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New Therapeutic Approaches for Osteosarcoma Treatment

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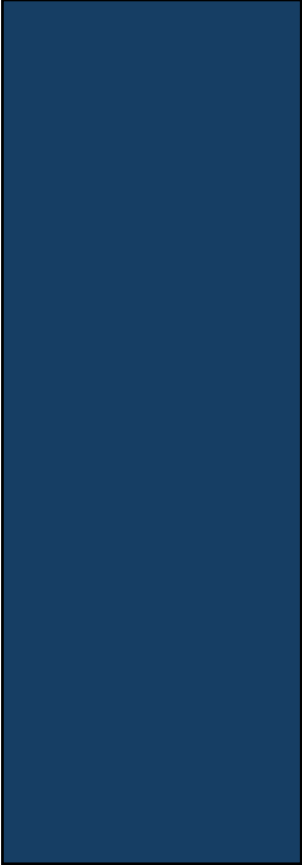
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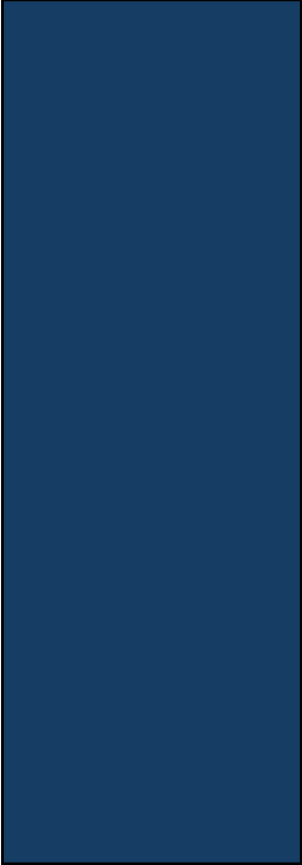
Chapter 1

“Aim and outline of the work”

Aim and outline of the work

Osteosarcoma has been the subject of extensive investigation over the last decades; nevertheless, therapeutic options for this disease remain limited, and clinical outcomes have shown little improvement, particularly in metastatic and relapsed patients. As will be discussed in **Chapter 2**, this lack of progress is not due to insufficient knowledge, but rather to the intrinsic biological complexity of osteosarcoma, which is characterised by marked genomic instability, early metastatic spread, and a tumour microenvironment that actively supports disease progression and therapeutic resistance. In this context, the present PhD thesis is aiming at exploring alternative and biologically informed strategies that may help overcome some of the current limitations of standard treatment.

Chapter 3 focuses on the evaluation of osteoblast-derived extracellular vesicles as carriers for conventional chemotherapeutic drugs. By loading these vesicles with methotrexate, doxorubicin, and cisplatin, we investigate whether this approach can improve drug selectivity and antitumour efficacy toward osteosarcoma cells, while reducing side effects on normal bone cells, both in vitro and in vivo. In **Chapter 4**, we shift attention to the molecular communication between tumour cells and their surrounding microenvironment, analysing next-generation sequencing data through bioinformatic approaches to identify signalling pathways and transcriptional programs involved in inflammation, extracellular vesicle-mediated communication, and tumour-stroma interactions. The impact of tumour-derived signals on bone cells is further addressed in **Chapter 5**, which examines the phenotypic effects of the osteosarcoma secretome on osteoblast function, including alterations in metabolism, differentiation, and matrix production, based on previously published work. Finally, **Chapter 6** summarizes the main conclusions of this PhD thesis and provides a critical discussion of the literature, highlighting future perspectives and the potential relevance of these findings for the development of more effective therapeutic approaches for osteosarcoma.



Chapter 2

“From bench to bedside: advancements in precision oncology and drug discovery for osteosarcoma”

Review submitted for publication to *Cancers*

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Abstract

Osteosarcoma remains a highly aggressive malignancy with limited therapeutic progress and poor outcomes, particularly in metastatic or recurrent cases. Conventional treatment approaches, primarily based on surgery and high-dose chemotherapy, are hindered by significant drawbacks, including severe toxicity, high relapse rates and drug resistance, underscoring the inadequacy of current standard approaches. This review examines emerging advances in precision medicine and drug discovery, including targeted inhibitors, immunomodulatory agents, combination treatments, and advanced biomaterials that promise to transform osteosarcoma care. Recent advances, such as combination of immune checkpoint inhibitors with novel agents or nanoparticle-based drug delivery systems, as well as CRISPR-Cas9 gene editing applications, offer new strategies to overcome the inherent challenges of conventional therapies. In addition, cutting-edge research leveraging multi-omics analyses and digital pathology is refining our understanding of the tumour microenvironment, paving the way for more individualised treatment strategies.

1. Introduction

Osteosarcoma (OS) is the most prevalent primary malignant bone tumour of childhood and adolescence. The average incidence is 0.3 per 100.000 per year, while it reaches 0.8-1 per 100.000 per year in adolescence [1]. The disease typically originates in the metaphyseal regions of long bones particularly the distal femur, proximal tibia, and proximal humerus where it induces rapid skeletal turnover and dynamic osteoblastic activity. This predilection reflects the tight coupling between OS biology and the physiology of bone growth [2], where proliferative stresses and microenvironmental cues may favour malignant transformation.

Despite substantial progress in diagnostic imaging and surgical management, clinical outcomes for OS have remained essentially unchanged for more than three decades. The introduction of multi-agent chemotherapy regimens in the 1970s and 1980s, most notably the Multi-Agent-Polychemotherapy (MAP) protocol (high-dose methotrexate, doxorubicin and cisplatin), initially improved survival for localised disease. However, five-year survival for non-metastatic OS stabilizes around 60–70%, while survival for patients presenting with metastatic disease remains below 30% [3]. This stagnation reflects the inability of current therapies to eradicate micrometastatic disease and the absence of effective systemic options at relapse.

The tightest challenge in OS management is represented by early metastatic dissemination. Although only 15–20% of patients display radiologically detectable metastases at diagnosis, biological evidence indicates that many more harbours occult micrometastases, particularly in the lungs [4]. These microscopic lesions are often resistant to chemotherapy and contribute to relapse. Indeed, lung metastases are responsible for the majority of OS-related deaths [5].

A major barrier to therapeutic progress lies in the biological complexity of OS, which is one of the most genomically unstable human malignancies, displaying widespread chromosomal aneuploidy, chromothripsis-like rearrangements, and extensive copy-number variations [6]. In contrast to tumour driven by recurrent targetable mutations, the genetic landscape of OS is characterised by uncontrolled structural alterations with relatively few recurrent, driver mutations. Alterations in tumour suppressors such as TP53, RB1, ATRX, and replication-associated DNA helicases are frequently observed [7], thus promoting aberrant proliferation, impaired DNA-damage responses, and genomic destabilization. This molecular heterogeneity translates into variable clinical behaviour and substantial interpatient differences in therapeutic sensitivity.

Several oncogenic signalling pathways are recurrently deregulated in OS, including PI3K/AKT/mTOR, Wnt/ β -catenin, FGFR, IGF-1R, and Notch. These pathways collectively regulate cellular proliferation, migration, metabolic rewiring, angiogenesis, and resistance to apoptotic and chemotherapeutic stress. Their coexistence and functional redundancy contribute to treatment resistance and complicate the development of targeted therapies. Efforts to inhibit single molecules or pathways have generally failed in clinical trials, as OS cells rapidly activate compensatory signalling networks that sustain survival [8].

In addition to intrinsic genetic alterations, the tumour microenvironment (TME) plays a pivotal role in OS progression and therapeutic resistance. Bone is a uniquely dynamic environment composed of osteoblasts, osteoclasts, immune cells, mesenchymal stem cells (MSCs), endothelial cells, and bone extracellular matrix components. Interactions between OS cells and the surrounding stroma promote tumour growth, immune evasion, and metastatic dissemination [9]. Osteoclastic bone resorption releases growth factors like TGF- β , which in turn stimulate tumour proliferation, EMT-like conversion, and osteolytic expansion [10]. MSCs within the TME enhance angiogenesis and metastatic competence through the secretion of cytokines such as IL-6, CCL2, and VEGF [11]. Moreover, immune dysregulation is a hallmark of OS: Tumour-Associated Macrophages (TAMs) frequently polarize toward an M2-like phenotype, promoting immunosuppression and metastatic progression [12].

A growing body of evidence has highlighted the central role of exosomes in mediating communication within the OS microenvironment (Figure 1). Tumour-derived exosomes carry oncogenic proteins, lipids, and RNAs, particularly microRNAs, that remodel stromal compartments, promote osteoclastogenesis, condition the pre-metastatic niche in the lungs, and suppress anti-tumour immune responses [13]. Parallel advances in the understanding of non-coding RNAs, especially microRNAs and long non-coding RNAs, have revealed complex regulatory networks that modulate gene expression, metastatic behaviour, and chemoresistance [14] (Figure 1).

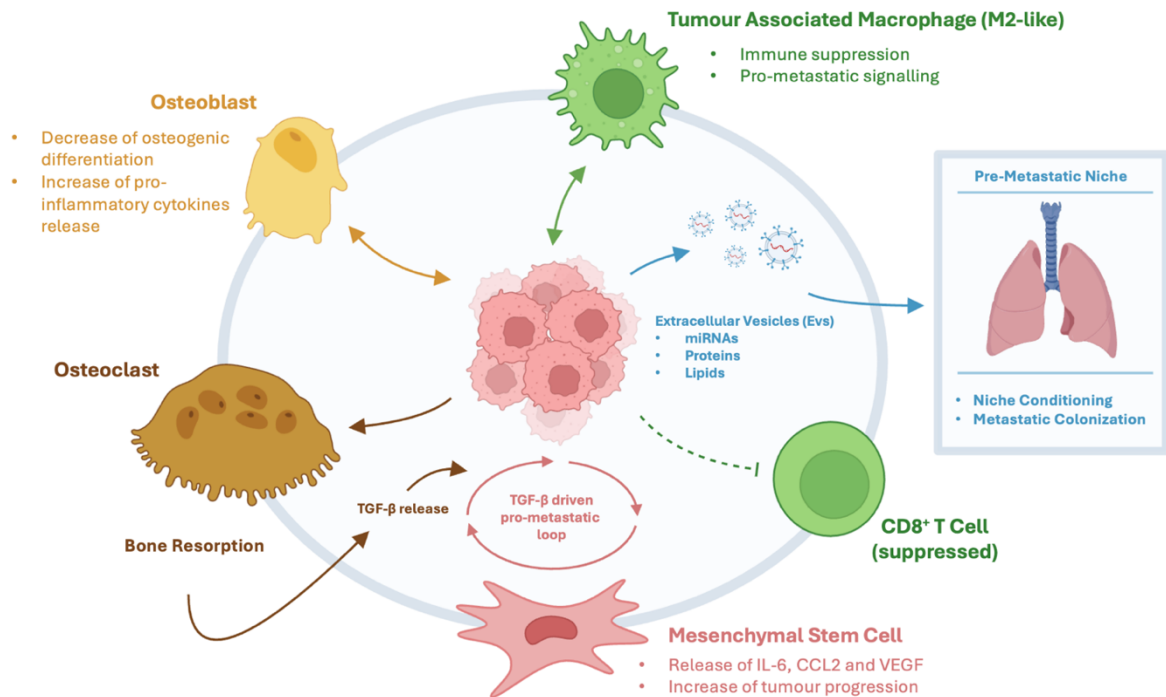


Figure 1. Osteosarcoma Tumour Microenvironment (TME). Tumour cells engage a reciprocal crosstalk with osteoblasts, leading to impaired osteogenic differentiation and inflammatory reprogramming. Osteoclast-mediated bone resorption results in the release of matrix-stored TGF- β , which activates pro-metastatic signalling pathways in osteosarcoma cells, reinforcing a feed-forward loop that promotes tumour aggressiveness. Mesenchymal stromal cells contribute to tumour progression through the secretion of cytokines and growth factors, including IL-6, CCL2, and VEGF, while tumour-associated macrophages with an M2-like phenotype foster immune suppression and pro-metastatic signalling. Tumour-derived extracellular vesicles transport bioactive molecules, such as miRNAs, proteins, and lipids, reshaping the local microenvironment and contributing to the conditioning of a permissive pre-metastatic niche in the lung. Together, these interconnected processes drive osteosarcoma growth, immune evasion, and early metastatic dissemination. Cartoon created with BioRender.com.

The convergence of genomic instability, dysregulated signalling, epigenetic reprogramming, and microenvironmental crosstalk explains why osteosarcoma remains such a challenging malignancy. Indeed, these biological insights have also opened new therapeutic opportunities. Precision oncology approaches leveraging multi-omics analyses, digital pathology, machine learning, and liquid biopsy technologies are beginning to refine patient stratification and enable more rational therapeutic design [15] [16]. In parallel, emerging fields such as immunotherapy, targeted molecular inhibitors, nanotechnology-based drug delivery, and biomaterials engineering offer promising avenues to overcome longstanding limitations associated with traditional chemotherapy [17].

2. Molecular pathogenesis and tumour microenvironment of osteosarcoma

Mechanistic studies have shown that OS pathogenesis involves the convergence of several deregulated oncogenic pathways, including PI3K/AKT/mTOR, Wnt/ β -catenin, MAPK, FGFR, IGF-1R, and Notch signalling. While Wnt/ β -catenin signalling physiologically promotes osteoblast differentiation, its aberrant and sustained activation in osteosarcoma disrupt the balance between proliferation and terminal osteogenic maturation, thereby maintaining tumour cells in an osteoprogenitor-like state [18]. This altered Wnt output increases proliferation and tumourigenicity rather than normal differentiation. In parallel, hyperactivation of PI3K/AKT enhances apoptosis resistance and contributes to chemoresistance, whereas dysregulated Notch signalling promotes angiogenesis, invasion, and tumour–stroma interactions that reinforce malignant progression [19].

This redundancy among pathways explains why molecularly targeted agents, when used individually, have generally failed to produce significant clinical benefit in OS. Tumour cells readily bypass inhibition of a single node by engaging compensatory signalling networks. Therefore, the molecular architecture of OS strongly supports the need for combination therapies or multimodal strategies that address multiple signalling axes simultaneously.

2.1 The Central Role of TGF- β Signalling in Osteosarcoma Progression

Among the many deregulated pathways in OS, transforming growth factor- β (TGF- β) signalling is uniquely significant due to its abundance in the bone microenvironment and its involvement in metastasis. TGF- β is released in substantial quantities during osteoclastic bone resorption, allowing tumour cells to exploit this physiologic process to support malignant expansion. Similarly to epithelial tumours where TGF- β may act as a tumour driver, also mesenchymal tumours such as OS respond to TGF- β predominantly through pro-oncogenic programs [20].

TGF- β enhances tumour cell proliferation, activates EMT-like transcriptional signatures, promotes migration and invasion, and regulates angiogenesis. Clinical studies demonstrate that circulating TGF- β levels are significantly increased in OS patients, especially in those with metastatic disease, and correlate with poor prognosis [21]. Mechanistically, TGF- β stimulates the expression of pro-metastatic and osteolytic mediators, including IL-11, MMP-1, CXCR4, CTGF, and PTHrP, all of which promoting bone degradation, extracellular matrix remodelling and metastatic colonisation of the lungs [22]. These effects are mediated through the canonical SMAD2/3 pathway as well as non-canonical branches involving JNK, p38 MAPK, and PI3K/AKT, providing multiple layers of signalling redundancy. This biological centrality has prompted significant interest in therapeutically targeting TGF- β [23] [24].

2.2 Epigenetic Regulation and Non-Coding RNA Networks

Epigenetic regulation has emerged as a major contributor to OS biology, complementing the genomic characteristic of the disease. DNA methylation changes, histone modifications, and especially the activity of non-coding RNAs including microRNAs

(miRNAs), long non-coding RNAs (lncRNAs) and circular RNAs shape the transcriptional landscape of OS cells.

MicroRNAs are among the most extensively studied epigenetic regulators in OS. Several tumour-suppressive miRNAs, such as miR-34a and miR-181c, are downregulated in OS, leading to overactivation of oncogenic pathways including TGF- β signalling. miR-34a promotes differentiation programs and restrains proliferation [25], whereas miR-181c suppresses invasion and metastasis by targeting SMAD7 and attenuating EMT-like transition [26]. Conversely, certain miRNAs act as oncogenic drivers; miR-95-3p, for example, has been associated with aggressive disease and unfavourable prognosis in specific patient cohorts [27]. Another miRNA associated with osteosarcoma and lung metastasis is miR-106b, by acting on G1/S transition. In vitro experiments on U2OS osteosarcoma cells showed an alteration of G1/S transition and a reduction of PI3K/AKT and PI3Kp100 pathways expression after miR-106b downregulation [28]. Additionally, miR-95 reduces proliferation, improves apoptosis and arrests U2OS cell cycle in G0/G1 phase. The miRNA target is the 3'-UTR of sodium channel epithelial 1 Subunit Alpha (SCNN1 α) [29]. Notably, some miRNAs can act on different targets and, at the same time, can be tumour suppressors and onco-miRNA. For instance, miR-23a has a dual role since it can inhibit special AT-rich-binding protein 1 (SATB1) and act as tumour suppressor in OS [30]. In vivo experiments conducted by He et al. exhibited a suppressor role of miR-23a; indeed, a downregulation of miR-23a led to an increase on RUNX2 and CXCL12 transcription, which had a role in OS cellular growth, invasion and migration [31]. Similarly, other studies showed that an increase of migration and invasion in osteosarcoma cell lines was associated with miR-23a downregulation. In fact, miR-23a decreased the 3'-untranslational regions of PTEN (phosphatase and tensin homolog) expression and improved migration and invasion in OS.

On the other hand, lncRNAs such as HOTAIR and MALAT1 have been shown to promote OS invasion and metastatic behaviour by recruiting chromatin-modifying complexes or by acting as competing endogenous RNAs that sequester tumour-suppressive miRNAs [32] [33]. Similarly, circRNAs including circHIPK3 and circPVT1 function as miRNA sponges, stabilising oncogenic transcripts and sustaining proliferative and invasive signalling [34] [35]. Through these mechanisms, non-coding RNAs contribute to chemoresistance, immune evasion, and metastatic competence. Owing to their pathway-specific and context-dependent activity, non-coding RNAs are increasingly recognised as promising biomarkers and therapeutic targets, with several preclinical studies exploring miRNA mimics, antagomiRs, and lncRNA-directed strategies.

2.3 Extracellular Vesicle–Mediated Intercellular Communication in Osteosarcoma

Extracellular vesicles (EVs), together with soluble components of the tumour secretome, represent a central mechanism through which OS cells communicate with the bone microenvironment and reshape its biological functions. Rather than acting as isolated carriers of single molecular species, EVs operate as integrated signalling platforms that convey inflammatory, matrix-remodelling, and angiogenic cues, thereby favouring tumour expansion [36].

Experimental studies conducted in our laboratory using EVs released by aggressive OS cells, such as the MNNG/HOS line, demonstrated that tumour-derived EVs are efficiently internalised by bone-resident cells and profoundly alter osteoblast function. In osteoblast cultures, exposure to MNNG/HOS-derived EVs suppresses metabolic activity and osteogenic differentiation, as reflected by reduced alkaline phosphatase (Alp) activity and impaired matrix-forming capacity [37]. Concomitantly, EV-treated osteoblasts acquire a pro-inflammatory and tumour-supportive secretory profile, characterised by increased expression and release of cytokines and chemokines involved in bone remodelling and tumour recruitment, including IL-6, IL-1 β , and selected CCL and CXCL family members, as well as matrix-remodelling enzymes such as MMP3 [38]. Importantly, these EVs do not directly induce osteoclast differentiation, indicating that their contribution to osteolysis is mediated indirectly through osteoblast-driven inflammatory and paracrine signalling rather than through direct activation of osteoclast precursors.

The biological effects of EVs are reinforced by the broader OS secretome. Prolonged exposure of osteoblasts to conditioned media from MNNG/HOS cells impairs collagen type I deposition and mineralised matrix formation, further weakening bone structural integrity. In addition, tumour-derived soluble factors reshape the protein composition of osteoblast-derived nanoparticles and vesicles, enriching pathways related to extracellular matrix organisation and tissue remodelling.

This suggests that osteosarcoma cells not only deliver instructive signals to bone cells but also reprogram the secretory and vesicular output of the microenvironment to amplify tumour-supportive processes [39].

