an immunodeficient organism (12).

The present review will discuss the main data available in the literature in favour of the existence of cancer stem-like cells in osteosarcoma, as well as their potential contribution to the enrichment of tumour heterogeneity. Their clinical impact in drug resistance will be also

discussed.

Clonal evolution of cancer cells in osteosarcoma: a combination of oncogenic and epigenetic events

In parallel to histological heterogeneity, osteosarcoma is one of the most complex oncologic diseases in terms of genetic aberration. In 2014, Reimann *et al*. found wide genomic rearrangements in the tumour exome of a single case of osteosarcoma (13). These authors detected 3,000 somatic single nucleotide variants, small indels and more than 2,000 copy number variants in diverse chromosomes. The osteosarcomas were thus characterized by a loss of heterozygosity. The complexity of the disease was confirmed by Bousquet *et al*. who studied a series of 44 osteosarcomas and observed recurrent somatic alterations to *TP53* and *RB1* and also detected 84 mutation points and 4 deletions related to 84 genes (14). Similarly, Smida *et al*. analyzed 160 osteosarcoma samples by whole-genome sequencing in order to identify somatic copy number alterations. They found specific unstable genomic regions in which numerous tumour suppressor genes were included (e.g. *TP53, RB1, WWOX, DLG2)* (15). This very high number of alterations perfectly illustrates the genomic complexity of osteosarcomas. The development of cancer is sustained by two main theories: i) the "linear model" theory, which is based on successive accumulations of oncogenic events in one cell leading to the development of a heterogeneous disease; ii) the branched evolution theory known as the "parallel model", characterized by the parallel evolution of subclones which accumulate DNA alterations and also lead to a polyclonal tumour mass (16, 17). Of course, the nature and number of these oncogenic events drive tumour initiation as has been shown by Funes *et al*. who transformed mesenchymal stem cells using genetic alterations (18). They observed that 4 oncogenic hits made possible the formation of colonies in agar, although only 5 oncogenes were able to induce tumour development in immunodeficient mice. They also suggested that tumourigenesis of modified mesenchymal stem cells was dependent on the nature of the oncogene. For instance, disruption to the RB pathway was enough to induce anchorage-independent growth of mesenchymal stem cells (18). In addition, one oncogenic hit sensitized mesenchymal stem cells to carcinogenic agents such as pesticides and may have led to tumour development in immunocompromised mice (19). In addition, the clonal evolution of cancers is tightly controlled by the selective pressure of the local micro-environment (e.g. immune infiltrate, hypoxia), and can be oriented under drug pressure toward resistant or tolerant cancer cells (20, 21). Regardless of what the first oncogenic event is, a permissive local micro-environment is obligatory for protecting cancer-initiating cells

against immune cells and fuelling these cells with adequate nutrients (22).

Both types of clonal evolution have been described in osteosarcoma (23). Wang *et al.* analyzed and compared 86 tumours in 10 osteosarcoma patients using whole exosome and genome sequencing. By analyzing the architecture and relationships of the cancer subclones, they demonstrated a dynamic mutational process and, for the first time, two patterns of lung metastases – with a linear model in 6 patients and a branched model in 4 patients. Based on the low number of patients included, the co-existence of both models in a same patient can be excluded. The tumour evolution model has recently been enriched by a "plasticity" model identified in Ewing sarcoma (24). In Ewing sarcoma, the plasticity model is based on equilibrium between various cancer cell subclones differentially expressing the chimeric EWS1/FLI1 transcription factor, leading to major modifications in cell migration and invasion properties. The two populations create an ecosystem with dynamic fluctuation in cells differentially expressing the fusion protein depending on the stage of the disease.