On the other hand, the impact of osteosarcoma-derived EVs is not confined to the local tumour microenvironment but extends systemically, favouring lung metastasis through the establishment of a pre-metastatic niche. Evidence from orthotopic, immunocompetent mouse models showed that tumour-derived EVs preferentially accumulate in the lung and are taken up by interstitial macrophages, where they trigger activation of the JAK2/STAT3 pathway and stimulate CXCL2 expression. This signalling cascade promotes the recruitment of granulocytic myeloid-derived suppressor cells, ultimately leading to an increased pulmonary metastatic burden. At the mechanistic level, this pro-metastatic effect was linked to the selective enrichment of S100A11 (a calcium-binding protein of the S100 family involved in inflammatory signalling and tumour progression) within tumour-derived EVs, since genetic depletion of S100A11 in osteosarcoma cells or pharmacological blockade of EV release markedly reduced macrophage reprogramming and lung metastasis [40].

Beyond immune modulation, osteosarcoma-derived EVs also directly influence lung stromal compartments. Both in vitro and in vivo studies demonstrated efficient uptake of tumour EVs by lung fibroblasts, resulting in their activation toward a cancer-associated fibroblast-like phenotype driven by TGF- β 1 signalling. EV-educated fibroblasts exhibited increased expression of α -SMA, fibronectin and collagen I, together with enhanced secretion of pro-inflammatory mediators, thereby contributing to extracellular matrix

remodelling and the creation of a lung microenvironment permissive to metastatic growth [41].

Taken together, these observations indicate that osteosarcoma-derived EVs function as long-range signalling entities that coordinate immune and stromal reprogramming in the lung, thereby supporting pre-metastatic niche formation and facilitating metastatic colonisation.

3. Mechanisms of metastasis and chemoresistance in osteosarcoma

Metastasis and chemoresistance represent the two major obstacles that continue to limit therapeutic success in OS. Although multimodal treatment increases survival for localized disease, most patients who present with metastases or who experience relapse ultimately succumb to progressive lung involvement. The biological processes that drive metastasis and the development of resistance to chemotherapy are multifactorial, involving intrinsic genetic programs, epigenetic alterations, microenvironmental crosstalk, and complex cellular adaptations to stress. Understanding these processes at a mechanistic level is crucial for the development of next-generation therapeutic strategies [42].

3.1 Early Dissemination and Pulmonary Tropism

Clinical and molecular evidence suggests that metastatic dissemination in OS often occurs early in the disease course, even before diagnosis. While only 15–20% of patients present with radiographically detectable metastases, it is widely believed that many harbour micrometastatic lesions at diagnosis [43] [44]. These small metastatic deposits, primarily located in the lungs, often evade chemotherapy and eventually give rise to overt metastatic disease. Pulmonary tropism is a defining feature of OS and is driven by both tumour-intrinsic properties and interactions with the lung microenvironment [45].

The dissemination process starts at the primary tumour site, where OS cells acquire motile and invasive phenotypes through EMT-like transitions driven by transcription factors such as Snail, Twist, and ZEB family proteins [46]. Although OS does not undergo classical EMT due to its mesenchymal origin, it activates analogous transcriptional programs that enhance motility, cytoskeletal reorganization, and extracellular matrix degradation. Upregulation of matrix metalloproteinases (MMP-2, MMP-9) and cathepsins enables tumour cells to degrade collagen-rich bone matrix and invade surrounding tissues [47] [48].

Once into the circulation, OS cells face hemodynamic stress and immune surveillance. Their survival is facilitated by resistance to anoikis, enhanced by PI3K/AKT and IGF-1R signalling, and by interactions with platelets, which shield tumour cells and promote extravasation. Upon reaching the lungs, tumour cells exploit a permissive microenvironment shaped by inflammation, vascular permeability, and the presence of tumour-derived exosomes that precondition the lung niche [49].

3.2 Molecular Drivers of Metastasis

Several molecular pathways play pivotal roles in promoting metastatic dissemination in OS. Among these, PI3K/AKT/mTOR axis emerges as a central driver by integrating survival, metabolic adaptation, and motility signals. Activation of PI3K/AKT enhances resistance to oxidative and anoikis-related stress, allowing circulating tumour cells to survive during blood dissemination [50]. In parallel, AKT signalling regulates focal adhesion turnover and integrin-dependent cytoskeletal dynamics, thereby facilitating cell migration, trans-endothelial passage, and extravasation into distant tissues. Dysregulation of this pathway is frequently observed in OS and reflects both upstream receptor activation and loss of tumour suppressive control, contributing to metastatic efficiency rather than simply to primary tumour growth [50].

The Wnt/ β -catenin pathway further reinforces metastatic competence by sustaining stemness-associated transcriptional programs and cellular plasticity [51]. Aberrant β -catenin signalling supports the maintenance of tumour-propagating subpopulations characterized by enhanced self-renewal, resistance to stress, and adaptability to foreign microenvironments. This stem-like state is particularly relevant during metastatic colonisation, where OS cells must rapidly adjust to non-bone niches, such as the lung parenchyma [52].

Non-coding RNAs represent an additional regulatory layer that fine-tunes metastatic behaviour. Several tumour-suppressive miRNAs, including miR-34a, miR-181c, and miR-143, are frequently downregulated in metastatic OS. Under physiological conditions, these miRNAs restrain invasion and dissemination by targeting genes involved in cytoskeletal remodelling, epithelial–mesenchymal transition–like programs, and TGF- β –dependent signalling cascades [53] [54] [55]. Their loss therefore removes critical brakes on cell motility and invasive capacity.

Conversely, a subset of oncogenic miRNAs actively promotes metastatic progression. miRNAs such as miR-1307, miR-501-3p, and miR-20a have been implicated in enhancing OS dissemination through complementary mechanisms that include modulation of extracellular matrix remodelling, activation of osteolytic and inflammatory programs, and adaptation to immune surveillance [56] [57] [58]. In particular, EV-associated oncogenic miRNAs contribute to the conditioning of a permissive metastatic niche by influencing bone remodelling and stromal responses, thereby linking tumour-intrinsic regulatory circuits to microenvironmental reprogramming [59].

3.3 Mechanisms of Chemoresistance

Chemotherapy resistance is a defining feature of OS relapse and metastatic progression and reflects the ability of tumour cells to activate multiple, partially overlapping survival strategies. Osteosarcoma cells display broad resistance mechanisms that involve genetic alterations, epigenetic reprogramming, and dynamic interactions with the tumour microenvironment, which collectively blunt the efficacy of cytotoxic therapies [60].

One of the most established mechanisms of resistance is the overexpression of ATP-binding cassette (ABC) transporters, particularly P-glycoprotein (ABCB1). Increased ABCB1 expression reduces intracellular accumulation of chemotherapeutic agents such as doxorubicin and paclitaxel, thereby lowering their cytotoxic efficacy. Clinically, elevated P-gp levels correlate with poor response to neoadjuvant chemotherapy and reduced overall survival [61].

Alterations in apoptotic signalling further contribute to chemoresistance. Upregulation of pro-survival proteins, including members of the Bcl-2 family and survivin, shifts the balance toward cell survival even in the presence of extensive DNA damage [62]. Concurrently, downregulation of pro-apoptotic factors impairs execution of chemotherapy-induced cell death. These effects are exacerbated by mutations or functional impairment of TP53, which are frequent in OS and compromise DNA damage sensing, cell-cycle arrest, and apoptotic responses under genotoxic stress [63].

Autophagy represents another key adaptive mechanism that could support survival during chemotherapy exposure. Although autophagy can exert context-dependent tumour-suppressive effects, in OS it often functions as a cytoprotective response to drug-induced stress. Enhanced autophagic flux enables tumour cells to recycle damaged organelles and macromolecules during treatment with agents such as cisplatin and doxorubicin. Inhibition of autophagy has been shown to restore chemosensitivity in multiple preclinical models, highlighting its role as a compensatory survival pathway rather than a bystander process [64].

Epigenetic mechanisms further reinforce resistance phenotypes. Changes in DNA methylation patterns and dysregulation of non-coding RNAs reshape transcriptional programs associated with survival and stress tolerance [65]. Loss of tumour-suppressive miRNAs, including miR-34a and miR-181c, leads to sustained activation of pro-survival and DNA repair pathways, while overexpression of oncogenic miRNAs promotes drug efflux, metabolic adaptation, and resistance to apoptosis. These epigenetic alterations provide plasticity, allowing OS cells to rapidly adapt to chemotherapy-induced selective pressure.

The tumour microenvironment plays a crucial amplifying role in chemoresistance. Dense extracellular matrix deposition limits drug penetration, while hypoxic conditions reduce reactive oxygen species-mediated cytotoxicity and favour activation of hypoxia-responsive survival programs. Moreover, crosstalk between OS cells and stromal or immune components, such as MSCs and M2-polarized macrophages, activates pro-survival signalling pathways including STAT3 and NF- κ B. These microenvironment-driven signals further protect tumour cells from chemotherapy-induced damage and contribute to the persistence of resistant subclones [66].

All together, these mechanisms highlight that chemoresistance in OS arises from the integration of tumour-intrinsic adaptations and microenvironmental support, rather than from a single dominant alteration. This complexity helps explain the limited benefit of chemotherapy intensification strategies and underscores the need for combinatorial approaches targeting both cancer cells and their supportive niche.

4. Current standard-of-care therapies and their limitations

Neoadjuvant chemotherapy or adjuvant chemotherapy combined with surgical resection of primary tumour is the standard treatment of OS for more than three decades. Established in the 1970s and 1980s, this multimodal approach improved survival in patients with no metastatic disease [67]. Differently, a poor long-term survival rate was associated with metastatic or recurrent OS. Outcomes for these patients remain unchanged despite refinements in surgical techniques and supportive care [68]. Chemotherapeutic regimen, commonly referred to as Multi-Agent-Polychemotherapy (MAP), include high-dose methotrexate (HD-MTX), doxorubicin, and cisplatin [69]. The overall survival in paediatric and adolescent patients is improved by high-dose methotrexate. However, the nephrotoxic, hepatotoxic and mucositis effects resulting from use of HD-MTX require vigilant monitoring during the treatment [70]. Moreover, cisplatin is associated with severe nephrotoxicity, ototoxicity, and neurotoxicity [71] while cumulative dose-dependent cardiotoxicity is caused by doxorubicin [72]. These toxicities impose significant limitations on treatment intensification, particularly in young patients.

Additional agents such as ifosfamide and etoposide are frequently used in patients with relapsed or refractory disease. These drugs are commonly administered in combination (ifosfamide–etoposide, IE, regimen) to exploit complementary mechanisms of DNA damage induction and cell-cycle interference [73]. While these regimens can induce temporary disease control or partial responses in a subset of patients, especially in the metastatic or recurrent cases, their overall impact on long-term survival remains limited. However, their clinical use is often accompanied by cumulative toxicity, including myelosuppression, nephrotoxicity, and secondary malignancy risk [74].

Despite these toxic regimens, a substantial proportion of tumours exhibit intrinsic or acquired chemoresistance. Histological evaluation of tumour necrosis following neoadjuvant chemotherapy remains one of the strongest prognostic factors in OS; patients with <90% necrosis (poor responders) have significantly lower survival rates compared to good responders. However, even in good responders, relapse remains common. Intensifying chemotherapy has not consistently improved outcomes, underscoring the complexity of resistance mechanisms and the inadequacy of cytotoxic escalation as a long-term strategy [75].

Looking at surgical resection, it remains essential for local control. Limb-salvage surgery, endoprosthetic reconstruction, and microsurgical techniques become a substitution of amputation and improve postoperative function [76]. Nevertheless, alone itself cannot eradicate micrometastatic disease, and local recurrence remains a concern in poor histologic responders or in cases where clear surgical margins cannot be reliably achieved [77]. Furthermore, the complicated resectability of axial or pelvic tumours contributes to poorer outcomes [78].

Currently, radiation therapy (RT) can be useful as adjuvant treatment in specific cases, such as postoperative treatment of close or positive surgical margins [79]. The limited role in the past of radiation therapy (RT) was associated with a radioresistance of OS

cells. Traditional radiotherapy uses high-energy X-rays to cause cells death through damages on DNA but highly DNA repairing seems to be present on patients [80]. Although new technologies, such as proton therapy or carbon-ion therapy, improve dose delivery and facilitate precise targeting of OS cells, the impact of RT remains modest in comparison to its success in other sarcomas [81].

Another major limitation of the current standard of care is the largely uniform application of this therapy across biologically heterogeneous tumours. The standard MAP regimen is applied in most of the cases, despite growing evidence that OS encompasses multiple molecular subtypes with distinct biological behaviours and treatment responses. The emergence of precision oncology, supported by genomic, transcriptomic, and epigenomic profiling, could represent a starting point for stratifying patients and identifying novel therapeutic agents. However, such approaches remain in early developmental stages for OS and are not yet integrated into standard clinical practice [82].

Disease relapse remains one of the most unsolved problems in OS. In most cases, recurrence develops within the first two years after diagnosis and is associated with a dismal prognosis, with long-term survival rates rarely exceeding 20%. Once relapse occurs, therapeutic options are few. Treatment often consists of reusing cytotoxic agents already administered during first-line therapy, empirical use of off-label targeted drugs, or referral to early-phase clinical trials [83]. Surgical removal of metastatic lesions, most commonly through lung metastasectomy, can provide a survival benefit in carefully selected patients, but it is seldom curative when systemic, therapy-resistant disease has already emerged [84].

Taken at face value, these limitations highlight that the lack of meaningful survival gains over recent decades cannot be attributed solely to the aggressive nature of the disease; it also reflects the inability of conventional treatment strategies to effectively control metastatic spread and acquired drug resistance. In this context, newer approaches such as rational combinations of targeted agents, immunomodulatory strategies, nanotechnology-based drug delivery systems, as well as tissue-engineering platforms, are being explored as potential ways to overcome these barriers and, over time, redefine the treatment landscape of osteosarcoma.

5. Targeted therapies in osteosarcoma

Osteosarcoma is marked, as mentioned before, by extensive genomic instability, a relatively low burden of recurrent point mutations, with the lack of a single dominant, druggable driver alteration. However, molecular profiling and functional studies are nowadays increasingly pointing to pathway dependencies and context-specific weaknesses that may be exploitable, especially when targeted agents are used in rational combinations rather than as stand-alone treatments.

5.1 Targeting Receptor Tyrosine Kinases and Downstream Pathways

Receptor tyrosine kinases (RTKs) such as IGF-1R, PDGFR, VEGFR, FGFR, and MET are frequently deregulated in OS, which contribute to tumour proliferation, angiogenesis, and metastatic competence. Several studies were promising on IGF-1R inhibition, given the strong preclinical evidence linking IGF-1R signalling to OS growth and survival [85]. However, multiple clinical trials testing monoclonal antibodies or small-molecule IGF-1R inhibitors failed to demonstrate significant clinical benefit, likely due to pathway redundancy and compensatory signalling through insulin receptor isoforms [86].

VEGFR and PDGFR inhibitors, including pazopanib and regorafenib, have demonstrated modest activity in relapsed OS. Regorafenib, a multikinase inhibitor targeting VEGFR1–3, KIT, RET, and RAF kinases, seems to allow an improvement in progression-free survival in randomized phase II trials and is now considered one of the few targeted agents with reproducible activity in refractory OS. Nevertheless, responses remain transient, and its benefit is primarily cytostatic rather than cytotoxic [87].

Sorafenib is another inhibitor that has been tested in OS for several years, largely because of its activity on multiple kinases that are relevant to bone tumours [88]. In practice, however, its use as a single agent has not translated into clear or durable clinical benefit. Most of the interest around sorafenib has come from combination studies rather than from monotherapy trials. For instance, when sorafenib was administered together with the mTOR inhibitor everolimus, some heavily pretreated patients experienced longer periods of disease stabilization [89]. Similar considerations could be extended to its combination with denosumab, which has been reported to produce clinical benefit in a limited number of cases [89]. These are not robust data and should be interpreted cautiously, but they reflect a recurring pattern seen in OS trials, where partial pathway inhibition is rarely sufficient.

From a biological standpoint, the PI3K/AKT/mTOR pathway represents an obvious target, given its frequent activation in OS patients. Nevertheless, clinical experience with mTOR inhibitors such as everolimus or temsirolimus has been variable. In some patients, short-term disease control has been observed, while in others the effect was minimal or absent. This heterogeneity likely reflects the ability of tumour cells to rapidly develop compensatory signalling mechanisms, a phenomenon that has been repeatedly observed in both preclinical models and clinical settings [90]. Therefore, current therapeutic efforts are increasing on combination strategies, including dual PI3K/mTOR inhibition or the addition of receptor tyrosine kinase inhibitors, rather than further evaluation of single agent mTOR blockade (Table 1).

5.2 Targeting TGF- β Signalling

TGF- β signalling, as previously described, represents one of the most promising targets in OS given its central role in metastasis, immunosuppression, and osteolytic activity. Several pharmacological approaches have been developed to interfere with TGF- β signalling, including neutralizing antibodies, ligand traps, antisense oligonucleotides, and small-molecule inhibitors targeting the type I receptor kinase (ALK5) [91]. Among these, the ALK5 inhibitor vactosertib is one of the most investigated agents; indeed, in

vivo experiments using murine and xenograft OS models showed that vactosertib treatment reduces primary tumour growth and lung metastatic burden while significantly inhibiting SMAD2 phosphorylation in tumour tissues [91]. Notably, vactosertib administration was also associated with increased infiltration of CD4⁺ and CD8⁺ T cells and natural killer cells, together with a reduction in regulatory T cells and M2-like macrophages within the tumour microenvironment, indicating a shift toward a less immunosuppressive milieu [91].

Looking at clinics, early-phase trials evaluating TGF- β pathway inhibitors across multiple solid tumours and hematologic malignancies suggest that these agents are generally tolerable. For example, vactosertib has demonstrated an acceptable safety profile in phase I/II studies, with the most frequently reported treatment-related adverse events including fatigue, abdominal pain, and transient elevations in liver enzymes, while serious toxicities were infrequent [92]. These safety data have provided the rationale for exploring combination strategies rather than single-agent use. In particular, combining TGF- β inhibition with immune checkpoint blockade has been proposed to counteract TGF- β -mediated immune exclusion, although early clinical experience in OS remains limited and objective responses have so far been modest [93] (Table 1).

5.3 Targeting Cell Cycle and DNA Damage Response Pathways

Osteosarcoma is characterised by extensive genomic instability, a feature that has naturally drawn attention to DNA damage response (DDR) pathways as potential therapeutic targets [94]. Rather than reflecting isolated defects, genomic profiling studies indicate that in many cases OS harbours alterations across multiple DDR components, including TP53, ATM, ATR, and genes involved in homologous recombination and replication stress regulation [95]. As a result, tumour cells often rely on compensatory repair mechanisms instead of complete loss of DDR function. This pattern of widespread but heterogeneous DDR dysfunction distinguishes OS from classical BRCA-driven tumours and makes direct application of established synthetic-lethal strategies more challenging [96].

PARP inhibitors are the most clinically advanced agents targeting DDR pathways, but their activity in OS remains uncertain. Canonical BRCA1 or BRCA2 mutations are uncommon in this disease; however, functional studies suggest that a subset of osteosarcomas displays impaired homologous recombination and elevated replication stress [97][98]. In preclinical models, PARP inhibition alone generally produces limited cytotoxic effects, whereas a clearer impact is observed when PARP inhibitors are combined with DNA-damaging treatments such as cisplatin or ionizing radiation [99].

Disruption of cell-cycle control represents another potential way to exploit DDR defects in OS. Alterations affecting the RB pathway, including RB1 loss or functional impairment of G1/S checkpoint regulation [100], are reported in a considerable fraction of tumours and contribute to unchecked proliferation and replication stress [101]. Based on this rationale, CDK4/6 inhibitors have been tested in OS preclinical models. These studies show that CDK4/6 blockade can induce G1 arrest, slow tumour cell proliferation, and increase sensitivity to DNA-damaging chemotherapy by restricting repair capacity during

replication [102]. At the same time, translation to the clinic remains uncertain, as OS shows marked heterogeneity in RB pathway status and preserved RB function appears necessary for sustained responses to CDK4/6 inhibition [103].

Overall, current data indicate that DDR- and cell-cycle-targeted strategies in OS are unlikely to provide meaningful benefit when used as single agents. Their potential appears greater in combination settings, particularly when treatments are designed to exploit replication stress, defective checkpoint control, or chemotherapy-induced DNA damage (Table 1).

5.4 Epigenetic Therapies: HDAC and DNMT Inhibitors

Among the different classes of epigenetic agents, histone deacetylase (HDAC) inhibitors have been extensively studied. Broad-spectrum compounds such as vorinostat and panobinostat consistently reduce OS cell viability in vitro, inducing cell-cycle arrest and apoptosis and markedly impairing clonogenic growth [104]. When evaluated as single agents in vivo, however, their antitumour effects are generally limited and often accompanied by systemic toxicity, a finding that is in line with the modest activity reported in early clinical experiences [105]. This has shifted attention toward more selective approaches and combination strategies. In this context, simultaneous inhibition of HDAC4 and HDAC8 using tasquinimod and PCI-34051 has been shown to significantly enhance the activity of doxorubicin in aggressive OS models. In SJSA-1 cells, this combination reduced cell viability by roughly 55–60% and nearly abolished colony formation, while increasing apoptotic markers such as cleaved caspase-3 and caspase-8 [106]. Notably, comparable effects were observed in three-dimensional spheroid systems, where combined treatment decreased spheroid volume by more than 30% and limited migratory behaviour, suggesting an impact that extends beyond simple growth inhibition [106]. At the molecular level, these effects were associated with reduced RUNX2 expression and attenuation of PI3K/AKT signalling, two pathways closely implicated in OS progression and resistance to chemotherapy [107].

DNA methyltransferase (DNMT) inhibitors have also been investigated in OS, particularly in models of drug resistance. As single agents, demethylating compounds such as decitabine or 5-aza-2'-deoxycytidine generally exert only modest antiproliferative effects. Their activity becomes more evident when they are combined with HDAC inhibitors, leading to a more sustained suppression of tumour cell growth. In chemo-resistant osteosarcoma cells, this combined epigenetic targeting has been shown to reactivate silenced apoptotic programs and partially restore sensitivity to cytotoxic stress, indicating that DNA methylation and histone acetylation cooperate in maintaining resistant phenotypes [108].

In addition to direct effects on tumour cell survival, DNMT inhibition appears to influence tumour immunogenicity. Interestingly, in OS models overexpression of DNMT1 has been associated with repression of CXCL12, TAP1, and LMP2, resulting in reduced CD8⁺ T-cell infiltration and immune escape [109], [110]. Pharmacological demethylation reverses these changes, restoring chemokine expression and enhancing antitumour immune responses in preclinical systems. Together, these observations provide a mechanistic

basis for combining epigenetic agents with immunotherapy or other immune-modulating strategies.

Overall, available experimental data suggest that epigenetic therapies do not deliver durable benefits as monotherapies in OS. Their main contribution could probably be achieved in combination settings, where epigenetic modulation can reshape transcriptional programs, weaken resistance mechanisms, and increase responsiveness to chemotherapy or immunotherapy. Hence, future translational efforts should focus on identifying epigenetic biomarkers and develop combination therapies (Table 1).

5.5 CRISPR-Cas9 Editing: Recent Advantages and Therapeutical Strategies

In OS, CRISPR–Cas9 technology has been used primarily as a functional approach rather than as a direct therapeutic tool. Because of the marked genomic instability of this disease and the absence of recurrent, dominant driver mutations, CRISPR-based gene editing has provided a practical way to identify genes that are truly required for tumour cell proliferation, survival, and drug response, going beyond correlative genomic observations.

An early application of CRISPR in OS addressed immune-related pathways. Using CRISPR/Cas9-mediated knockout of PD-L1, Liao et al. edited OS cell lines including KHOS and MNNG/HOS. PD-L1 loss did not significantly affect cell migration or invasion, but it resulted in a clear reduction in cell proliferation and clonogenic growth [111]. Importantly, PD-L1-deficient cells showed increased sensitivity to standard chemotherapeutic agents. In these models, the IC₅₀ for doxorubicin decreased from approximately 0.09 μ M in control cells to about 0.03 μ M after PD-L1 deletion, while sensitivity to paclitaxel was similarly enhanced [111]. These findings indicated that PD-L1 contributes to chemoresistance through cell-intrinsic mechanisms, independently of its role in immune checkpoint signalling, and highlighted the value of CRISPR editing in uncovering non-obvious functional dependencies.

More recently, larger-scale CRISPR screening approaches have been applied to systematically map essential genes in OS. In a kinome-wide CRISPR–Cas9 loss-of-function screen performed across multiple OS models, including U2OS, Saos-2, and OS-732 cells, Wang et al. identified Polo-like kinase 1 (PLK1) as the top common and essential gene across the osteosarcoma cell lines. Genetic disruption of PLK1 has a strong antiproliferative effect, markedly reducing clonogenic survival in vitro and inducing G2/M cell-cycle arrest. This was accompanied by accumulation of DNA damage markers, including γ -H2AX, and activation of caspase-dependent apoptosis [112].

The relevance of PLK1 dependency was confirmed in vivo. CRISPR-mediated PLK1 knockout significantly suppressed tumour growth in xenograft and patient-derived xenograft models, with tumour volume and weight reduced by approximately 50–60% compared to control tumours. In addition, PLK1 loss was associated with a reduction in pulmonary metastatic burden. Importantly, no significant histological toxicity was observed in major organs [112].

These genetic findings were further validated pharmacologically. Treatment with the PLK1 inhibitor volasertib recapitulated the effects of genetic PLK1 deletion, inducing G2/M arrest, increasing γ -H2AX and CHK2 phosphorylation, and promoting apoptosis [113]. In vivo, weekly administration of volasertib led to a marked reduction in tumour growth, decreased Ki-67 staining, and increased TUNEL positivity, again without overt systemic toxicity [112]. The close correspondence between genetic and pharmacological inhibition reinforces the translational relevance of the CRISPR screen and supports PLK1 as a functionally therapeutic target in osteosarcoma.

However, despite these advances, direct therapeutic genome editing remains difficult to be soon applied in clinics for OS. Efficient tumour-specific delivery, off-target effects, and long-term safety remain substantial challenges. At present, the most realistic contribution of CRISPR technology relies on defining functional gene dependencies and prioritizing druggable targets, rather than in direct genome editing of tumour cells. In this context, CRISPR-based functional screening represents a powerful strategy to bridge the gap between genomic complexity and rational therapeutic development in OS (Table 1).

Table 1. Targeted therapies and signalling pathways in osteosarcoma.

Target / Pathway	Representative agents	Preclinical evidence	Clinical evidence	Main limitations
IGF-1R	Monoclonal antibodies, small-molecule inhibitors	Reduced OS cell proliferation and tumour growth in preclinical models	Limited clinical benefit in early-phase trials	Pathway redundancy and compensatory signalling via insulin receptor
VEGFR / PDGFR (multikinase)	Regorafenib, pazopanib, sorafenib	Anti-angiogenic activity and delayed tumour progression in vivo	Improved progression-free survival in phase II trials	Predominantly cytostatic effects; transient responses
PI3K/AKT/mTOR	Everolimus, temsirolimus	Growth inhibition and enhanced	Variable and inconsistent	Strong pathway redundancy; rapid compensatory

		chemosensitivity in selected models	activity as monotherapy	signalling; tumour heterogeneity
TGF- β / ALK5	Small-molecule ALK5 inhibitors	Reduced primary tumour growth and metastatic burden; immune modulation	Acceptable safety in early-phase trials	Limited efficacy as single agents; need for rational combinations
DNA damage response	PARP inhibitors	Enhanced cytotoxicity when combined with DNA-damaging agents	No clear benefit as monotherapy	Lack of classical homologous recombination deficiency
Epigenetic regulators	HDAC and DNMT inhibitors	Reduced viability and clonogenicity; resensitization to chemotherapy in combination settings	Modest clinical activity as single agents	Systemic toxicity; absence of predictive biomarkers
CRISPR-identified functional dependencies (e.g. PLK1)	CRISPR–Cas9 screening; PLK1 inhibitors (e.g. volasertib)	PLK1 knockout or inhibition induces G2/M arrest, DNA damage and apoptosis, reducing tumour growth and metastasis in OS models	No OS-specific clinical trials; PLK1 inhibitors tested in other solid tumours	Not a direct therapeutic approach; delivery and safety issues; requires pharmacological translation

6. Immunotherapy and immune modulation in osteosarcoma

Immunotherapy has transformed the treatment of several cancers, but similar progress has not been achieved in OS. Although there is evidence that OS cells can express tumour-associated antigens and that immune cells are present within the tumour tissue, clinical responses to immunotherapeutic approaches have been largely failed. In practice, osteosarcoma's behaviour induces the generation of an "immune-cold tumour", with limited cytotoxic T-cell infiltration, dominant immunosuppressive signals, and a tumour microenvironment that actively restricts effective immune activation. This mismatch between apparent immunogenic features and poor therapeutic responsiveness highlights how the immune biology of osteosarcoma is still not fully elucidated.

6.1 The Immunological Landscape of Osteosarcoma

The immune microenvironment of OS is characterised by the presence of different immunosuppressive cellular populations: among these, tumour-associated macrophages (TAMs) represent one of the most abundant immune subsets [114]. Immunohistochemical and transcriptomic analyses consistently show a prevalence of macrophages polarized toward an M2-like phenotype, which actively contribute to immune suppression and tumour progression. In clinical cohorts of OS patients, high M2 macrophage infiltration correlates with reduced overall survival and increased metastatic risk, whereas tumours enriched in M1-polarized macrophages display improved antigen presentation capacity and higher expression of T-cell-activating signals [114].

Comprehensive immunogenomic profiling has further clarified the extent of immune dysfunction in OS. In an integrated multi-omics analysis of primary, recurrent, and metastatic OS samples, low immune infiltration scores have been reported when compared with tumours known to respond to immune checkpoint blockade. In this cohort, CD8⁺ T cells accounted for only a minor fraction of infiltrating immune cells, and T-cell receptor (TCR) analysis revealed limited clonality, indicating poor antigen-driven T-cell expansion. Notably, fewer than one third of predicted neoantigens were transcriptionally expressed. This was mainly explained by increased nonsense-mediated decay, which degraded neo-antigenic transcripts and thereby reduced antigen presentation, even in the presence of extensive structural genomic alterations [115].

In parallel, regulatory T cells were consistently enriched within the OS microenvironment and contributed to immune suppression through sustained IL-2 consumption and secretion of inhibitory cytokines such as TGF- β . This imbalance between effector and regulatory lymphocyte populations was accompanied by impaired dendritic cell and natural killer (NK) cell activity. Functional analyses showed reduced expression of antigen-processing and presentation machinery, including TAP1 and LMP2, as well as increased expression of inhibitory ligands on tumour cells, further limiting immune effectiveness [115].

6.2 Immune Checkpoint Inhibitors: Clinical Evidence and Biological Limitations

Although immune checkpoint inhibitors (ICIs) have transformed the management of several solid malignancies, their translation into meaningful clinical benefit for OS patients has been challenging. Clinical trials evaluating PD-1/PD-L1 or CTLA-4 blockade have consistently shown limited efficacy. For instance, in the SARC028 study, pembrolizumab induced clear responses in only a small fraction of OS patients, with most individuals experiencing disease stabilization or progression rather than tumour regression [116]. Comparable findings have been reported with other agents such as nivolumab, atezolizumab, and ipilimumab, which demonstrated acceptable safety profiles but minimal therapeutic impact when used as monotherapy. These outcomes clearly indicate that, unlike in immunologically responsive tumours, checkpoint inhibition alone is insufficient to overcome OS immune resistance [117].

The modest clinical activity of ICIs can be largely explained by the unique biological features of OS. This tumour is generally considered immunologically “cold,” characterised by a low tumour mutational burden and, consequently, a limited repertoire of neoantigens capable of eliciting robust T-cell responses. In parallel, defects in antigen processing and presentation often due to reduced MHC class I expression, further impair immune recognition [118]. Beyond tumour-intrinsic factors, the OS microenvironment plays a central role in limiting immunotherapy efficacy. It is dominated by immunosuppressive cell populations, including regulatory T cells, myeloid-derived suppressor cells, and tumour-associated macrophages, which collectively dampen cytotoxic T-cell function and promote immune escape. Moreover, PD-L1 expression is highly heterogeneous and frequently low, reducing the possibility of a consistent response to PD-1/PD-L1 blockade [118] (Table 2).

6.3 Macrophage-Targeted Immunotherapy

Macrophages constitute a major immune component of the OS microenvironment and exert a profound influence on disease behaviour. Their abundance and functional plasticity have progressively shifted therapeutic interest from strategies that exploit macrophage biology to counteract tumour progression [119], [120]. Within this framework, mifamurtide (liposomal muramyl tripeptide phosphatidylethanolamine, L-MTP-PE) represents the most established macrophage-directed intervention in OS. Acting as an immunostimulatory agent derived from bacterial wall structures, mifamurtide activates monocytes and macrophages and induces the production of pro-inflammatory cytokines, including TNF- α and IL-1 β [121]. This activation enhances macrophage-mediated anti-tumoral activity and, when mifamurtide is administered alongside conventional chemotherapy, has been associated with improved overall survival and a favourable trend in event-free survival among osteosarcoma patients [121].

Other pharmacological strategies focus on limiting the accumulation or pro-tumoral activity of tumour-associated macrophages (TAMs). Interfering with the CSF-1/CSF-1R signalling pathway, which is essential for macrophage recruitment and survival, has emerged as a means to reduce TAM presence within the tumour microenvironment and

to improve responses to additional anticancer treatments. At the same time, the inherent adaptability of macrophages has opened the door to reprogramming approaches aimed at redirecting TAMs away from an M2-like, immunosuppressive state [120]. Experimental evidence indicates that such phenotypic shifts can restrain OS growth and metastatic spread. In this setting, all-trans retinoic acid (ATRA) has been shown to suppress M2 polarization and limit metastatic progression, whereas metformin has been reported to modify macrophage polarization and metabolic programs, ultimately contributing to tumour growth inhibition [122].

A further macrophage-centred therapeutic strategy targets the CD47/SIRPα innate immune checkpoint. CD47 functions as a “don’t eat me” signal that prevents macrophage-mediated phagocytosis and is frequently upregulated in OS cells. Disrupting the interaction between CD47 and SIRPα restores macrophage phagocytic capacity and reduces tumour growth in preclinical OS models. Despite the compelling biological rationale, clinical investigations targeting this pathway in OS patients have not yet been elucidated [123] (Table 2).

Overall, these approaches underscore the central role of macrophages in shaping the OS microenvironment and support their relevance as therapeutic targets.

6.3 CAR-T and CAR-NK Cell Therapies

Cellular immunotherapy has dramatically reshaped the treatment of haematological cancers, however translating these advances to solid tumours remains one of the major challenges. Osteosarcoma (OS) highlights many of the intrinsic limitations of this approach, since one of the most persistent obstacles is antigen selection: many surface molecules expressed by OS cells are not tumour-exclusive and are also detected, to varying degrees, in normal tissues, which inevitably raises limitations about on-target off-tumour toxicity [117], [124]. Even when candidate antigens are identified, therapeutic efficacy is further compromised by tumour heterogeneity, inefficient trafficking of infused cells, early functional exhaustion, and poor long-term persistence within the strongly immunosuppressive OS microenvironment [124].

Within this challenging landscape, several tumour-associated antigens have nonetheless been pursued as CAR-T targets: notably, HER2, GD2, and B7-H3 (CD276). Clinical experience with HER2-directed CAR-T cells in osteosarcoma and related sarcomas has consistently demonstrated an acceptable safety profile, with no evidence of severe cytokine release syndrome or clinically meaningful off-tumour toxicity. However, antitumour activity has generally been limited, and durable responses have been uncommon, a finding that has been largely attributed to antigen heterogeneity and progressive CAR-T cell dysfunction following tumour infiltration [125].

GD2, a disialoganglioside enriched in paediatric solid tumours including osteosarcoma, has provided a strong biological rationale for CAR targeting. While GD2-specific CAR-T cells showed pronounced cytotoxicity in preclinical models, early clinical evaluation revealed only transient tumour regression in a subset of patients, without sustained benefit. These results underscore the relevance of immune escape mechanisms and insufficient in vivo persistence as major barriers to effective clinical translation [126].

In contrast, increasing attention has focused on B7-H3, which is highly and consistently expressed in osteosarcoma while showing limited distribution in normal tissues. Preclinical studies on paediatric solid tumour models (Patient and Cell Line-Derived Xenograft, PDX and CDX) have reported marked tumour regression in osteosarcoma models treated with B7-H3-targeted CAR-T cells [127]. The translational relevance of this antigen is further supported by data from B7-H3-directed antibody-drug conjugates, which achieved an overall response rate of 92.3%, with 61.5% of paediatric solid tumour models maintaining complete responses. Early-phase clinical trials investigating B7-H3 CAR-T cells are currently underway, and preliminary observations suggest biological activity, although long-term efficacy remains to be determined [127].

Beyond CAR-T strategies, CAR-engineered natural killer (CAR-NK) cells are increasingly being explored as an alternative adoptive cell platform for osteosarcoma. Compared with CAR-T cells, CAR-NK cells are associated with a lower risk of cytokine release syndrome and graft-versus-host disease and retain intrinsic cytotoxic activity that is less dependent on antigen presentation through major histocompatibility complex molecules [128]. These properties are particularly relevant in osteosarcoma, which is typically characterized by a low tumour mutational burden and frequent defects in antigen presentation. Preclinical studies have demonstrated that CAR-NK cells targeting osteosarcoma-associated antigens such as B7-H3, CD70, and MCAM can effectively suppress tumour growth. Nevertheless, the short lifespan and limited in vivo persistence of adoptively transferred NK cells continue to represent significant challenges [128].

Taken together, current evidence suggests that CAR-based cellular therapies hold considerable promise for osteosarcoma but remain constrained by biological resistance mechanisms intrinsic to solid tumours. Progress in this field will likely depend on improved antigen selection, strategies to enhance immune cell trafficking and persistence, and the rational integration of CAR-T and CAR-NK approaches into combination therapeutic regimens (Table 2).

Table 2. Immunotherapy and immune-modulating strategies in osteosarcoma.

Strategy	Target / Mechanism	Experimental evidence	Clinical evidence	Key challenges
Immune checkpoint inhibition	PD-1 / PD-L1, CTLA-4	Limited immune activation in OS models	Low objective response rates in clinical trials	Immune-suppressed tumour microenvironment; low neoantigen expression
Macrophage activation	Mifamurtide (L-MTP-PE)	Enhanced macrophage-	Improved overall survival when	Benefit restricted to selected patient subsets

		mediated antitumour activity	combined with chemotherapy	
Macrophage reprogramming	CSF-1R inhibition, ATRA, metabolic modulators	Reduced tumour growth and metastasis in preclinical models	Limited or absent clinical validation in OS	Macrophage plasticity; context-dependent effects
Innate immune checkpoint blockade	CD47–SIRPα axis	Increased macrophage- mediated phagocytosis in OS models	No clinical data currently available in OS	Potential hematologic toxicity; target ubiquity
CAR-T cell therapy	GD2, B7-H3 (HER2 in selected settings)	Potent cytotoxicity in vitro and xenograft models	Limited and transient responses in early clinical experience	Antigen heterogeneity; poor persistence and trafficking
CAR-NK cell therapy	B7-H3, CD70, MCAM	Effective tumour suppression in preclinical models	Early developmental stage	Short in vivo lifespan; limited persistence

7. Nanotechnology-enabled therapeutic strategies for osteosarcoma treatment and bone reconstruction

In recent years, nanotechnology has gained increasing attention as a potential driver of therapeutic innovation in OS. This interest is linked to its capacity to address several persistent limitations of conventional treatments, including systemic toxicity, inefficient drug penetration into tumour tissue, the emergence of chemoresistance, and the difficulty of controlling micrometastatic disease. Instead of relying only on dose escalation or new cytotoxic agents, nanotechnology-based approaches introduce a

fundamentally different strategy centred on improving how therapies are delivered and distributed within the body.

A key strength of nanotechnology is the ability of nanoscale carriers to preferentially accumulate within tumour tissues and to release therapeutic drugs in a more controlled and localised manner. By exploiting distinctive physicochemical features, such as size, surface charge, and functionalization, nanoparticles can be engineered to enhance drug stability, prolong circulation time, and reduce off-target exposure. In addition, these platforms offer the possibility of integrating therapeutic and diagnostic functions within a single system, enabling real-time imaging alongside treatment and supporting the growing concept of theragnostic.

In the context of OS, where standard chemotherapy remains limited to dose toxicities and suboptimal biodistribution, nanotechnology-based strategies provide an opportunity to redirect precision treatment. By improving tumour selectivity and minimizing systemic adverse effects, these approaches have the potential to enhance therapeutic efficacy while preserving patient safety.

7.1 Magnetic Nanoparticles (SPIONs) and Magnetic Hyperthermia Therapies

Superparamagnetic iron oxide nanoparticles (SPIONs) have attracted considerable interest as multifunctional nanoplatforms for OS treatment, mainly because of their magnetic responsiveness, acceptable biocompatibility, and ease of surface modification. When subjected to an alternating magnetic field, typically using a frequency in the range of 100–500 kHz and a field strength of approximately 10–20 kA/m, SPIONs are able to generate heat through Néel and Brownian relaxation processes. This localized temperature increase generally falls within the hyperthermic window of 39–45 °C, which is sufficient to affect tumour cells while limiting damage to surrounding healthy tissues [129].