Very recently, Gambera *et al*. established multicolour (RGB) $p53^{-/-}$ $Rb^{-/-}$ mouse mesenchymal stem cells (25) that can form osteosarcomas when inoculated into bone micro-environment cells. They also deciphered the clonal evolution during tumour progression (26). They identified two main steps in tumour progression. At an early stage of development (25 days), tumour growth is characterized by polyclonal expansion with no modification to the proportions of the coloured cells injected. At a late stage of tumour growth (50 days), Gambera *et al*. observed the emergence of dominant clones at the periphery of the tumour mass, corresponding to clonal evolution of the disease. Overall, these data provided evidence of marked clonal modifications in cancer cells from a polyclonal context to the formation of dominant clones which were oligoclonal and exhibited similar tumourigenesis properties (25). In addition, the metastatic process to the lung was associated with an oligoclonal and monoclonal dynamic. Although this model cannot be transposed to humans, there is some evidence of dominant clones in osteosarcoma. In 2015, Kovac *et al*. investigated the evolutionary landscape using exosome sequencing in 31 osteosarcoma samples (27). They identified 14 genes associated with a BRCAness signature as the main drivers for tumour development not exclusively expressed in all subclones. *TP53* mutations were frequently observed in subclones. These authors hypothesized that osteosarcoma could be initiated by a mutation in *TP53* or *RB* in one specific subclone (monoclonal disease), leading to chromosal instability and chromatide breakages, and to new oncogenic events in various subclones

(polyclonal disease). PARP inhibitors may then be a therapeutic option in osteosarcoma (28). The existence of dominant subclones was confirmed by Chen *et al*. by studying a case report of a chemoresistant osteosarcoma sample in which they identified a clone associated with a new *TP53-KPN3* translocation (29). The three models for cancer cell evolution are responsible for the considerable heterogeneity found in osteosarcoma and the emergence of dominant clones which evolve in a dynamic manner and in perfect symbiosis with their permissive ecosystem.

As shown in Ewing sarcoma, for which the heterogeneity of DNA methylation is a reflection of the spectrum of the disease (30), epigenetic genetic alterations are observed in osteosarcomas and are associated with its pathogenesis (e.g. tumour growth, metastatic process) (31-33). Epigenetic modulations can regulate osteosarcoma cell differentiation and can concomitantly interfere with their micro-environment (34-36). For instance, Lamoureux *et al*. demonstrated that selective inhibition of bromodomain epigenetic signalling induced an inhibitory effect in primary tumour growth and simultaneously in osteoblasts and osteoclasts, two cell types found in the local micro-environment (35). More recently, Li *et al*. gave evidence of epigenetic downregulation in osteosarcoma cells of CXCL12 (SDF-1) via DNA methyltransferase-1, related to their ability to form lung metastases and, interestingly, to their impairment of cytotoxic T-cells homing in on the tumour mass (36). They found a correlation between CXCL12 expression and the overall survival of osteosarcoma patients. Tumour heterogeneity and clonal evolution of osteosarcomas are thus regulated by epigenetic events.

Presence of cancer stem cells in osteosarcoma: their functional impact

The conventional theories for clonal evolution described above can be completed by the "cell origin" theory. In this theory, the first oncogenic event may occur in a cancer stem cell or in a cell in the non-side population (37), with cancer as the end result of successive cell divisions in stem cells with cumulative DNA replication errors (e.g. mutations, epigenetic mistakes) making possible both the self-renewal of "differentiated" cancer cells and the maintenance of undifferentiated cells. The presence of a side population that excludes Hoechst 33342 dye has been demonstrated on osteosarcoma cell lines and in human primary osteosarcoma (38, 39). These cells are able to regenerate both side- and non-side cells, show higher clonogenicity than non-side populations and sustained tumourigenicity. They have also shown increased multi-drug resistance and are phenotypically similar to stem cells thanks to the expression of Oct-4 and nanog for instance. Through analogy with embryonic stem cells,

this side population has been called cancer stem cells or stem-like cells. However, their immune-tolerant property, their low cycling characteristic and drug resistance have led to this population also being referred to as dormant, quiescent, tolerant and persister cells (21). It has been suggested that cancer stem cells are unique subclones within a tumour, responsible for tumour progression, resistance to therapies and the initiation of metastases. This definition is supported by clinical cases showing metastases more than 20 years after complete remission (40) or a local recurrent disease after inoculation of adipose tissue 13 years after complete remission (41).