Experimental studies in OS models have shown that magnetothermal treatment maintaining temperatures around 42–43 °C can markedly reduce tumour cell viability. These effects are associated with the activation of caspase-dependent apoptosis, mitochondrial dysfunction, and increased intracellular reactive oxygen species. Notably, magnetic hyperthermia has been reported to act synergistically with conventional chemotherapeutic agents, including cisplatin and doxorubicin, leading to enhanced cytotoxic effects at lower drug doses than those required for chemotherapy alone [130].

From a materials perspective, surface coating plays a crucial role in determining the biological behaviour of SPIONs. Functionalization with polymers or biomaterials such as dextran, polyethylene glycol, chitosan, or silica is commonly employed to improve colloidal stability, reduce magnetic aggregation, and modulate surface charge under physiological conditions. In parallel, SPIONs act as efficient T2/T2* MRI contrast agents because their superparamagnetic core creates local magnetic susceptibility effects that shorten transverse relaxation times and cause signal loss on T2- and T2*-weighted images. This imaging capability enables the use of SPIONs in theragnostic strategies, combining diagnosis and therapy [131].

Despite these advantages, *in vivo* studies consistently indicate preferential accumulation of SPIONs in reticuloendothelial organs, particularly the liver and spleen, highlighting the importance of optimizing surface chemistry and pharmacokinetics to address long-term clearance and translational safety in osteosarcoma therapy (Table 3).

7.2 Mesoporous Bioactive Glasses (MBGs): Ion Release, Drug Delivery, and Bone Regeneration

Mesoporous bioactive glasses (MBGs) have emerged as particularly attractive materials in the context of OS due to their intrinsic bioactivity and their ability to act simultaneously as therapeutic carriers and ion-releasing platforms [132]. A defining feature of MBGs is their ordered mesoporous structure, with reported pore sizes ranging from 2 to 50 nm, coupled with very high specific surface areas that can approach 1000 m²/g [132][133]. These structural characteristics directly translate into a high capacity for drug loading and finely tuneable release behaviour.

Importantly, MBGs are not biologically inert carriers. Their therapeutic relevance also derives from the gradual dissolution of the glass matrix and the consequent release of bioactive ions. Calcium and silicon ions released from MBGs are well known to support osteogenic processes, promoting osteoblast differentiation and matrix mineralization [134], [135]. At the same time, the incorporation of additional components within MBG-based systems can confer anti-tumoral effects. In OS models, composite MBG platforms containing magnetic or metal-based elements have demonstrated direct cytotoxic activity, as exemplified by a Mc-Fe₃O₄-MBG system showing an IC₅₀ value of 12.19 µg/mL against MG63 OS cells [136].

The mesoporous architecture of MBGs also enables highly efficient drug delivery. Several MBG-based formulations have been reported to achieve doxorubicin loading efficiencies up to 90%, while other systems display incorporation values exceeding 80%, or more moderate loading levels such as 77.59%, 63%, or 35%, depending on pore organization and surface chemistry [137], [138]. Drug release from these platforms is strongly influenced by environmental conditions. In particular, acidic environments promote enhanced drug release, with tuneable profiles observed across a wide pH range from 4.4 to 10, a behaviour that is especially relevant given the altered pH conditions typically associated with OS lesions [137].

Beyond their role in drug delivery, MBGs are well suited for post-surgical applications. MBG-based scaffolds combining sustained ion release with high drug-loading capacity have shown the ability, in preclinical models, to suppress residual tumour cell activity while simultaneously supporting new bone formation and scaffold integration [132]. This combination of localized anti-tumoral action and osteogenic stimulation addresses a key clinical challenge in OS treatment, where effective control of residual disease must be achieved alongside reconstruction of large bone defects following tumour resection (Table 3).

7.3 Biodegradable Metal-Based Scaffolds for Treatment and Bone Reconstruction

Biodegradable metallic scaffolds have attracted growing interest in OS research because they are able to provide temporary mechanical-loading support while progressively degrading in vivo, thereby reducing the long-term complications associated with permanent metallic implants. For this reason, an ideal bioresorbable scaffold must degrade at a rate that is well matched to the process of new bone formation. If degradation is too fast, mechanical stability is lost; if it is too slow, the material may interfere with bone regeneration. Importantly, this balance strongly depends on the material used [139]. Magnesium-based alloys are generally characterized by relatively rapid corrosion, zinc-based systems exhibit slower degradation behaviour, and iron-based materials degrade very slowly, making precise control of degradation kinetics a central design challenge [140].

Iron, in particular, combines favourable mechanical properties with limited intrinsic corrosion, which has stimulated the development of strategies aimed at accelerating its degradation. Micro-alloying approaches have been proved to be effective in this regard. For example, Fe–Se alloys with selenium contents in the range of 0.2–1.0 wt% have been reported to inhibit OS cell proliferation while maintaining good compatibility with healthy cells. In vitro studies on human 143B OS cells showed a marked reduction in cell viability (<50%) upon exposure to Fe–Se extracts ranging from 0.6–1.0, whereas pre-osteoblastic MC3T3–E1 cells retained viability above 75–100%, indicating a favourable therapeutic window [141]. Mechanistically, the anti-tumoral effect was associated with Se^{4+} ion release and a significant increase in intracellular reactive oxygen species, leading to apoptosis and loss of mitochondrial function in OS cells.

Magnesium-based platforms have been evaluated more extensively in OS-oriented experimental settings. Anti-tumoral effects of Mg-based pellets have been assessed in both 2D and 3D in vitro culture systems, and these findings have been further validated in tumour-bearing mouse models, with mechanistic analyses performed at the molecular and cellular levels. Beyond the effects associated with magnesium corrosion alone, Mg implants can also be engineered into multifunctional systems. An illustrative example is provided by AZ31 Mg alloys surface-modified with Fe–OH, which have been shown to enable combined photothermal and chemo-dynamic therapeutic effects [142].

More complex composite designs have also been explored. A low-temperature 3D-printed Poly (lactic-co-glycolic acid) or PLGA/Mg multifunctional scaffold was evaluated in vivo using a rat distal femur model, where near-infrared laser irradiation was employed to eliminate residual tumour cells [134], [143]. In this system, the magnesium component contributed both to the photothermal response and to the promotion of bone regeneration. All together, these metal-based scaffolds are particularly attractive because they integrate structural support with time-dependent degradation and can be further optimized through surface modification or composite engineering to meet the dual clinical demands of OS treatment: effective local tumour control and reconstruction of large bone defects following resection (Table 3).

7.4 Injectable Hydrogels for Local Therapy and Bone Regeneration

Injectable hydrogel systems have been explored as localized therapeutic platforms for OS, particularly for use within bone defects following tumour resection. Several formulations are designed to undergo rapid in situ gelation and display self-healing behaviour, with reported stress relaxation half-times in the range of 30–40 s, allowing them to conform efficiently to irregular surgical cavities. These properties further prolonged retention of therapeutic agents at the target site while limiting systemic dispersion [144].

From a therapeutic point of view, hydrogel-based delivery has demonstrated sustained release profiles lasting approximately 14–21 days, resulting in markedly improved local efficacy compared with free-drug administration. In murine OS models involving incomplete tumour resection, drug-loaded hydrogels implants have been associated with tumour growth inhibition rates of about 85–90%, whereas substantially lower inhibition is observed with single-agent or non-localized treatments. Survival analyses in these models further indicate a benefit for localized hydrogel-based therapies, with median survival times extended by roughly 10–15 days relative to control groups [145].

Hydrogels have also been engineered to support bone regeneration alongside tumour suppression. Micro-computed tomography performed 3–4 weeks after implantation has revealed clear increases in bone mineral density and bone mineral content within hydrogel-treated defects. At the molecular level, expression of osteogenic markers including ALP, COL1, RUNX2, and BGLAP has been reported to rise by approximately 3–5-fold, indicating active osteogenic differentiation in the regenerated tissue [145].

In addition, in literature has been well highlighted that hydrogel degradation profiles can be tuned over several weeks, aligning with the temporal requirements of bone healing, while maintaining favourable cytocompatibility, with in vitro cell viability typically exceeding 80–85% [146]. Taken together, these findings support the use of hydrogel-based systems as localized therapeutic matrices capable of integrating effective tumour control with bone regeneration in osteosarcoma (Table 3).

Table 3. Nanotechnology-based and biomaterial platforms for osteosarcoma therapy and bone reconstruction.

Platform	Primary function	Experimental models	Key advantages	Main limitations
Superparamagnetic iron oxide nanoparticles (SPIONs)	Magnetic hyperthermia and imaging	OS cell lines; murine tumour models	Local heat generation; MRI contrast capability;	Preferential accumulation in liver and spleen;

			synergy with chemotherapy	long-term clearance concerns
Mesoporous bioactive glasses (MBGs)	Ion release and drug delivery	In vitro OS models; scaffold implantation	High drug-loading capacity; pH- responsive release; osteogenic ion delivery	Control of degradation kinetics and ion dosage
Biodegradable metal- based scaffolds (Mg, Fe, Zn)	Load-bearing support and local antitumour effects	2D/3D cultures; rodent bone defect models	Mechanical strength; time-dependent degradation; suitability for large defects	Precise corrosion- rate control; gas formation (Mg); slow degradation (Fe)
Injectable hydrogels	Local drug depot and bone regeneration	Murine post- resection OS models	In situ gelation; sustained release (≈14–21 days); combined tumour control and repair	Limited penetration depth; formulation- dependent stability

8. Conclusions and future perspectives

Osteosarcoma remains a particularly difficult disease to treat, not because therapeutic options are lacking, but because its biology consistently overcomes new treatment strategies. Despite decades of clinical experience and sustained research efforts, survival rates have improved only marginally, especially for patients with metastatic or recurrent disease. This persistent stagnation reflects the limits of conventional

approaches and highlights how deeply the disease is shaped by early dissemination, extensive heterogeneity, and adaptive resistance.

One of the most important lessons emerging from recent studies is that OS does not lend itself to single-target solutions. Genomic instability, overlapping signalling networks, epigenetic plasticity, and continuous interaction with the bone microenvironment collectively support tumour progression and therapeutic escape. In this context, the repeated failure of single-agent strategies is not unexpected. Instead, future progress will likely depend on combination approaches that are biologically informed and designed to interfere with multiple vulnerabilities at once, rather than focusing on isolated molecular alterations.

At the same time, the way OS is investigated is gradually changing. Advances in molecular profiling, functional genomics, and integrative analytical tools are beginning to move the field beyond purely descriptive classifications. In particular, CRISPR–Cas9–based functional studies have provided a powerful tool to identify new therapeutic targets which are not easily discoverable from sequencing data alone, helping to prioritize targets that are genuinely required for tumour maintenance and progression. These approaches shift the focus from static molecular landscapes to functional vulnerabilities, which may be relevant for therapeutic development.

Immunotherapy represents another area where expectations must be carefully aligned with biological reality. The limited efficacy of immune checkpoint inhibitors in OS reflects a profoundly immunosuppressed tumour microenvironment, which moves away from the idea of an inherent lack of immunogenicity. Understanding this distinction has important implications: indeed, rather than abandoning immunotherapy altogether, current evidence supports the exploration of strategies that reshape the immune context, including modulation of macrophage polarization, targeting of innate immune checkpoints, and rational integration of immunotherapy into combination regimens. Similarly, adoptive cell therapies highlight both the potential and the unresolved challenges of immune-based approaches in solid tumours, emphasizing the need to overcome limited persistence, suboptimal tumour infiltration, and strong suppressive signals within the tumour microenvironment.

Technological innovation adds a further layer to this evolving landscape. Nanotechnology-based delivery systems and biomaterial platforms offer practical solutions to problems that have long limited OS treatment, such as poor drug biodistribution, systemic toxicity, and the management of large post-resection bone defects. The possibility of combining local tumour control with skeletal regeneration is particularly relevant in OS and highlights the importance of approaches that integrate oncological efficacy with functional reconstruction, rather than treating these goals separately.

Nevertheless, significant challenges remain before these advances can be translated into routine clinical practice. Tumour heterogeneity, the absence of robust predictive biomarkers, limited availability of representative models, continue to slow clinical validation.

In conclusion, the future of OS therapy is unlikely to be defined by a single transformative breakthrough. Probably, progress will depend on the gradual integration of biological insight, functional precision, immune modulation, and advanced delivery technologies. Bridging the gap between bench and bedside remains a complex and demanding task, but it also represents the most realistic path toward overcoming the current therapeutic plateau and achieving meaningful improvements in patient outcomes.

9. References

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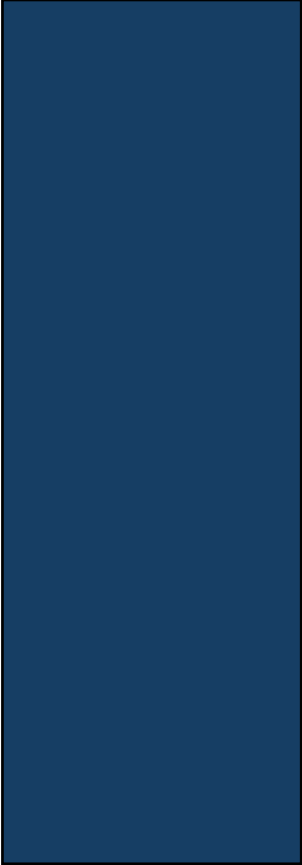
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Chapter 3

“Anti-tumoral effect of osteoblast-derived extracellular vesicles loaded with methotrexate, doxorubicin, and cisplatin on MNNG/HOS osteosarcoma cells”

Manuscript in preparation

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Abstract

Osteosarcoma remains a clinical challenge due to its aggressive nature, high metastatic potential, and poor outcomes in relapsed and refractory cases. Conventional chemotherapy has improved survival but is limited by systemic toxicity, low tumour selectivity and the frequent development of chemoresistance. The advent of extracellular vesicles (EVs) as natural biological carriers offers new perspectives for drug delivery, combining nanoscale size, intrinsic biocompatibility, and the ability to mediate selective intercellular communication. In this study, we explored human fetal osteoblast-derived EVs (hFOB-EVs) as vehicle for targeted delivery of chemotherapeutics to osteosarcoma cells. We first demonstrated that hFOB-EVs are efficiently internalised by MNNG/HOS osteosarcoma cells; next, we successfully loaded these EVs with methotrexate (MTX), doxorubicin (DXR), or cisplatin (CPT) by employing optimised passive and indirect loading strategies, achieving encapsulated drug levels ranging from 0.7 µg/mL for MTX to 2.9 µg/mL for CPT and 20.4 µg/mL for DXR. Interestingly, treatment of MNNG/HOS with MTX, DXR or CPT – loaded hFOB-EVs reduced cell viability and proliferation, while enhancing apoptosis, *in vitro*. Conversely, treatment of normal cells (i.e. osteoblasts) with drug-loaded-EVs failed to affect their homeostasis *in vitro*, thus indicating the lack of any toxic effect. Finally, in an *in vivo* osteosarcoma model established through paratibial injection of MNNG/HOS cells in balb/c nude mice, MTX-loaded hFOB-EVs markedly reduced tumour growth, assessed through bioluminescence analysis, when compared to hFOB-EVs treatment. Nevertheless, MTX-loaded hFOB-EVs demonstrated higher efficacy in reducing tumour growth when compared to free MTX (5mg/kg), with a dose nearly 2800-fold lower. In conclusion, these findings highlight hFOB-EVs as safe and functionally competent nanocarriers capable of enhancing the antitumour efficacy of standard chemotherapeutics, opening new avenues for the development of EVs-based therapeutic strategies against osteosarcoma.

1. Introduction

Osteosarcoma is the most frequent primary malignant bone tumour, predominantly affecting children and young adults, likely in conjunction with the rapid skeletal growth occurring during this age range. Population-based data estimate an age-standardised incidence of approximately 0.3 per 100,000 person-years in the general population, peaking at 0.8–1.1 per 100,000 among adolescents aged 15–19 years, with a male-to-female ratio of about 1.4:1 [1]. Despite significant advances in imaging, surgical techniques, and multimodal therapy, the prognosis for patients presenting metastatic or recurrent disease remains poor, with five-year survival not exceeding 25–30 % [2]. Nowadays, conventional chemotherapy for localised disease relies on the combination of high-dose methotrexate (MTX), doxorubicin (DXR) and cisplatin (CPT), administered before and after surgical resection [3–5]. This multidrug approach highly increased survival in the last decades, but its effectiveness is counterbalanced by severe systemic toxicities, including cardiotoxicity, nephrotoxicity, hepatotoxicity, and myelosuppression, which limit dose escalation and long-term tolerability [4–6]. Nevertheless, the lack of tumour-specific uptake often results in suboptimal drug concentrations at the target site, finally leading to multidrug resistance and metastatic relapse [7]. Collectively, these challenges suggest an urgent need for therapeutical

approaches that enhance tumour selectivity, sustain therapeutic efficacy, and mitigate systemic burden.

As a matter of fact, in the last years nanocarriers have been widely investigated as innovative tools for cancer therapy. Among these, extracellular vesicles (EVs) – nanosized, membrane-enclosed structures secreted by most cell types – have emerged as versatile and physiologically compatible vectors for drug delivery [8,9]. According to the MISEV 2023 guidelines [10], EVs can be rigorously characterised by size distribution, morphology, and protein markers such as CD63, CD81, and Annexin II, ensuring reproducibility and comparability across studies. Focusing on their structure, the lipid bilayer protects the encapsulated cargo from enzymatic degradation, prolong circulation time, and mediate intercellular communication through receptor–ligand and membrane fusion mechanisms. Moreover, compared with synthetic nanoparticles, EVs exhibit low immunogenicity, enhanced stability in biological fluids, and intrinsic targeting potential that mirrors the tropism of their parental cells [11].

When examining bone biology, the bone microenvironment is finely regulated by the activity of osteoblasts (bone-forming cells) and osteoclasts (bone-resorbing cells), which communicate with each other and with surrounding cell types also through the release of EVs. In fact, recent studies from our team demonstrate that osteoblast-derived vesicles can modulate osteosarcoma cell aggressiveness, mitochondrial bioenergetics, and redox homeostasis, highlighting the existence of a crosstalk between bone-forming cells and tumour [12]. This communication suggests that osteoblast-derived EVs may be selected as natural nanocarriers for the delivery of drugs, taking advantage of their bone tropism and biological compatibility.

Interestingly, when compared with synthetic nanoparticles, EVs exhibit higher biodistribution, reduced immune clearance, and a biological content that reflects the phenotype of their parental cells [13–15]. Various studies have optimized drug-loading methods, including passive incubation, sonication, and preconditioning of donor cells, to incorporate chemotherapeutics into EVs [16,17]. Although these strategies have been successfully explored in malignancies such as breast, lung, and pancreatic cancer, their application into osteosarcoma treatment remains relatively underexplored. Hence, we aimed to investigate human fetal osteoblast-derived EVs (hFOB-EVs) as nanocarriers for the delivery of conventional chemotherapeutic drugs, providing a foundation for the development of EV-based systems to enhance drug efficacy and reduce systemic toxicity in osteosarcoma therapy. In this study, we successfully generated drug-loaded hFOB-derived EVs by identifying the most suitable strategy of loading for each chemotherapeutic. We demonstrated that these drug-loaded EVs exert potent antitumour activity against osteosarcoma cells both *in vitro* and *in vivo*, while they are ineffective against normal osteoblast-like cells.

2. Materials and Methods

2.1. Materials

Dulbecco's Modified Minimum Essential Medium (DMEM, cat. #ECB7501L), Phosphate-Buffered Saline (PBS, cat. #ECB4004L), fetal bovine serum (FBS, cat. #ECS0180L), Hank's Balanced Salt Solution (HBSS, cat. #ECB4007L), L-Glutamine 100× (cat. #ECB3000D), penicillin/streptomycin (Pen/Strep, cat. #ECB3001D), trypsin/EDTA (cat. #ECB3052D) and all sterile disposable plasticware were supplied by Euroclone (Milan, Italy). The EdU

(5-ethynyl-2'-deoxyuridine) HTS Kit 488 (cat. #BCK-HTS488) was purchased from BaseClick GmbH (Munich, Germany); the [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay reagent (MTT, cat. #V13154) was obtained from Invitrogen (Waltham, MA, USA). Methotrexate (L-4-amino-N¹⁰-methylpteroylglutamic acid, cat. #M8407), cisplatin (cis-diammineplatinum(II) dichloride, cat. #479306), Triton X-100 (cat. #T9284), and dimethyl sulfoxide (DMSO, cat. #D8418) were purchased from Merck KGaA (Darmstadt, Germany). Doxorubicin (cat. #S1208) was supplied by Selleck Chemicals (Cologne, Germany). The Annexin V-DY-634/PI Apoptosis Staining Detection Kit (cat. #AB214484) was purchased from Abcam Ltd (Cambridge, UK). Gene Pulser® electroporation cuvettes (cat. #1652088) were provided by Bio-Rad Laboratories (Hercules, CA, USA). PKH67 fluorescent dye (cat. #MINI26 1KT) was purchased from Sigma-Aldrich (St. Louis, MO, USA) while CMFDA fluorescent dye (cat. #C2925) was obtained from Thermo Fisher Scientific (Waltham, MA, USA).

2.2 Cell cultures

The human fetal osteoblast cell line (hFOB 1.19, from now hFOB) and the human osteosarcoma cell line MNNG/HOS were obtained from the European Collection of Authenticated Cell Cultures (ECACC, Salisbury, UK). Both cell lines were grown at 37 °C, 5% CO₂ in DMEM supplemented with 10% FBS, 100 IU/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine.

2.3 Extracellular Vesicles (EVs) isolation

hFOB derived EVs (hFOB-EVs) were isolated according to well established protocols [12]. Upon reaching 75-80% of confluence, hFOB cells were washed in DPBS and starved in serum-free DMEM for 24 h, then the conditioned medium (CM) was collected and sequentially centrifuged at 300 x g, 4 °C for 5 min to remove dead cells and at 5,000 x g, 4 °C for 25 min for membrane debris removal. The supernatant was collected and transferred to a Beckman L7-65 ultracentrifuge in a Beckman SW41-Ti or SW28 rotor and centrifuged at 100,000 x g, at 9 °C for 70 min. Supernatant was discarded, while the pellet, containing EVs, was resuspended in DMEM for cell treatments. To quantify hFOB-EVs, they were subjected to nanoparticle tracking analysis as well as to protein extraction, the latter giving a yield of 2.4 µg/12 ml CM. Freshly-isolated EVs were used for all the subsequent experiments.

2.4 Nanoparticle tracking analysis

Extracellular vesicles were isolated from hFOB CM (12 ml collected from one 175cm² flask of 80% confluent cells) and resuspended in 100µl of nanofiltered DPBS. EVs were then diluted 1:100 and used for nanoparticle tracking analysis using a nanosight NS300 NTA apparatus. Flow and camera gain were adjusted following the manufacturer's instructions based on the NS300 quality control parameters. Five camera acquisitions of 60 seconds each were analysed for every biological replicate.

2.5 Transmission electron microscopy (TEM)

Extracellular vesicles were isolated from hFOB CM (12 ml collected from one 175cm² flask of 80% confluent cells) and resuspended in 2% PFA. Five µl of EVs were put onto Formvar-coated grids and allowed to adsorb for 20 min in a dry environment. Grids were washed in PBS and fixed in 1% glutaraldehyde for 5 min. Samples were washed in distilled

water and contrasted with 4% uranyl-oxalate solution for 5 min. Grids were air-dried for 10 min and observed under a Philips CM 30 TEM, 80 kV.

2.6 Western blot analysis

For cells and EVs protein extraction, samples were lysed in RIPA buffer (50 mM Tris HCl pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) containing protease inhibitors. Six µg of EVs protein lysates were resolved by 10-15% SDS-PAGE under reducing conditions and transferred to nitrocellulose membranes. Blots were incubated 2 h in 5% nonfat dry milk in TBS-T, probed with the primary antibody in 1% milk O/N at 4°C or for 1 h at room temperature (RT), washed and incubated with the appropriate HorseRadish Peroxidase (HRP)-conjugated secondary antibody for 1 h at RT. After washing, protein bands were revealed by the SuperSignal Chemiluminescence substrate, according to the manufacturer's instructions, and acquired using a BioRad Gel Doc XR+ Imaging System. The analysis of band intensities was performed using the NIH ImageJ tool.

2.7 EV internalisation assay

Extracellular vesicles derived from hFOB CM (12 ml collected from one 175cm² flask of 80% confluent cells) were incubated at 37°C with the membrane-permeant green-fluorescent dye 5-chloromethylfluoresceindiacetate (CMFDA) for 30 min followed by 5 min at 37°C with the red fluorescent membrane-labelling dye PKH26. Then, the EVs were washed in PBS and ultracentrifuged at 100,000 x g, at 4°C for 70 min. Finally, EVs were resuspended in PBS for the treatment. Target cells were incubated with stained or unstained EVs for 24h before microscopic assessment of internalisation. Nuclei were counterstained with DAPI.

2.8 Drug loading

To evaluate the optimal strategy for drug encapsulation within hFOB-derived EVs, three different loading methods were tested: passive diffusion, electroporation, and indirect loading. Each approach was optimised for methotrexate (MTX), doxorubicin (DXR), and cisplatin (CIS).

2.8.1 Passive loading

EVs isolated from hFOB CM were resuspended in drug solutions prepared as follows:

- MTX, 44 mM dissolved in 0.1 M NaOH in H₂O.
- DXR, 0.3 mM dissolved in DPBS.
- CPT, 3.3 mM dissolved in DPBS.

EV suspensions were incubated for 30 min at 37 °C to promote diffusion of each compound across the vesicle membrane. After incubation, samples were subjected to two sequential ultracentrifugation in DPBS at 100,000 × g for 70 min at 8 °C to remove unincorporated drug.

The final EV pellets were resuspended in serum-free DMEM and used to treat MNNG/HOS cells.

2.8.2 Electroporation

EVs isolated from hFOB-CM were resuspended in DXR 0.3 mM dissolved in DPBS and pre-incubated at 4 °C for 30 min. The EVs were then diluted 1:1 (v/v) with the electroporation buffer (400 mM sucrose in DPBS) and transferred to 0.4 cm Gene Pulser® cuvettes. Electroporation was performed using a Gene Pulser® Xcell (Bio-Rad) at 250 V and 125 μ F. Immediately after the pulse, EVs were incubated for 30 min at 37 °C and 5% CO₂ to allow membrane recovery, then washed twice in DPBS by ultracentrifugation (100,000 $\times$ g, 70 min, 8 °C) to remove residual, unincorporated drug. Finally, EVs were resuspended in serum-free DMEM and used for MNNG/HOS treatment.

2.8.3 Indirect loading

For indirect loading, hFOB cells were starved with serum-free DMEM supplemented with either DXR 8 μ M or CPT 100 μ M for 24 h at 37 °C, 5% CO₂. Next, cells were washed twice with DPBS and maintained for an additional 24 h in serum-free medium to allow secretion of drug-containing EVs. The conditioned medium was collected, and EVs were isolated following the standard ultracentrifugation procedure. The resulting drug-loaded EVs were resuspended in serum-free DMEM and used to treat MNNG/HOS cells.

2.9 MNNG/HOS cells treatment

For each test employed to assess any effect on tumour phenotype, MNNG/HOS cells were treated for 48 hours as follow:

- Vehicle (serum-free DMEM),
- Free drugs (positive controls): MTX 0.5 μ M, DXR 4 μ M, CPT 100 μ M,
- Unloaded EVs (hFOB-EVs),
- Drug-loaded EVs (hFOB-EVs+MTX, hFOB-EVs+DXR, hFOB-EVs+CPT),

2.10 Determination of drug loading efficiency

For quantitative analysis of MTX and DXR, EVs isolated from hFOB cultures (144 mL total CM, corresponding to twelve 175 cm² flasks with 80% confluent cells) were subjected to drug loading and isolation as described above. The final EV pellets were resuspended in 500 μ L of acidified isopropanol (90% isopropanol, 0.075 M HCl) and analysed by high-performance liquid chromatography (HPLC) equipped with UV/Vis detection. Drug concentrations were determined through specific calibration curves generated by increasing concentrations for each compound and expressed as μ g of drug per mL of lysis solution.

As for CPT, content within hFOB-derived EVs was quantified through a colorimetric assay based on 3,3',5,5'-tetramethylbenzidine (TMB) oxidation in the presence of hydrogen peroxide (H₂O₂). Briefly, to generate the calibration curve, increasing concentrations of CPT standard solutions were prepared in DPBS. For each 100 μ L of standard, 50 μ L TMB reagent (0.1 mg/mL in citrate–phosphate buffer, pH 5.0) was added together with 50 μ L of H₂O₂ (3% w/v) to initiate the reaction, in a final volume of 200 μ L per well in a 96-well plate. Samples were incubated overnight at RT in the dark to allow complete colour development. For EV samples, drug-loaded EVs were lysed in DPBS containing 0.1% Triton X-100 to release incorporated CPT. The TMB reagent was then added at the same final concentration as used for the standards, followed by H₂O₂ (3%), and incubated overnight at RT under identical conditions. Unloaded EVs were processed in parallel as

background controls. Absorbance at 650 nm was finally recorded and samples O.D. were interpolated with standard curve to calculate the amount of encapsulated drug.

2.11 MTT assay

Metabolic activity of MNNG/HOS cells was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) bromide reduction assay. Briefly, cells were plated in 96-well plates at a density of 9.0×10^3 cells/cm² in 96-well plates in DMEM + 10% FBS and incubated for 24 h at 37 °C, 5% CO₂. Cells were then treated as mentioned in section 2.9. MTT was dissolved in DPBS at 5 mg/ml concentration and added at 1:6 (v/v) ratio directly into the cell supernatant. Three hours later, medium was removed and DMSO was added to dissolve the precipitated formazan salts arising from the reaction. Plates were shaken at 160 rpm on an orbital shaker for 10 min, then absorbance at 595 nm was recorded and plotted as X-fold to the vehicle.

2.12 EdU (5- ethynyl-2'-deoxyuridine) cell proliferation assay

Cell proliferation assay was assessed by the EdU HTS Kit 488. Briefly, MNNG/HOS cells plated and at a density of 9.0×10^3 cells/cm² in 96-well plates in DMEM + 10% FBS and incubated for 24 h at 37 °C, 5% CO₂. Cells were then treated as aforementioned and, after 48 hours, incubated with 5 µM EdU for 5 hours: then, cells were washed in DPBS and fixed in 4% paraformaldehyde (PFA) for 10 min. EdU incorporation was detected using the EdU HTS Kit: cells were permeabilised with 0.5% Triton X-100 in PBS, incubated with the click assay cocktail for 30 min at RT, protected from light, and then washed twice in 1X rinse solution. Nuclei were counterstained with DAPI. Cell proliferation was assessed under a fluorescence microscope and expressed as the percentage of EdU-positive cells over the total number of cells.

2.13 Apoptosis

Apoptosis was evaluated in MNNG/HOS cells using Annexin V/Propidium Iodide (PI) flow-cytometric analysis. MNNG/HOS cells were seeded in 10-cm dishes at a density of 6.0×10^6 cells/cm² and incubated for 24 h at 37 °C in 5 % CO₂. Next, cells were treated as above described. At the end of the treatment, the CM containing detached cells was collected and transferred into a 15 mL tube, while adherent cells were detached and the resulting suspension was combined with the previously collected supernatants to recover both floating and adherent cell populations. Cells were centrifuged at $300 \times g$ for 5 min at RT, resuspended in 5 mL of DMEM + 10 % FBS, and a 20 µL aliquot was used for viability assessment by trypan blue staining. Cells were then washed twice and resuspended in 1× binding buffer at a cell density of 1×10^6 cells/mL. For staining, 100 µL of cell suspension were transferred to FACS tubes and incubated with 5 µL of Annexin V DY-634 conjugate and 5 µL of Propidium Iodide staining solution for 15 min at RT in the dark. After incubation, 400 µL of 1× binding buffer were added to each sample, and fluorescence was analysed within 1 h using a flow cytometer (BD FACSAria III).

Cells were classified as:

- Annexin V⁻/PI⁻ = viable,
- Annexin V⁺/PI⁻ = early apoptotic,
- Annexin V⁺/PI⁺ = late apoptotic or necrotic,
- Annexin V⁻/PI⁺ = necrotic.

Data were expressed as the percentage of Annexin V-positive cells relative to total events.

2.14 Osteosarcoma *in vivo* model

All procedures involving animals and their care, were conducted according with national and international laws and policies (European Economic Community Council Directive 86/609, OJ L 358, 1, 12 December 1987; Italian Legislative Decree 4.03.2014, n.26, *Gazzetta Ufficiale della Repubblica Italiana* no. 61, 4 March 2014) and the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines. Five-week-old female Balb-c nu/nu mice (ENVIGO Laboratories) were housed under pathogen-free conditions in the animal facility of the University of L'Aquila, Italy, at the following parameters: temperature: 24 °C, humidity: 60%, dark/light cycle: 12/12 h. They had access to food and water *ad libitum* and were fed with a standard diet (Mucedola, Milan, Italy. Code: 4RF21) composed of 60.8% carbohydrates, 21% proteins, 3.45% fat, 6.8% fibers, 7.95% trace elements, and 12% humidity.

An orthotopic osteosarcoma model has been established according to Heymann et al. [18, 19] by paratibial injection of MNNG/HOS-Luc⁺ (1x10⁶ cells/40µL of DPBS) in both tibiae of 5-week-old Balb-c nu/nu mice after deep anaesthesia with intraperitoneal injection of 80 mg/kg ketamine and 10 mg/kg xylazine. Mice were randomised to 4 experimental groups: vehicle (DPBS), unloaded hFOB-EVs (hFOB-EVs), MTX 5mg/kg, hFOB-EVs + MTX (N = 5 for vehicle and unloaded hFOB-EVs, N = 10 for MTX 5mg/kg and hFOB-EVs + MTX). All treatments were performed intraperitoneally (i.p.) and began one day after tumour cell inoculation. Mice were treated twice per week until sacrifice (day 18) and, during this period, were routinely monitored for general health, behaviour, body weight, and signs of drug-related toxicity throughout the study. Tumour volume was evaluated by caliper measurement twice a week, with tumour volume calculated as $\frac{4}{3}\pi(a \times b \times c)$. At the endpoint (day 18), mice were euthanised by CO₂ inhalation and blood has been collected by intracardiac puncture. After 45 minutes at RT, blood was centrifuged at 20000 x g for 15 minutes at 4 °C to collect serum. Finally, soft organs (lungs, heart, spleen, liver, kidneys) and both left and right hindlimbs (tumours included) were fixed in PFA 4%.

2.15 Bioluminescence analysis

Tumour growth was monitored *in itinere* by bioluminescence imaging, using 2D-imaging Hamamatsu Photonics. Briefly, at days 9, 16, and 18 from MNNG/HOS cells injection, mice were deeply anaesthetised through intraperitoneal injection (i.p.) of 80 mg/kg ketamine and 10 mg/kg xylazine. A second i.p. injection of 40 mg/kg of D-luciferin was then performed, and images were acquired 5 minutes later using exposure time of 1 minute and 15 minutes. Quantitative analysis was performed on 1-minute exposure images by manually drawing a region of interest (ROI) around the brightest tumour area and calculating the resulting bioluminescence signal (BRI photon intensity, a.u.) for each bioluminescent hindlimb.

2.16 Micro Computed Tomography (µCT) Analysis

Images of tibiae previously fixed in 4% PFA were acquired using a SkyScan 1174 system (Bruker, Billerica, MA, USA) with an isotropic voxel size of 12 µm (X-ray voltage: 50 kV). Image reconstruction was performed using a modified Feldkamp–Davis–Kress (FDK) algorithm implemented in the Skyscan NRecon software. Three-dimensional (3D) and two-dimensional (2D) morphometric parameters were analysed over the entire tibia.

Three-dimensional measurements were obtained from a Marching Cubes–based surface rendering, whereas 2D areas and perimeters were computed using the Pratt edge-detection algorithm. To assess ectopic bone growth, a dedicated volume of interest (VOI) was manually segmented along the scanned tibia surface wherever ectopic mineralized tissue was visible. Ectopic bone volume (EBV) was extracted from this VOI and normalized to the total bone volume (TBV) of the corresponding tibia and expressed as *ectopic bone volume / total bone volume (%)*.

Osteolytic areas were quantified by measuring cortical discontinuities consistent with bone resorption. For each tibia, regions of missing cortical bone were manually delineated using ImageJ (NIH, USA) on the bone surface. The cumulative osteolytic area was then normalized to the total bone surface and expressed as *osteolytic area / bone surface (%)*.

2.17 Statistical analysis

Data are expressed as mean $\pm$ standard deviation (SD). Statistical analysis was conducted using GraphPad Prism. Data normality was verified by Shapiro–Wilk test. Differences among groups were analysed by paired-*t*-test. A *p*-value < 0.05 was considered statistically significant.

3. Results

3.1 hFOB-derived EVs characterisation and evaluation of their internalisation in recipient MNNG/HOS cells

We first characterised the EVs isolated from the CM of the human fetal osteoblast cell line hFOB (hFOB-EVs). Extracellular vesicle size was confirmed by NanoSight, which also allowed to determine the EV concentration per preparation (Fig.1A). The vesicular nature of the isolated particles was confirmed by TEM, which also showed the EV membrane integrity (Fig.1B); moreover, the typical positive EV biomarkers, CD81, CD63 and Annexin II (Anxa II), were expressed in the EV protein lysates (Fig.1C). Altogether, these results fulfil the requirements described in the MISEV2023 guidelines to define a vesicular fraction as EVs. We next investigated whether OB-EVs were actively internalised by MNNG/HOS cells. To this aim, hFOB-EVs were double-labelled with the intra-vesicular green-fluorescent dye, CMFDA, and with the red-fluorescent membrane dye, PKH26. Fluorescence microscopy demonstrated that both fluorophores were detectable in osteosarcoma cells, suggesting their ability to internalise hFOB-EVs (Fig.1D).

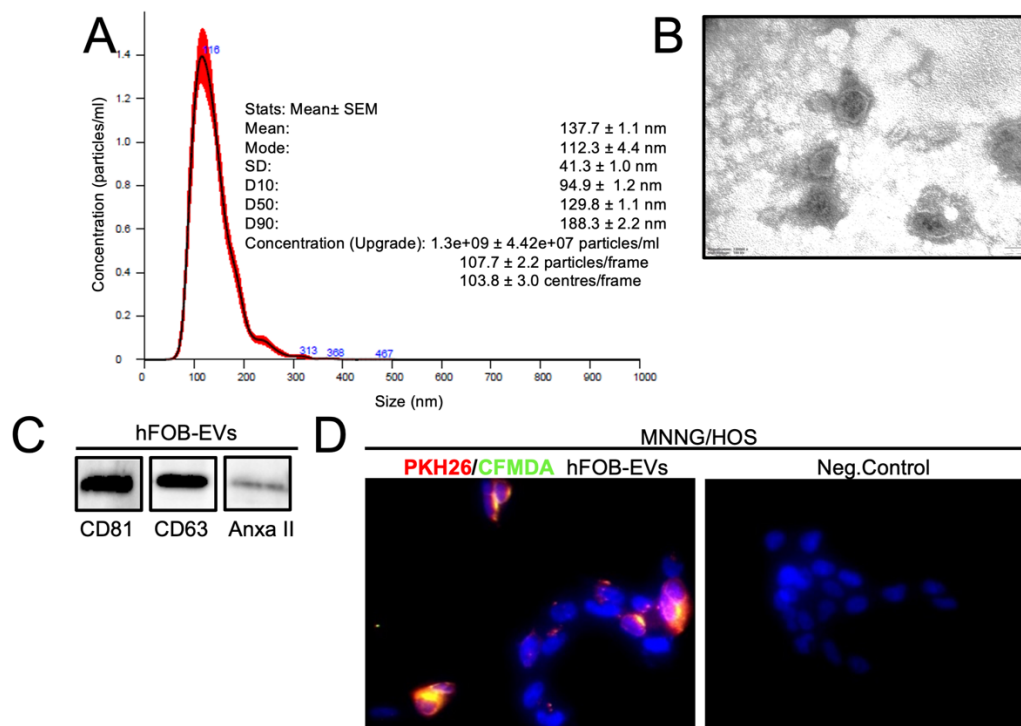


Figure 1. Characterisation of hFOB-derived EVs. The human fetal osteoblast cell line hFOB was starved in serum-free DMEM for 24 hours and EVs were isolated from conditioned medium (CM) by ultracentrifugation. (A) Size and concentration determination of hFOB-EVs by nanoparticle tracking analysis (NanoSight NS300). (B) Transmission Electron Microscopy (TEM) evaluation of OB-EVs (arrows, original magnification 130000x). (C) Western blot for the EV positive markers CD81, CD63, Annexin II (Anxa II), in protein lysates extracted from hFOB (50 μg) and hFOB-EVs (6 μg). (D) Left panel: representative fluorescence micrographs of MNNG/HOS cells incubated with hFOB-EVs previously labelled with CMFDA (green cytoplasmic dye) and PKH26 (red lipophilic dye) for 48 hours. Right panel: representative image of the negative control performed on MNNG/HOS cells incubated with unlabelled OB-EVs. Cells were also stained with the nuclear dye DAPI. (scale bar=50 μm). Data are representative of 3 independent preparations.