In the last few decades, numerous works have tried to identify specific markers and the properties of cancer stem cells in osteosarcoma (Table 1). Osteosarcoma cancer stem cells are supported by sox2, a stem cell transcription factor which inhibits the Hippo pathway (42, 43). In addition to the expression of the stemness markers shared with embryonic stem cells, osteosarcoma cancer stem cells have been characterized by their ability to form cell spheroids *in vitro*, which are highly tumourigenic *in vivo* (38, 39). Cancer stem-like cells expressed high levels of aldehyde deshydrogenase-1 (ALDH1) (44, 45). ALDH-1 expression was associated with resistance to chemotherapy (44) and the metastatic potential of cancer cells (45). The receptors for stem cell growth factor (CD117) and stro-1 expressed by mesenchymal stem cells are expressed by osteosarcoma cancer stem cells and were associated with metastasis and drug resistance (49). CD133 was also linked to the stem-cell phenotype in osteosarcoma (50-54). CD133 or prominin-1 is a pentaspan transmembrane glycoprotein localized in cellular protrusions (55). Like $ALDH1^+$ cells, CD133-expressing osteosarcoma cells displayed high tumourigenicity *in vivo* (51). Its high expression in patients predicted lung metastases and consequently correlated with poor prognosis (52-54). Tian *et al*. demonstrated the expression of CD271, a low-affinity nerve growth factor receptor, by osteosarcoma cancer stem cells (56) and defective autophagy led to the suppression of the stem-like properties of CD271+ (57). Numerous other factors (CBX3, KLF4, SATB2, etc) summarized in Table 1 controlled the biological properties/maintenance of stem cells. The biology of osteosarcoma cancer stem cells is also under the control of epigenetic networks. Several recently-identified microRNAs regulate stem cell phenotype and their invasion and migration properties by targeting specific molecular pathways, such as PTEN, POU5F1 Wnt or Jagged1 (68-74).

Osteosarcoma cancer stem cells are resistant to chemotherapy and radiotherapy, and can drive cancer recurrence (75-76). Consequently, conventional chemotherapy impacts cancer

stem cells and enriches the tumour mass in stem cells (77). They play a significant role in tumour heterogeneity through permanent enrichment of new mutated cancer cells and dominant subclones, and by regulating their local micro-environment. Cancer cells dialogue permanently with locally-based partners. These communications include direct exchanges of small mediators using channels of the gap junction type (78), with some selectivity. For instance, entothelial cells use gap junctions to communicate with osteosarcoma cells, and cancer cells do not communicate using this mode of communication with undifferentiated mesenchymal stem cells, unlike mesenchymal stem cells, which initiate their differentiation toward the osteoblast lineage. The dialogue between both cell types is controlled by acidosis. Acid-activated mesenchymal stem cells influence osteosarcoma cell behaviour such as their stemness properties (79). Mesenchymal stem cells and cancer cells can also dialogue through the release of extracellular vesicles (80, 81). Baglio *et al*. recently demonstrated the role of exosomes in osteosarcoma development and, more interestingly, that tumour cells educated mesenchymal stem cells by paracrine activity associating extracellular vesicles (81). Tumour exosomes containing both IL-1 and TGFβ educated mesenchymal stem cells, which in turn promoted tumour growth and the development of lung metastases. In addition to identifying new therapeutic targets, these works show that osteosarcoma cells can regulate their micro-environment qualitatively and consequently enrich tumour heterogeneity. These data are reinforced by publications that underline the role of TGFβ in the stemness of osteosarcoma cells (82-84). TGFβ1 is thus crucial for the differentiation of osteosarcoma cells for cancer toward cancer stem cells (82). The second key molecular pathway for stemness in osteosarcoma is Wnt/beta catenin signalling, which supports stem cell formation (83). Crosstalk between both pathways has been observed in both chronic inflammation and carcinogenesis (84). Local immunity is also controlled by cell communications leading to an increase in tumour heterogeneity (7). In parallel to the heterogeneity of cancer cells, immune heterogeneity with tumour-associated macrophages (85) and tumour-infiltrating lymphocytes (86) has been established and defines an immune tolerant niche.

Tumour heterogeneity, cancer stem cells and new therapeutic options

Based on the data available in the literature, blocking agents have been developed as new therapeutic options for osteosarcoma patients to overcome drug resistance (10, 87, 89, 90). Thus, pimozide and resveratrol inhibit osteosarcoma cancer stem cells (91, 92). Pimozide blocks the epithelial-to-mesenchymal transition and both drugs interrupt the STAT-3 (IL-6