3.2 hFOB-EVs loaded with MTX impair MNNG/HOS cells tumour phenotype.

We first treated MNNG/HOS cells with different concentration of MTX, to choose the best dose of free MTX to be used as positive control for the downstream functional analyses, which resulted to be 0.5 μ M (Fig.2).

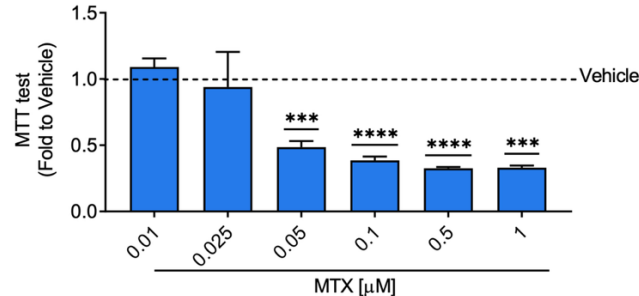


Figure 2: Effect of MTX on MNNG/HOS cells. MNNG/HOS cells were starved in serum-free DMEM overnight, then they were treated with different concentration (indicated in the abscissa) of MTX. Cell metabolic activity was assessed by MTT assay after 48 hours of treatment (N=3; ***p<0.001, ****p<0.0001 vs Vehicle; one sample t-test).

Next, we loaded these hFOB-EVs with MTX (hFOB-EVs-MTX) by passive incubation, as described in the materials and method section; MTX incorporated by hFOB-EVs was assessed by High-Performance Liquid Chromatography (HPLC) which showed its detectability, reaching a concentration of 0.7 μ g/ml (Fig.3A). Interestingly, MNNG/HOS treatment with hFOB-EVs-MTX significantly reduced cell metabolic activity assessed by MTT assay (Fig.3B), as well as cell number, evaluated by counting the number of DAPI positive nuclei (Fig.3C) compared to MNNG/HOS treated with unloaded hFOB-EVs (hFOB-EVs) or with vehicle. Consistently the number of live cells, evaluated by trypan blue exclusion assay, was also significantly reduced by hFOB-EVs-MTX (Fig.3D). These effects were similar to that of free MTX (MTX 0.5 μ M).

To explain the reduction of cell number, we assessed the percentage of Annexin V positive cells as a marker of apoptosis, finding that it was increased by treatment with free MTX and hFOB-EVs-MTX (Fig.3E,F); conversely, the same treatments significantly reduced cell proliferation (Fig 3G,H).

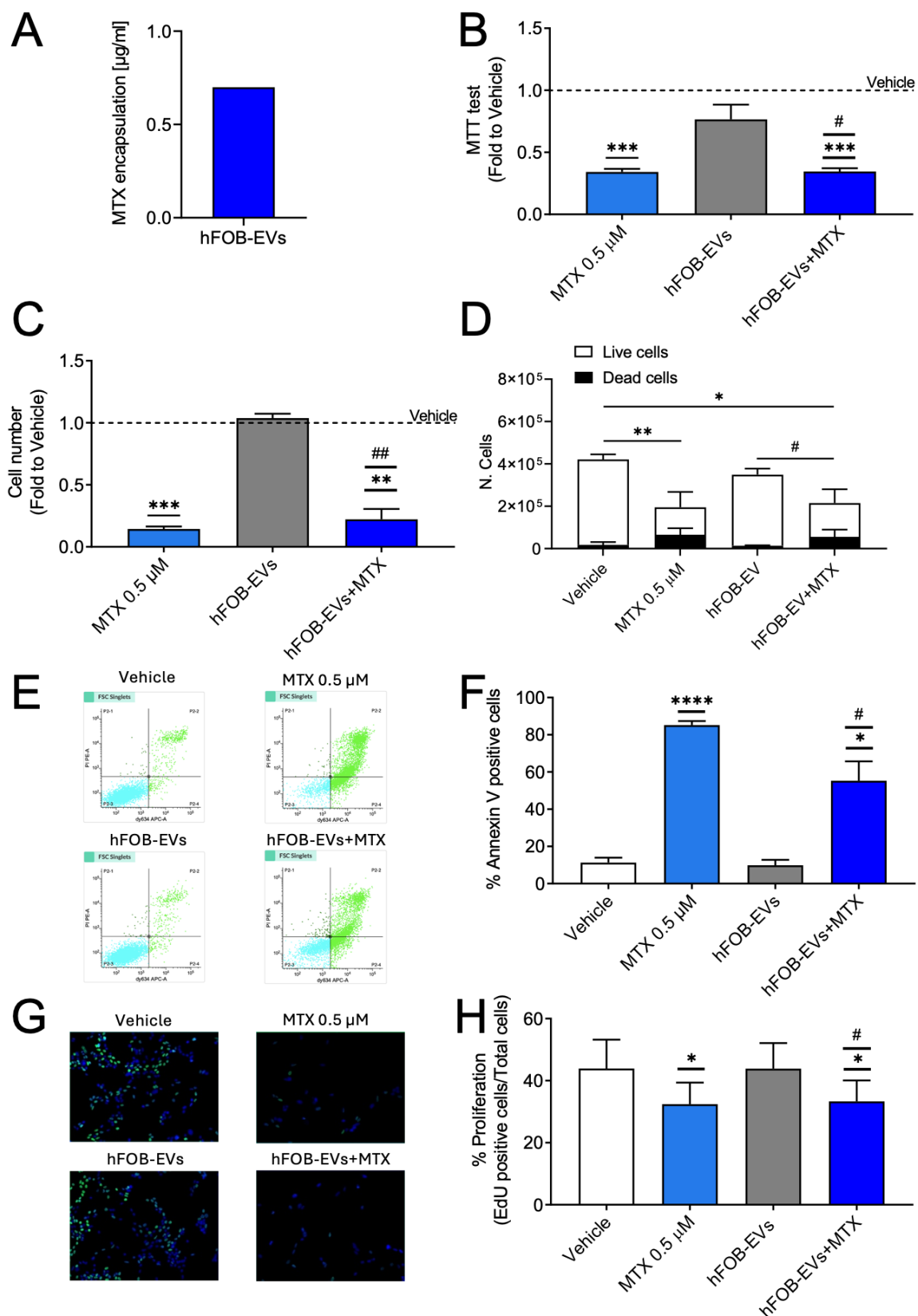


Figure 3: Effect of hFOB-EVs loaded with Methotrexate (MTX) on MNNG/HOS cells phenotype. hFOB-EVs pellet was resuspended in 44mM MTX in 0.1M NaOH and incubated at 37°C for 30 min. EVs were then recovered by ultracentrifugation and (A) resuspended in acidified isopropyl alcohol (90% isopropanol; 0.075M HCl) to quantify MTX by High-Performance Liquid Chromatography (HPLC) or (B-G) resuspended in DMEM serum-free for MNNG/HOS treatment. MNNG/HOS cells were starved in serum-free DMEM overnight, then treated with Vehicle, 0.5 μM free-MTX, hFOB-EVs, or hFOB-EVs loaded with MTX (hFOB-EVs+MTX).

EVs+MTX). (B) Cells metabolic activity assessed by MTT assay (N=3; *** $p < 0.001$ vs Vehicle, one sample t test; # $p < 0.05$ vs hFOB-EVs, paired t-test). (C) Cell number evaluated by DAPI nuclear staining (N=3; ** $p < 0.01$ and *** $p < 0.001$ vs Vehicle, one sample t test; ## $p < 0.01$ vs hFOB-EVs, paired t-test). (D) Trypan blue exclusion assay showing the percentage of viable and non-viable cells after treatment (N=3; $p < 0.05$ and $p < 0.01$ vs Vehicle; # $p < 0.05$ vs hFOB-EVs; paired t-test). (E) Representative images of cells populations assessed by flow cytometry analysis after Annexin V and PI staining. (F) Percentage of apoptotic cells assessed by flow cytometry analysis of Annexin V positive cells (N=3; * $p < 0.05$ and *** $p < 0.0001$ vs Vehicle, # $p < 0.05$ vs hFOB-EVs; paired t-test). (G) Representative images of EdU assay (scale bar = 50 μ m). (H) Percentage of cells proliferation/total cells assessed by EdU assay (N=3; * $p < 0.05$ vs Vehicle, # $p < 0.05$ vs hFOB-EVs; paired t-test).

3.3 hFOB-EVs loaded with DXR impair MNNG/HOS cells tumour phenotype.

Following the results of MTX, we moved to test DXR loading in hFOB-EVs by employing the passive loading protocol; however, MTT assay revealed that free DXR employed at a concentration of 4 μ M, as suggested by the dose response curve (Fig.4A), significantly reduced metabolic activity while treatment with hFOB-EVs+DXR was ineffective (Fig.4B). Therefore, we attempted another approach usually employed to load EVs, that is electroporation; however, also in this case hFOB-EVs+DXR failed to reduce MNNG/HOS metabolic activity, compared to both vehicle and unloaded OB-EVs (Fig.4C).

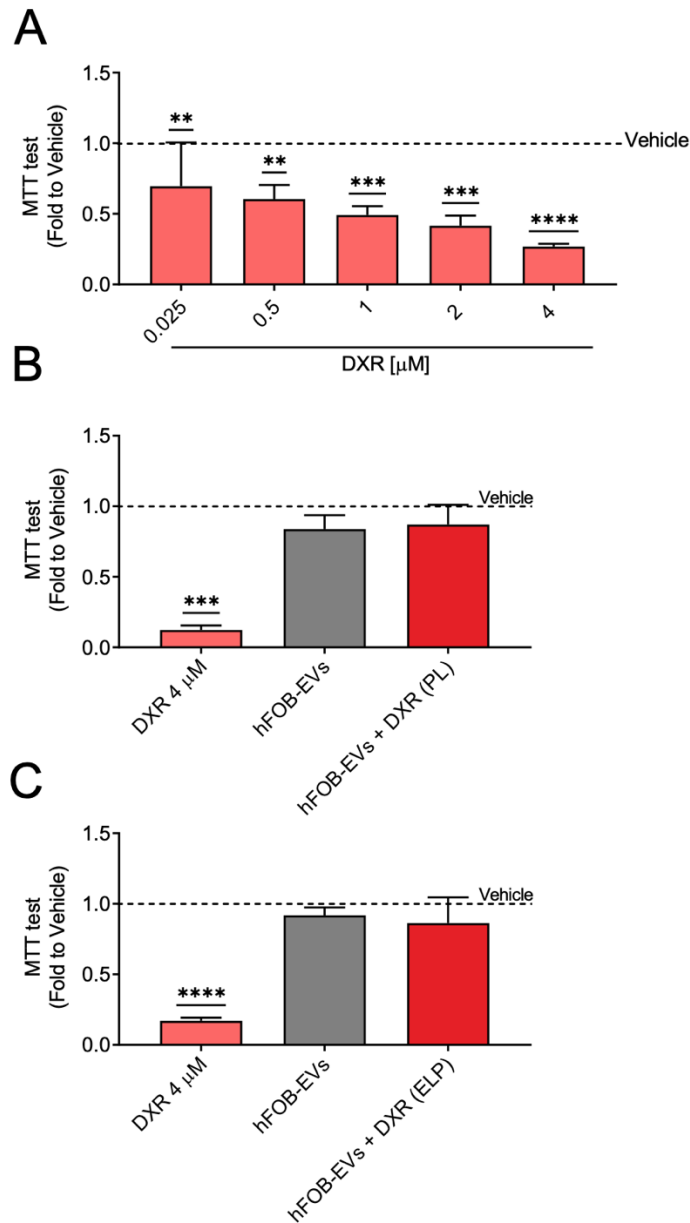


Figure 4: Effect of free Doxorubicin on MNNG/HOS cells and setting up of drug loading into hFOB-EVs. (A) MNNG/HOS cells were starved in serum-free DMEM overnight, then they were treated different concentration (indicated in the abscissa) of free DXR. Cells metabolic activity was assessed by MTT assay after 48 hours of treatment (N=3; **p<0.01, ***p<0.001, ****p<0.0001 vs Vehicle; one sample t-test). For passive loading set up, hFOB-EVs pellet was resuspended in 0.3 mM DXR in DPBS and incubated at 37°C for 30 min. EVs were then recovered by ultracentrifugation and resuspended in DMEM serum-free. MNNG/HOS cells were starved in serum-free DMEM overnight, then treated with 4 μ M free-DXR, hFOB-EVs or hFOB-EVs loaded with DXR [FOB-EVs+DXR (PL)]. (B) Cells metabolic activity was assessed by MTT assay (N=3; ***p<0.001 vs Vehicle; one sample t- test). For electroporation set up, hFOB-EVs pellet was resuspended in 0.3 mM DXR in DPBS and incubated at 4°C for 30 min. EVs were then diluted 1:1 (v/v) with the 400 mM sucrose in DPBS and electroporated in cuvettes at 250 V and 125 μ F. Immediately after the pulse, EVs were incubated for 30 min at 37 °C, washed twice in DPBS by ultracentrifugation and resuspended in serum-free DMEM. MNNG/HOS cells were starved in serum-free DMEM overnight, then treated with 4 μ M free-DXR, hFOB-EVs or hFOB-EVs loaded with DXR [FOB-EVs+DXR (ELP)]. (C) Cells metabolic activity was assessed by MTT assay (N=3; ****p<0.0001 vs Vehicle; one sample t- test).

The last and successful approach tested for DXR encapsulation was the indirect loading method, in which hFOB cells were treated with a sub-toxic concentration of DXR. Briefly, a dose–response curve was first generated by treating hFOB cells with increasing DXR concentrations (1, 2, 4, 8, 16, 32, and 64 μ M). MTT assay performed after 24 hours of treatment indicated that 8 μ M represented the most suitable concentration, as it resulted in approximately 20–30% reduction in cell viability (data not shown).

Next, hFOB cells were exposed to DXR (8 μ M) for 24 hours, after which the drug-containing medium was replaced with fresh and drug-free medium to allow the cells to release DXR-enriched EVs. Following this recovery period, CM were collected and EVs were isolated. Notably, HPLC analysis confirmed the successful incorporation of DXR into the vesicles, revealing measurable amounts of drug corresponding to 20.4 μ g/mL of encapsulated DXR (Fig. 5A). Treatment of MNNG/HOS cells with hFOB-EVs+DXR resulted in a significant reduction of metabolic activity compared to vehicle (Fig. 5B). This inhibitory effect was comparable to that produced by free DXR (4 μ M), as confirmed by MTT assay and by cell counting following DAPI staining (Fig. 5C). Also in this case, the number of live cells was significantly reduced by hFOB-EVs-DXR when compared to the vehicle, while a trend to reduction was observed with hFOB-EVs (Fig. 5D). Furthermore, flow-cytometric analysis revealed a significant increase in Annexin V⁺ cells upon treatment with hFOB-EVs+DXR, consistent with enhanced apoptosis when compared with both vehicle and hFOB-EVs (Fig. 5E, F). The magnitude of apoptotic induction was like what we observed following treatment with free DXR. Lastly, the percentage of EdU-positive nuclei markedly decreased after exposure to hFOB-EVs+DXR when compared with both vehicle and hFOB-EVs, indicating a strong antiproliferative effect (Fig. 5G,H).

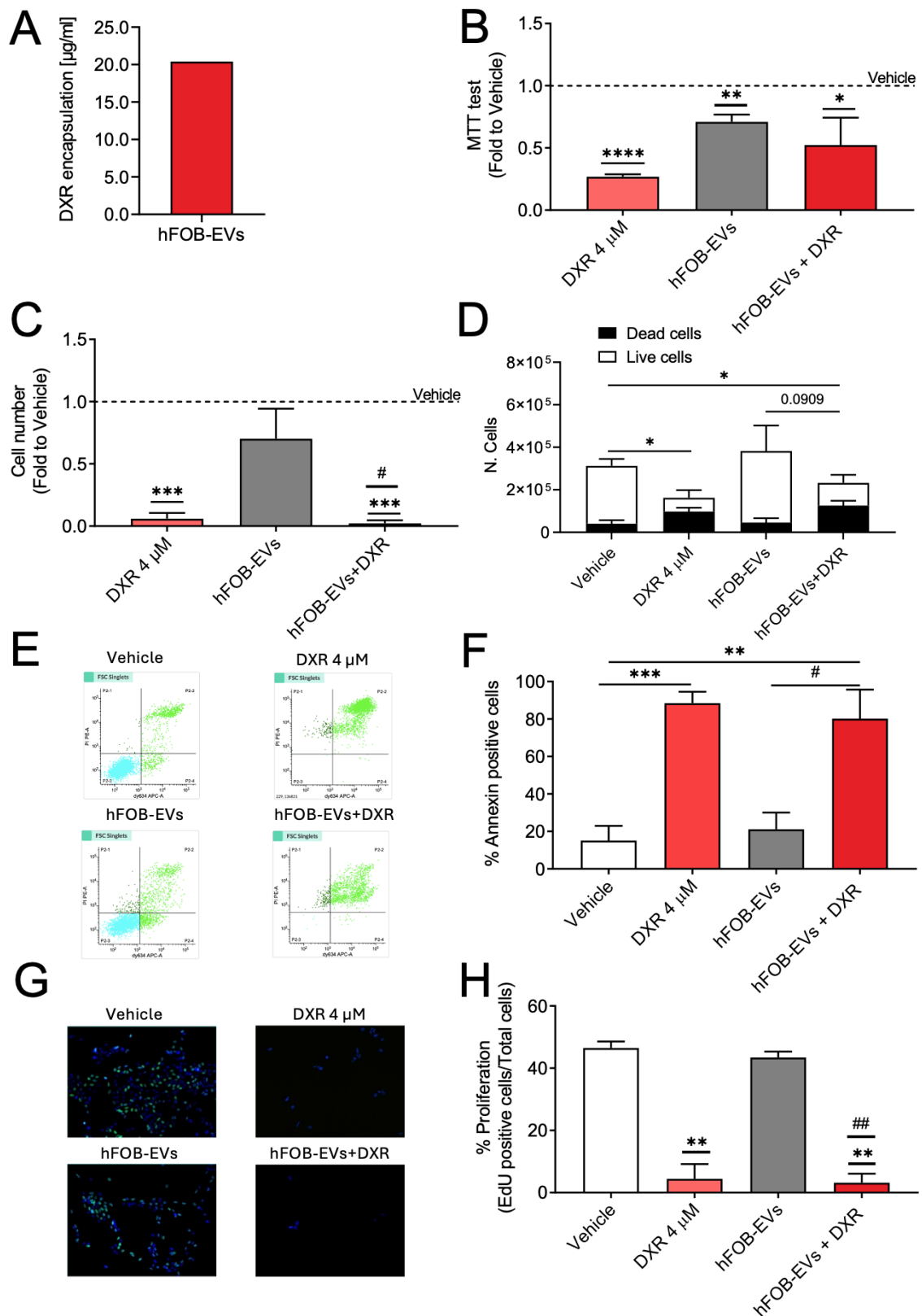


Figure 5: Effect of hFOB-EVs loaded with Doxorubicin (DXR) on MNNG/HOS cells phenotype. hFOB cells were treated with 8 μM DXR for 24h, then they were starved in serum-free DMEM for 24h and EVs were isolated from their CM by ultracentrifugation. Recovered hFOB-EVs were resuspended in (A) acidified isopropyl alcohol (90% isopropanol; 0.075M HCl) to quantify DXR by High-Performance Liquid Chromatography (HPLC) or (B-G) in DMEM serum-free for MNNG/HOS treatment. MNNG/HOS cells were starved in serum-free DMEM overnight, then treated with 4 μM free-DXR, hFOB-EVs or hFOB-EVs loaded

with DXR (FOB-EVs+DXR). (B) Cells metabolic activity was assessed by MTT assay (N=4; * $p<0.05$, ** $p<0.01$ and **** $p<0.0001$ vs Vehicle; one sample t- test). (C) Cell number evaluated by DAPI nuclear staining (N=3; *** $p<0.001$ vs Vehicle, one sample t-test; # $p<0.05$ vs hFOB-EVs, paired t-test). (D) Trypan blue exclusion assay showing the percentage of viable and non-viable cells after treatment (N=3; $p<0.05$, vs Vehicle; paired t-test). (E) Representative images of cells populations assessed by flow cytometry analysis after Annexin V and PI staining. (F) Percentage of apoptotic cells assessed by flow cytometry analysis of Annexin V positive cells (N=3; ** $p<0.01$ and *** $p<0.001$ vs Vehicle, # $p<0.05$ vs hFOB-EVs; paired t-test). (G) Representative images of EdU assay (scale bar = 50 μm). (H) Percentage of cells proliferation/total cells assessed by EdU assay (N=3; ** $p<0.01$ vs Vehicle, ## $p<0.01$ vs hFOB-EVs; paired t-test).