signalling pathways) and Wnt-β/catenin signalling modulated by TGBβ. Numerous therapeutic options target the TGFβ pathway. Miao *et al*. have recently developed single-walled carbon nanotubes to specifically inhibit TGFβ-induced osteosarcoma cell dedifferentiation and prevent the acquisition of the stem cell phenotype (93). Targeting the wnt/beta catenin pathway may be a therapeutic alternative (94). Martins-Neves *et al*. used IWR-1, an ankyrase inhibitor to attenuate Wnt/beta catenin signalling in osteosarcoma cancer stem cells with promising results in pre-clinical mouse models (94). Blockading N-cadherin/NF-KB signalling also appears interesting with the administration of metformin to inhibiting the stem cell phenotype (95). Shang *et al*. revealed that metformin increased the sensitivity of stem cells to conventional chemotherapy and confirmed the advantages of this drug in the treatment of osteosarcoma (96). Metformin should be considered as a metabolic modulator of osteosarcoma cancer stem cells (97). Targeting EGFR and CD133 (98), Sox9 (99), TSSSC3 and Src/AKt pathways (100) FGFR2 (101) has also been assessed recently in osteosarcoma and exhibited high efficacy by repressing the self-renewal of stem cells, tumour growth and the metastatic process. Immunotherapies are interesting potential future options (10, 102), as shown recently by Mesiano *et al*. (102) and D'Angelo *et al.* (103). Mesiano *et al.* used cytokine-induced killer cells which are effective against cancer stem cells in sarcoma (102). D'Angelo *et al*. developed autologous T cells expressing NY-ESO-1^{c259} expressed by synovial sarcomas (103), and showed that patients with metastases treated with an affinity-enhanced T-cell receptor recognizing an HLA-A2-restricted NY-ESO-1/LAGE1a-derived peptide, increased the anti-tumour response by around 50%. In addition, circulating NY-ESO-1^{c259} T cells were detectable in blood for at least 6 months in all responders, and most administered NY-ESO-1^{c259} T cells exhibited an effector memory phenotype following *ex vivo* expansion (103).

Conclusion

Cancer stem cells, which should be called cancer "stem-like" cells, are detectable in osteosarcoma. They contribute markedly to tumour heterogeneity and are responsible for drug resistance. Dialogue is established between cancer stem-like cells and their local micro-environment, and they are able to educate to facilitate their maintenance and development. This dialogue is a future potential target and there is drug development in combination with conventional chemotherapies (Figure 1). Better characterization of cancer stem-like cells in osteosarcoma and their role in the clonal evolution of the disease is mandatory for improving

the therapeutic response of poor responders, as well as for improving the overall survival of osteosarcoma patients which has changed little in the last four decades.