3.4 hFOB-EVs loaded with CPT impair MNNG/HOS cells tumour phenotype.

Following the optimization of MTX and DXR loading, we finally tested cisplatin (CPT) loading into hFOB-EVs. Both passive and electroporation-based loading protocols yielded negligible drug effect on MNNG/HOS cells vitality (data not shown). Conversely, indirect loading proved effective.

Briefly, we first treated MNNG/HOS cells with different concentration of CPT, to choose the best dose of free CPT to be used as positive control for the downstream functional analyses, which resulted in 100 μM (Fig.6).

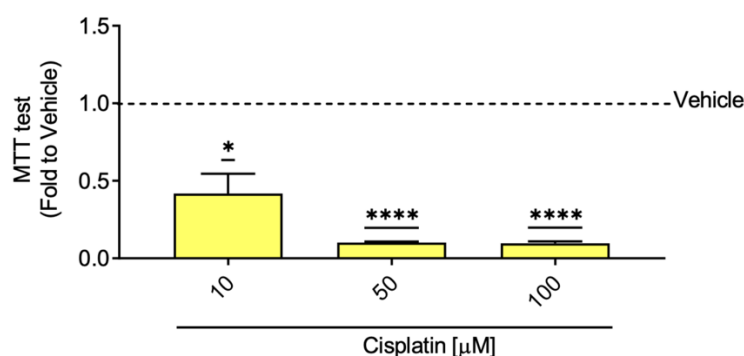


Figure 6: Effect of free CPT on MNNG/HOS cells. MNNG/HOS cells were starved in serum-free DMEM overnight, then they were treated different concentration (indicated in the abscissa) of free CPT. Cells metabolic activity was assessed by MTT assay after 48 hours of treatment (N=3; * $p<0.05$, **** $p<0.0001$ vs Vehicle; one sample t-test).

Next, a dose–response curve was generated by treating hFOB cells with increasing CPT concentrations (50, 100, 125, 250, 500 μM ; 1, 2 and 3 mM). MTT assay performed after 24 hours of treatment indicated that 100 μM represented the most suitable concentration, as resulted in a sub-optimal reduction in cell viability of approximately 20–30% (data not shown).

hFOB cells were therefore pre-treated with CPT (100 μM) for 24 hours, following the same strategy adopted for DXR loading. After treatment, the CPT-containing medium was replaced with fresh, drug-free medium to allow the cells to recover and release CPT-enriched EVs. Conditioned media were then collected, and EVs were isolated. Under

these conditions, a CPT concentration of 2.9 $\mu\text{g/mL}$ was detected in the EV preparation, as quantified by TMB colorimetric assay (Fig. 7A). Treatment of MNNG/HOS cells with hFOB-EVs+CPT decreased metabolic activity compared with vehicle ($p=0.06$, Fig. 7B). This inhibitory effect was comparable to that produced by free, as confirmed by cell counting following DAPI staining, with a significant reduction when compared with both vehicle and hFOB-EVs (Fig. 7C). Of note, the number of live cells was also significantly reduced by hFOB-EVs-CPT when compared with both vehicle and hFOB-EVs, although this time free CPT showed higher efficacy (Fig. 7D). Consistent with these results, Annexin V/PI staining revealed a pronounced apoptotic response following treatment with hFOB-EVs+CPT, once again comparable to that induced by free CPT (Fig. 7E,F). Moreover, EdU assay showed a marked decrease in proliferation by approximately 50% compared to both vehicle and hFOB-EVs (Fig. 7G,H).

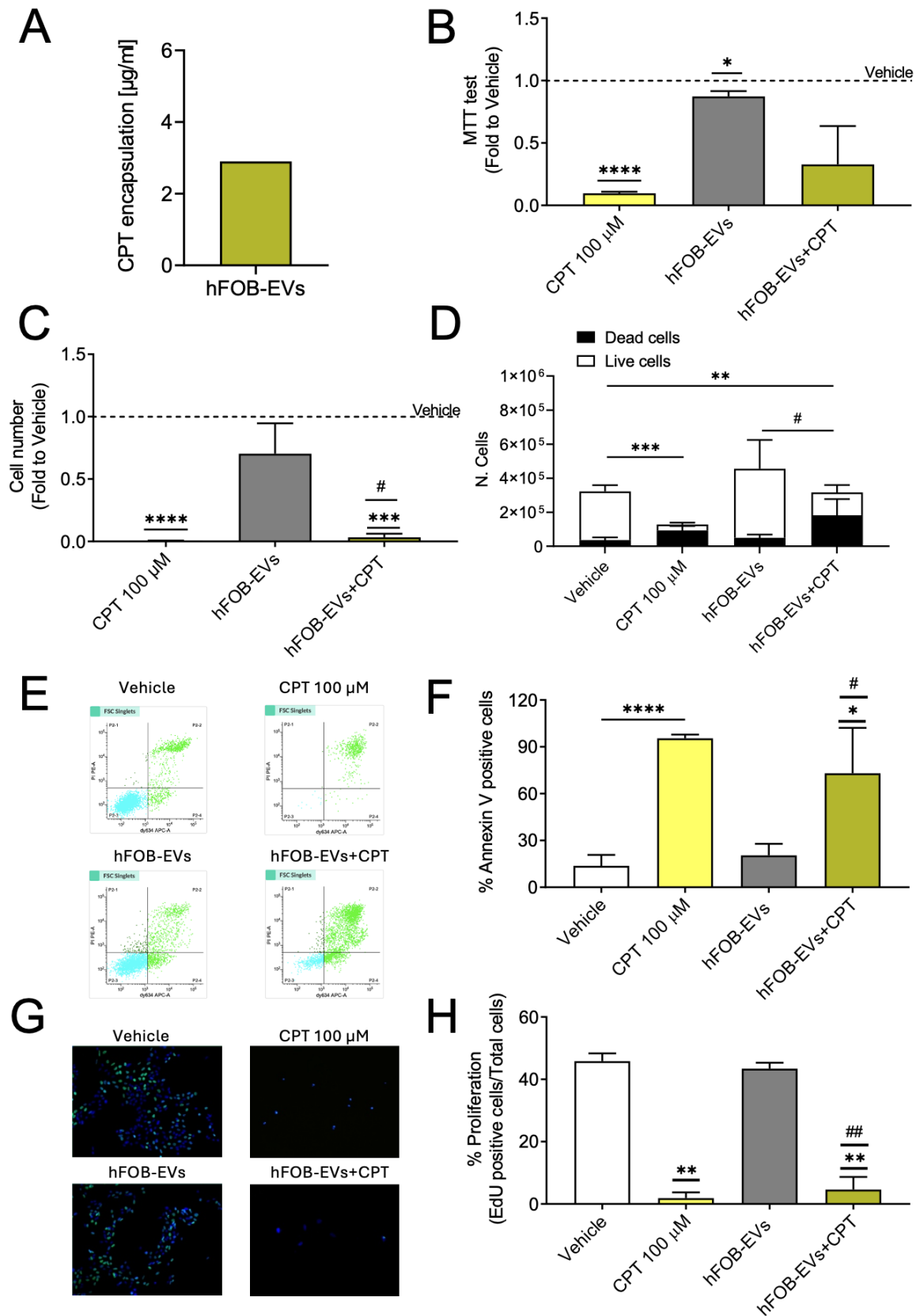


Figure 7: Effect of hFOB-EVs loaded with Cisplatin (CPT) on MNNG/HOS cells phenotype. hFOB cells were treated with 100 μM CPT for 24h, then they were starved in serum-free DMEM for 24h and EVs were isolated from their CM by ultracentrifugation. Recovered hFOB-EVs were resuspended in (A) in DPBS containing 0.1% Triton X-100 to quantify CPT by 3,3',5,5'-tetramethylbenzidine (TMB) based colorimetric assay or (B-G) in DMEM serum-free for MNNG/HOS treatment. MNNG/HOS cells were starved in serum-free DMEM overnight, then treated with 100 μM free-CPT, hFOB-EVs or hFOB-EVs loaded with CPT (FOB-EVs+CPT). (B)

Cells metabolic activity was assessed by MTT assay (N=4; * $p<0.05$ and **** $p<0.0001$ vs Vehicle; one sample t- test). (C) Cell number evaluated by DAPI nuclear staining (N=3; *** $p<0.001$ and **** $p<0.0001$ vs Vehicle, one sample t-test; # $p<0.05$ vs hFOB-EVs, paired t-test). (D) Trypan blue exclusion assay showing the percentage of viable and non-viable cells after treatment (N=3; ** $p<0.01$ and *** $p<0.001$ vs Vehicle; # $p<0.05$ vs hFOB-EVs, paired t-test). (E) Representative images of cells populations assessed by flow cytometry analysis after Annexin V and PI staining. (F) Percentage of apoptotic cells assessed by flow cytometry analysis of Annexin V positive cells (N=3; $p<0.05$ and **** $p<0.0001$ vs Vehicle, # $p<0.05$ vs hFOB-EVs; paired t-test). (G) Representative images of EdU assay (scale bar = 50 μm). (H) Percentage of cells proliferation/total cells assessed by EdU assay (N=3; ** $p<0.01$ vs Vehicle, ## $p<0.01$ vs hFOB-EVs; paired t-test).

3.5. Drug-loaded EVs do not affect hFOB cells behaviour

Having demonstrated the ability of drug-loaded-hFOB-EVs to counteract tumour phenotype, with an effect comparable to that of the free drug, we next tested the response of a normal cell line, that is hFOB donor cells, to this treatment. As shown in figure 8, at the highest concentration employed for MTX (Fig. 8A) , DXR (Fig. 8B) and CPT (Fig. 8C) there is a significant decrease of cell metabolic activity, but this is not the case for drugs loaded-hFOB-EVs, thus suggesting that these EVs-loaded chemotherapeutics while efficacious in impairing tumour cells phenotype, are harmless against normal cells.

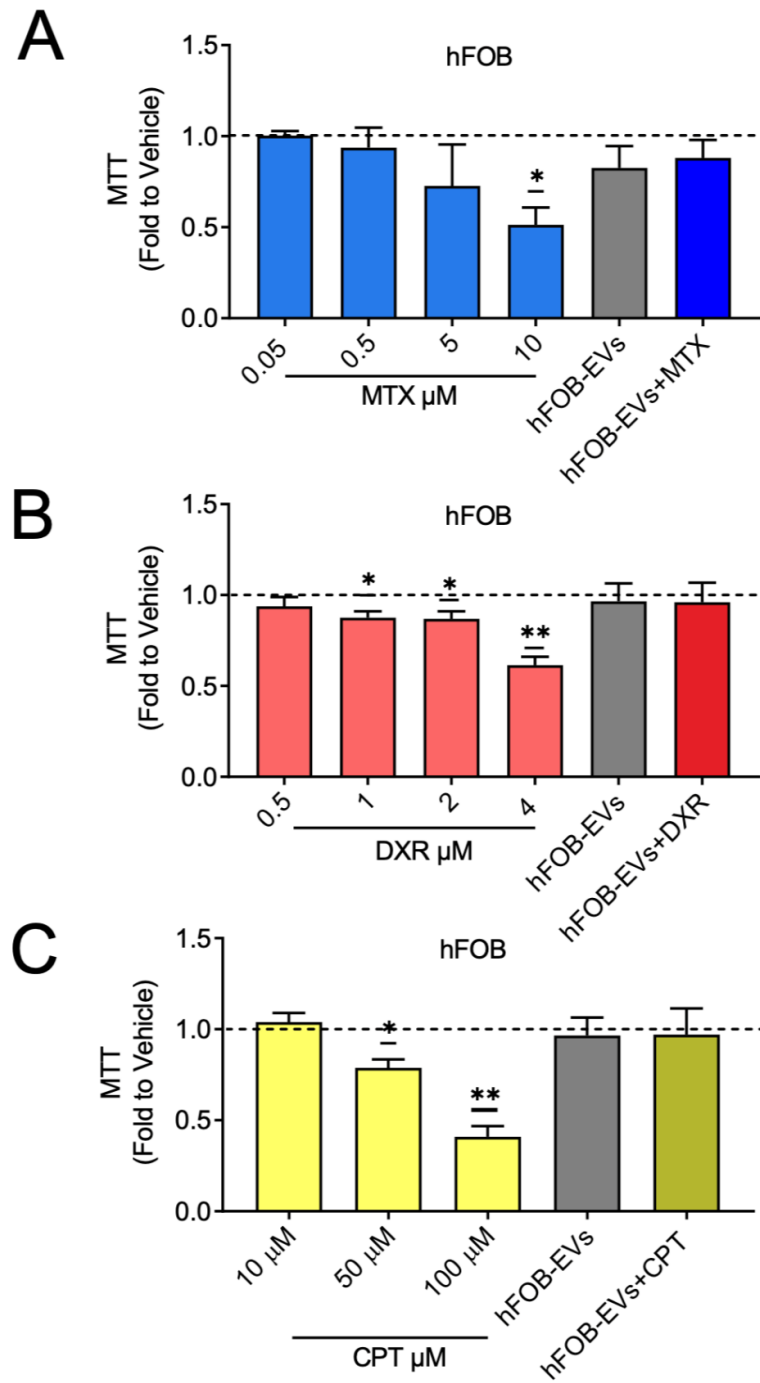


Figure 8: Effect of drug-loaded-hFOB-EVs on hFOB cells. hFOB cells were starved in serum-free DMEM overnight, then they were treated with hFOB-EVs loaded with (A) MTX, (B) DXR or (C) CPT, with hFOB-EVs, with different concentration (indicated in the abscissa) of free drugs and with vehicle (dotted line). Cells metabolic activity was assessed by MTT assay after 48 hours of treatment (N=3; *p<0.05 and **p<0.01 vs vehicle; one sample t-test).

3.6 In vivo antitumour effect of MTX-loaded hFOB-EVs

To determine whether the antitumour effects observed in vitro translated into a relevant biological response in vivo, we employed a paratibial xenograft model using MNNG/HOS-Luc⁺ osteosarcoma cells injected into Balb-c nu/nu mice. Treatments with vehicle, unloaded hFOB-EVs, hFOB-EVs+MTX, or free MTX (5 mg/kg) were administered twice a week, starting the day after tumour cell implantation and continuing until day 18 (Fig. 9A). Across all treated groups, mice receiving vehicle, unloaded or drug-loaded EVs showed normal behaviour, preserved motility and stable body weight throughout the study. Of note, body weight was significantly lower only in mice treated with free MTX compared to unloaded-EVs, with no significant differences between mice treated with either vehicle or MTX-loaded hFOB-EVs (Fig. 9B).

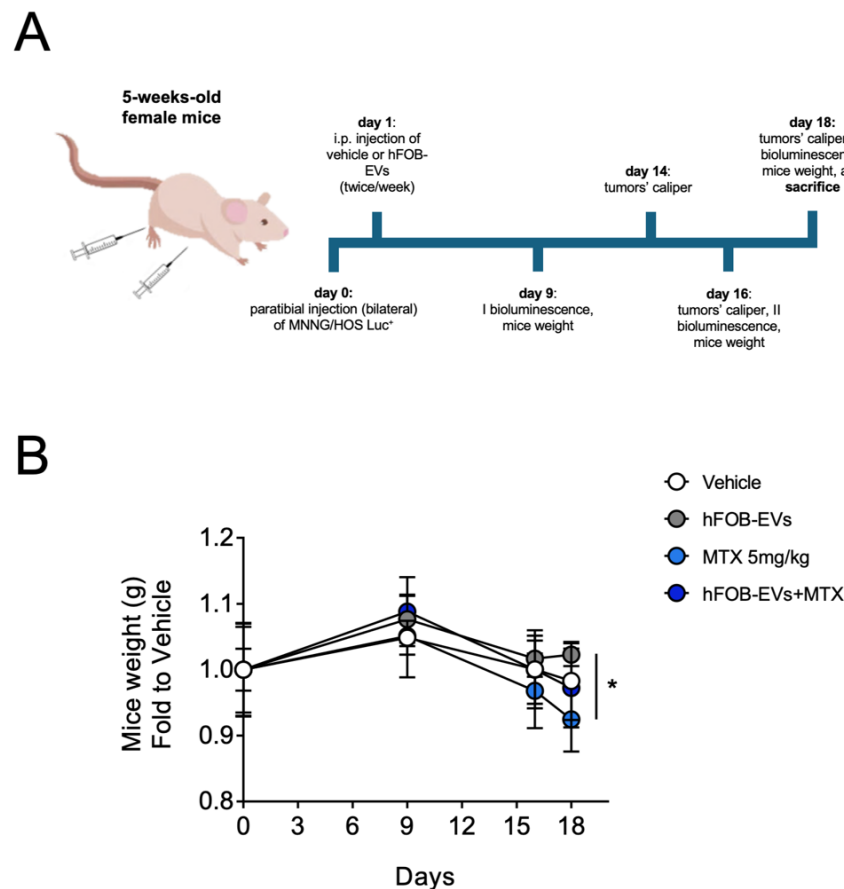


Figure 9. In vivo experimental design and mice weight monitoring. (A) Schematic representation of the in vivo experimental plan. Five-week-old female Balb/c nu/nu mice were injected paratibially (bilaterally) with 1×10^6 MNNG/HOS-Luc⁺ cells (day 0). From day 1, mice were treated twice per week with vehicle (DPBS), unloaded hFOB-EVs, free drug (MTX 5 mg/kg) or drug-loaded EVs (hFOB-EVs+MTX). Tumour growth was assessed by bioluminescence on days 9, 16 and 18 and by caliper measurement on days 14, 16 and 18. Mice body weight was assessed with a precision balance on days 0 (before MNNG/HOS Luc⁺ cells injection), 9, 16 and 18. (B) Effect of free MTX and MTX-loaded EVs on body weight compared with their respective controls (vehicle and hFOB-EVs) (N = 5 for vehicle and unloaded hFOB-EVs, N = 10 for MTX 5mg/kg and hFOB-EVs + MTX; *p<0.05, vs hFOB-EVs; nonlinear regression).

Looking at bioluminescence, treatment with hFOB-EVs+MTX significantly reduced tumour photon emission at day 16 compared to vehicle and even outperformed free MTX

(Fig. 10A,B). Importantly, free MTX itself did not show significant tumour reduction compared with vehicle. Consistently, bioluminescence analysis conducted at day 18 revealed a significant decrease of tumour photon emission in mice treated with hFOB-EVs+MTX compared to unloaded-EVs, once again demonstrating higher efficacy than free MTX. Moving to tumour calipers, hFOB-EVs+MTX did not significantly reduced tumour volume compared to vehicle, unloaded EVs, or free MTX which is in sharp contrast with what we observed by bioluminescence (Fig. 10C).