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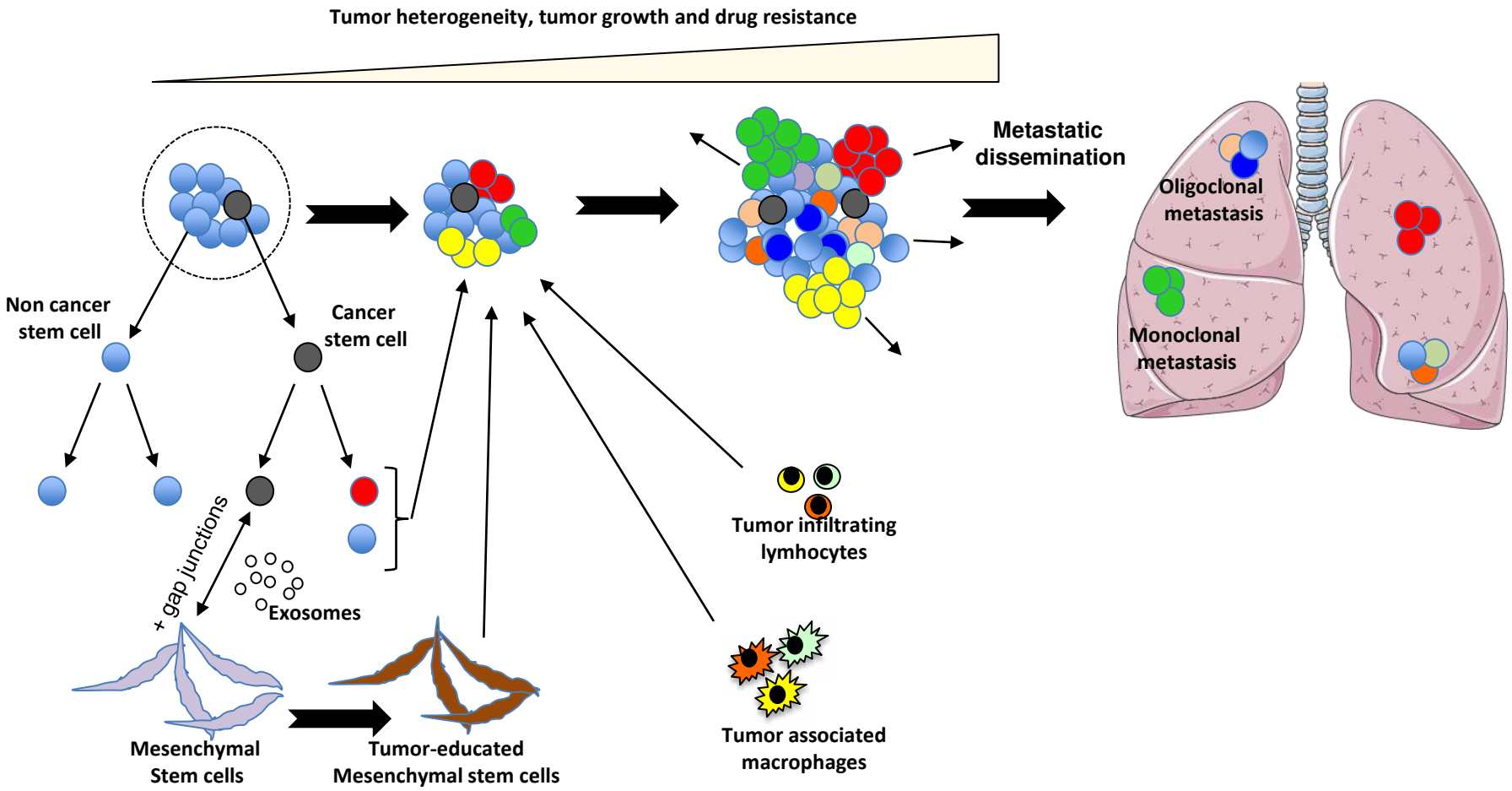
Table 1: Main markers expressed by osteosarcoma cancer-stem cells

Markers	References
Oct4 (octamer-binding transcription factor 4)**,** Nanog (Nanog Homeobox), transcription factors, stemness markers	38, 39
Sox2 (SRY-related HMG-box-2) transcription factor, Stemness markers	42, 43
ALDH1 (Aldehyde desyhdrignease-1)	44-46
CD24 (cell adhesion molecule)	47
CD44 (receptor of hyaluronic acid)	48
CD117 (receptor of stem cell growth factor)	49
Stro-1 (marker of mesenchymal stem cells)	49
CD133 (prominin-1)	50-55
CD271 (low-affinity nerve growth factor receptor)	56, 57
CBX3 (Chromobox protein homolog 3)	54, 58
ABCA5 (ATP-binding cassette, sub-family A, member 5)	58
KLF4 (Kruppel Like Factor 4)	60-62
SATB2 (Special AT-rich sequence-binding protein 2)	63
RAB39A (Rab small GTPase) **-RXRB** (Retinoid X Receptor Beta)	64
TB1XR1 (Transducin (beta)-like 1 ×-linked receptor 1)	65
SENP1 (Sentrin Specific Protease-1)	66
hTERT (human telomerase reverse transcriptase)	67

Figure Legend

Figure 1: Clonal evolution of osteosarcoma cells and their role in tumour heterogeneity. Initially formed by mono- or oligoclonal subclones, dominant clones appear progressively, resulting in marked heterogeneity in the tumour mass. Of these cancer cells, a subpopulation exhibits stemness markers and educates mesenchymal stem cells to release exosomes, which in turn increase the stem cell phenotype and upmodulate tumour growth and the development of metastases. These two-way communications enrich tumour heterogeneity and increase the risk of drug resistance. Immune cells, with their diversity and induction of local immune tolerance, complete the heterogeneity of the tumour mass.

Figure 1


Tumor heterogeneity, tumor growth and drug resistance
Non cancer stem cell
Cancer stem cell
Metastatic dissemination
Oligoclonal metastasis
Monoclonal metastasis
+ gap junctions
Exosomes
Tumor infiltrating lymhocytes
Mesenchymal Stem cells
Tumor-educated Mesenchymal stem cells
Tumor associated macrophages