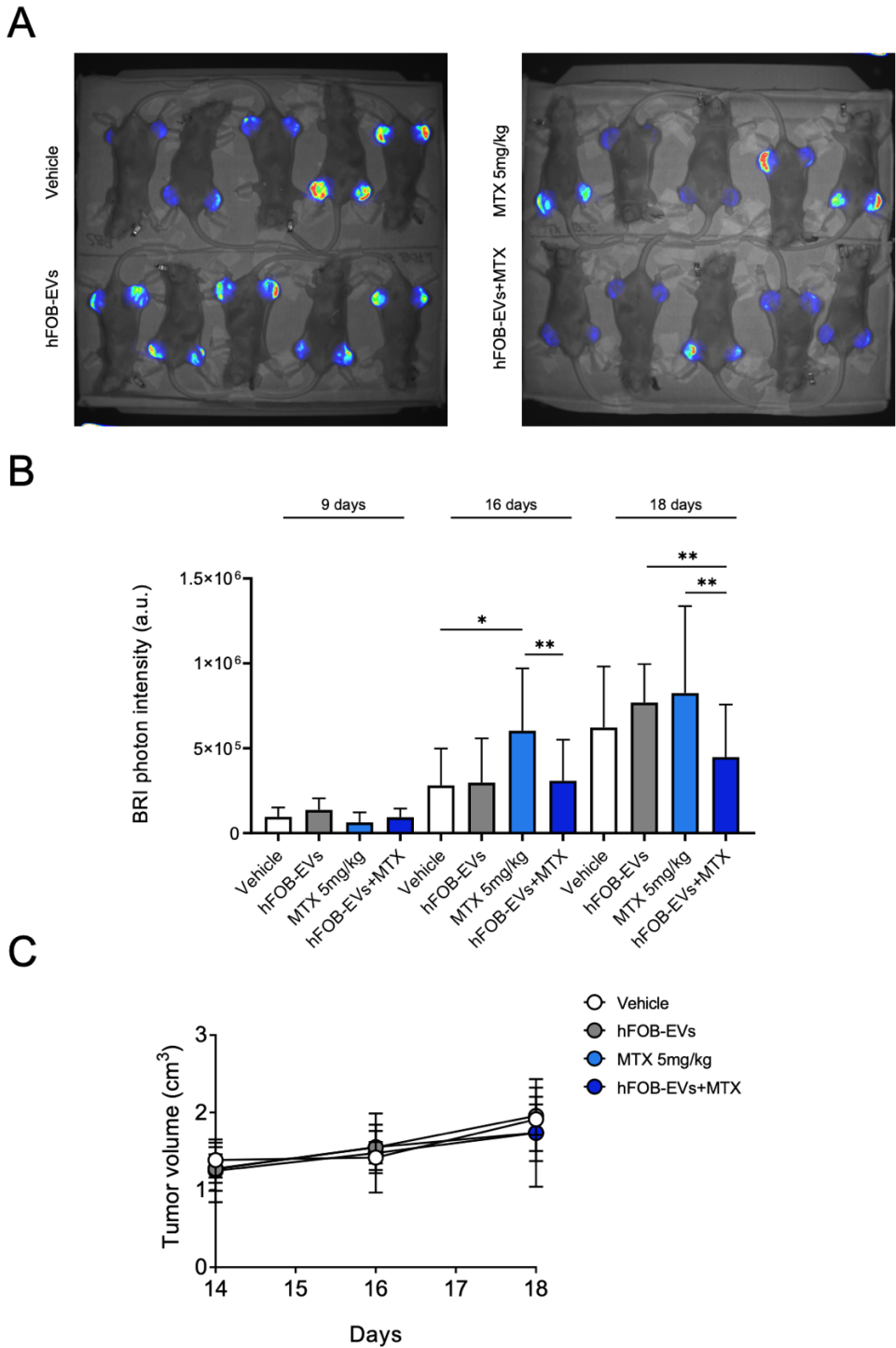


Figure 10. Bioluminescence imaging of tumour growth following treatment. Mice were weekly subjected to deep anaesthesia (intraperitoneal injection of 80 mg/kg ketamine and 10 mg/kg xylazine) followed by

bioluminescence imaging (intraperitoneal injection of 40 mg/kg D-luciferin) to monitor osteosarcoma onset and progression. (A) Representative bioluminescence images acquired 18 days post-injection of mice treated with vehicle, unloaded-hFOB-EVs, MTX 5mg/kg or hFOB-EVs+MTX. (B) Bioluminescence (BRI) photon intensity (a.u.) of mice hindlimbs at 9, 16 and 18 days post injection, analysed through Photonics-Wasabi software (Hamamatsu) (N = 5 for vehicle and unloaded hFOB-EVs, N = 10 for MTX 5mg/kg and hFOB-EV+MTX; * $p < 0.05$, ** $p < 0.01$; unpaired t-test). (C) Tumour growth was monitored by caliper measurements performed on days 14, 16 and 18 post- (N = 5 for vehicle and unloaded hFOB-EVs, N = 10 for MTX 5mg/kg and hFOB-EVs + MTX; nonlinear regression).

Bone histomorphometry analysis by microCT provided additional evidence of therapeutic benefit. As expected, paratibial MNNG/HOS lesions produced a mixed osteolytic/osteogenic phenotype, which was markedly counteracted by hFOB-EVs+MTX (Fig. 11A). Treatment with these loaded EVs significantly decreased the percentage of ectopic bone over total bone volume compared to unloaded EVs, with an effect comparable to free MTX (Fig. 11B). Most importantly, hFOB-EVs+MTX was also able to reduce osteolytic area when compared with all treatment groups, whereas free MTX failed to exert this effect (Fig. 11C).

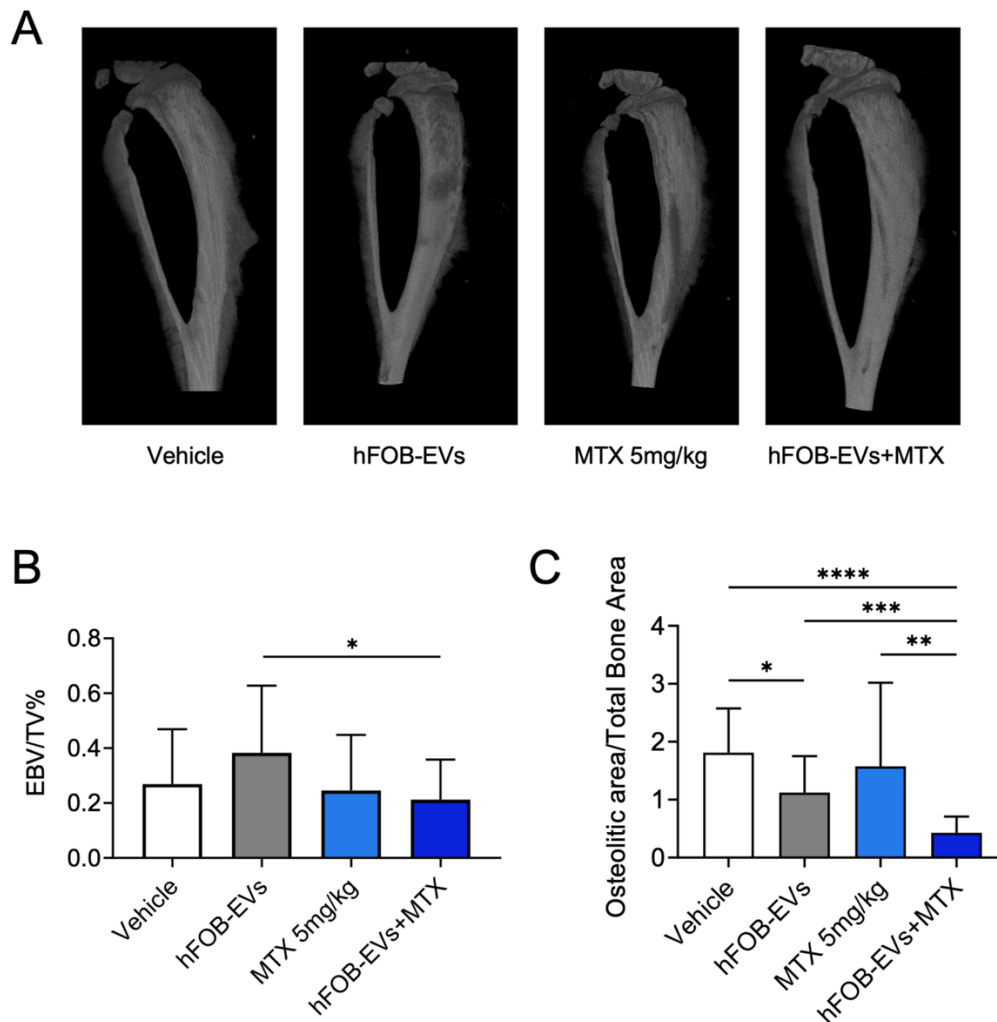


Figure 11: Effect of hFOB-EVs loaded with MTX on bone physiology. (A) Representative microCT images of tibiae from mice treated with vehicle, unloaded hFOB-EVs, free MTX (5 mg/kg), or MTX-loaded hFOB-EVs (hFOB-EVs+MTX) (B) Percentage of Ectopic Bone Volume (EBV) over Tissue Volume (TV) assessed by MicroCT analysis of tibiae (N = 5 for vehicle and unloaded hFOB-EVs, N = 10 for MTX 5mg/kg and hFOB-EV+MTX; * $p < 0.05$; unpaired t-test). (C) Percentage of Osteolytic Area over Total Bone Area assessed by ImageJ analysis of tibiae (N = 5 for vehicle and unloaded hFOB-EVs, N = 10 for MTX 5mg/kg and hFOB-EV+MTX; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$; unpaired t-test).

4. Discussion

In this work we demonstrate that extracellular vesicles released by the human fetal osteoblast cell line hFOB are effective and safe nanocarriers for three conventional osteosarcoma chemotherapeutics, that is methotrexate, doxorubicin and cisplatin, both in vitro and in vivo. By systematically comparing different loading strategies, we identified passive diffusion as the most suitable approach for MTX, whereas indirect loading of donor cells was necessary to obtain functionally active DXR- and CPT-loaded EVs. Drug-loaded hFOB-EVs reduced proliferation and viability of MNNG/HOS osteosarcoma cells and increased apoptosis to an extent comparable to, or greater than, free drugs, while sparing normal osteoblasts. In an orthotopic xenograft model, EV-mediated delivery of MTX inhibited tumour growth with drug doses that were orders of magnitude lower than conventional regimens, and without detectable systemic toxicity.

Our findings are consistent with a growing body of evidence supporting EVs as attractive drug-delivery systems in oncology. Several studies have shown that EVs can be loaded with chemotherapeutic drugs such as DXR, paclitaxel, cisplatin or MTX and can enhance antitumour activity while reducing off-target effects compared with free drugs [20,21]. For instance, MSC-, tumour- and milk-derived EVs loaded with DXR through passive incubation, electroporation or sonication have been reported to increase intracellular accumulation of the drug and to potentiate cytotoxicity in breast, lung and colon cancer models [20,22,23,24]. Similarly, EV-based formulations carrying MTX or CPT have been explored in models of leukaemia and solid tumours, achieving improved therapeutic effects [25,26,27,28]. However, most of these studies employed EVs derived from mesenchymal stem cells or tumour cells and focused on non-bone malignancies. In osteosarcoma, only a few reports have investigated EV-mediated delivery of chemotherapeutics, mainly using MSC-derived exosomes or hybrid exosome–liposome systems to transport DXR [29,30]. To our knowledge, our study is the first to exploit osteoblast-derived EVs as carriers for standard osteosarcoma drugs and to demonstrate their efficacy in an orthotopic bone tumour model.

The choice of osteoblast-derived EVs is biologically relevant. Osteoblasts are key cellular players within the bone microenvironment, and their EVs participate in the complex crosstalk with osteoclasts, endothelial cells and tumour cells [12,31,32]. Previous work from our group showed that osteoblast EVs can modulate osteosarcoma cell metabolism, redox homeostasis and aggressiveness, and display an inherent tropism for bone tissue [12]. These characteristics may confer a natural targeting ability that is particularly advantageous for osteosarcoma, a tumour arising within bone and frequently associated with local bone destruction and aberrant osteogenesis. In line with this concept, hFOB-EVs in our study were readily internalised by MNNG/HOS cells and, when loaded with drugs, were able to inhibit tumour growth and bone pathology in vivo.

A major novelty of our work is the comparative analysis of loading techniques for three clinically used drugs. Numerous reviews have summarised the available strategies for exogenous loading of EVs, simple incubation, electroporation, sonication, extrusion, freeze–thaw cycles, saponin permeabilization and others, highlighting that loading efficiency and EV integrity strongly depend on the physicochemical properties of both cargo and vesicles [33, 34]. Yet, only a limited number of studies systematically compare different methods using the same EV source and drug. For DXR, several groups have obtained efficient loading using electroporation or sonication of MSC- or tumour-derived EVs, reporting improved drug entrapment and enhanced cytotoxicity compared with passive incubation [20,22,35]. In contrast, our data show that both passive loading and

electroporation were ineffective in generating DXR-loaded hFOB-EVs with functional antitumour activity, despite employing conditions similar to those previously described. This discrepancy suggests that optimal parameters for electroporation and other active methods may be highly EV-source specific, and protocols developed for one vesicle type cannot be directly translated to another.

By adopting an indirect loading approach, preconditioning donor osteoblasts with sub-toxic concentrations of DXR or CPT, we successfully obtained EVs containing therapeutically relevant amounts of both drugs. Indirect loading strategies have been increasingly explored as an alternative to physical methods, exploiting the natural pathways of EV biogenesis and cargo sorting [36]. Tumour- or MSC-derived EVs have been shown to incorporate chemotherapeutics, tyrosine kinase inhibitors, or small RNAs after exposure of donor cells to these agents, and to deliver them to recipient cells with preserved vesicle integrity [37,38]. In our system, 24-hour exposure of hFOB cells to DXR or CPT followed by a drug-free period allowed the secretion of EVs carrying 20.4 µg/mL of DXR and 2.9 µg/mL of CPT, respectively, which were sufficient to significantly reduce MNNG/HOS viability and proliferation. These results reinforce the idea that indirect loading may represent a more gentle and efficient strategy for certain combinations of EV source and cargo, especially when physical methods compromise membrane integrity or cause drug precipitation.

Conversely, MTX could be successfully loaded into hFOB-EVs by passive incubation, achieving detectable levels (0.7 µg/mL) and robust functional activity. Passive diffusion across the EV membrane has been described for small hydrophobic or weakly amphipathic molecules [39,40]. Several reports have loaded MTX into different nanocarriers, including synthetic nanoparticles and EVs, showing that even modest loading efficiencies can translate into significant therapeutic effects owing to improved pharmacokinetics and tumour accumulation [7,25,41]. Our data fit within this framework and highlight that drug chemistry, such as MTX amphiphilicity and ionisation state at the incubation pH, may favour spontaneous partitioning into the EV lipid bilayer, unlike the more hydrophilic and positively charged DXR and CPT, which required biosynthetic incorporation.

An important aspect of our study is the favourable safety profile of drug-loaded hFOB-EVs. While MTX, DXR and CPT at the highest doses tested markedly reduced viability of donor hFOB cells, the corresponding drug-loaded EVs did not impair osteoblast metabolic activity, indicating that EV-mediated delivery may preferentially target tumour cells while limiting toxicity on normal bone-forming cells. Similar observations have been reported for MSC- or plant-derived EVs carrying DXR or other chemotherapeutics, where EV formulations decreased cardiac, hepatic or haematological toxicity compared with free drugs [42, 43]. Consistently, in vivo body weight monitoring revealed a modest but significant reduction only in mice treated with free MTX when compared to unloaded EVs, whereas no significant differences were observed between all the other treatment groups. Although weight loss did not significantly differ between free MTX and MTX-loaded EVs, the selective reduction observed in the free MTX group relative to unloaded EVs may be due to early systemic toxicity associated with conventional drug administration. In contrast, the preservation of body weight in mice treated with MTX-loaded hFOB-EVs supports the notion that EV-mediated delivery improves tolerability, potentially by modulating drug biodistribution and limiting off-target exposure.

Moreover, the in vivo efficacy of MTX-loaded hFOB-EVs is even higher remarkable when considering that EV-delivered MTX achieved a more pronounced reduction in tumour

bioluminescence than free MTX, despite being administered at a substantially lower dose. Interestingly, based on HPLC analysis we estimated that the MTX dose delivered via hFOB-EVs was approximately 0.03 µg, which is substantially lower than the 80 µg of free MTX (5 mg/kg). However, this reduction in bioluminescent signal was not accompanied by a significant decrease in tumour volume as assessed by caliper measurements over the same time frame. This apparent discrepancy likely reflects the different nature of the two readouts. While bioluminescence imaging provides a sensitive and dynamic measure of tumour cell viability and metabolic activity, tumour volume measurements also account for non-viable components, including necrotic tissue, extracellular matrix deposition and inflammatory infiltrates. In fast-growing osteosarcoma models, treatment-induced tumour cell death can therefore reduce bioluminescent output without immediately translating into macroscopic tumour shrinkage, particularly at early time points. Ongoing histological analyses will be essential to further dissect the cellular and structural composition of the tumour mass and to clarify the relative contribution of viable tumour cells, necrosis and stromal components to the observed volumetric measurements.

Nanocarrier-based strategies for osteosarcoma therapy, including polymeric nanoparticles, liposomes, hydrogels and mesoporous silica systems, typically aim at more moderate dose reductions or improved local retention [6,14,18,44,45,46]. Our results suggest that osteoblast-derived EVs may provide particularly efficient drug delivery to the bone tumour niche, perhaps because of their endogenous homing properties and ability to cross biological barriers. Moreover, micro-CT analysis showed that hFOB-EVs+MTX not only reduced ectopic bone formation but also significantly decreased osteolytic lesions, an effect not observed with the corresponding free drug. This dual impact on tumour burden and bone remodelling is highly relevant for osteosarcoma, where pathological bone turnover contributes to pain, fracture risk and metastatic dissemination [14,47].

Despite these strengths, several limitations of our study should be acknowledged. First, we used a single osteosarcoma cell line, MNNG/HOS, which, although widely employed, may not fully capture the heterogeneity of human disease. Future studies should validate our findings in additional osteosarcoma models, including patient-derived xenografts and metastasis-prone cell lines. Second, we did not perform detailed pharmacokinetic or biodistribution analyses to directly demonstrate preferential accumulation of hFOB-EVs in bone lesions versus other organs. Labelling approaches combined with in vivo imaging, as used in other EV-based delivery studies [48,49], will be important to confirm the targeting properties of osteoblast-derived EVs and to refine dosing schedules. Third, the mechanisms underlying the selective cytotoxicity towards tumour cells remain to be elucidated. It is conceivable that tumour-specific uptake pathways, differences in intracellular trafficking, or interactions between drug cargo and endogenous EV components contribute to the observed effects. Transcriptomic and proteomic profiling of drug-loaded versus unloaded hFOB-EVs, as well as of treated tumour cells, may provide insight into these mechanisms.

Finally, from a translational perspective, the use of fetal osteoblasts raises practical questions about scalability and clinical applicability. While hFOB cells are convenient for proof-of-concept experiments, future developments will likely require EVs derived from clinically compatible sources, such as primary osteoblasts, induced pluripotent stem

cell-derived osteogenic cells, or bioengineered nanovesicles mimicking osteoblast EV composition [14,17,50,51].

5. Conclusions

In conclusion, this study provides experimental evidence that osteoblast-derived EVs are promising nanocarriers for conventional osteosarcoma chemotherapeutics. By directly comparing passive, electroporation and indirect loading approaches, we show that loading strategy must be tailored to each drug and EV source, and that indirect loading of donor osteoblasts is particularly suitable for DXR and CPT, whereas MTX can be efficiently incorporated by passive incubation. Drug-loaded hFOB-EVs exert potent antitumour effects against osteosarcoma cells in vitro and in vivo (limited to MTX-loaded hFOB-EVs), while sparing normal osteoblasts and markedly reducing systemic toxicity and required drug doses. These results, together with previous reports on EV-mediated drug delivery in cancer and osteosarcoma [20–22,27–31], support further exploration of bone-derived EVs as a platform to repurpose existing chemotherapeutics and to design combination therapies that integrate cytotoxic drugs with biological agents or nucleic acids therapies.

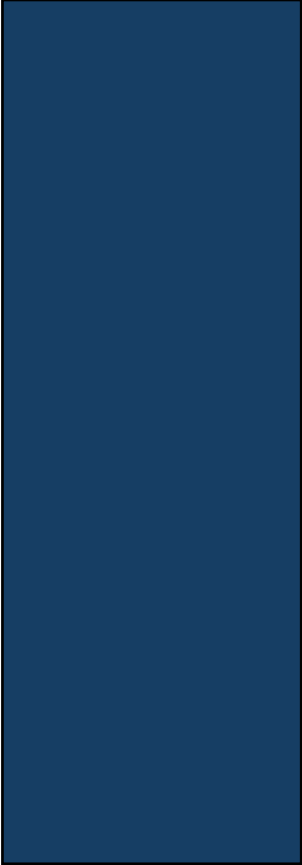
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Chapter 4

“Effect of Osteoblast-Derived Extracellular Vesicles on Osteosarcoma Cells Transcriptional Profile: Role of Shuttled miRNAs”

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Abstract

Background/Objectives: Osteosarcoma is the most common primary malignant bone tumour, affecting children and young adults. Recent evidence suggests that extracellular vesicles (EVs), small membrane-bound nanoparticles released by all cell types, play a key role in intercellular communication within the tumour microenvironment. Therefore, we aimed to characterise the transcriptional and miRNA-mediated mechanisms underlying these effects. **Methods:** Phenotypic assays were performed to assess metabolic activity, proliferation, apoptosis and invasion of human osteosarcoma cell lines after treatment with osteoblast-derived EVs (OB-EVs). Illumina-based RNAseq was conducted on RNA isolated from OB-EVs-treated cells, and qRT-PCR using commercially available TaqMan miRNA cards on RNA isolated from OB-EVs. **Results:** In Saos-2 and U2OS cells, OB-EVs reduced metabolic activity and differentially affected proliferation, apoptosis and invasive capacity. MNNG/HOS cells treated with OB-EVs for 48 hours showed substantial transcriptomic changes, with 296 differentially expressed genes (97 up- and 199 down-regulated in OB-EVs treated cells versus untreated cells), indicating a direct impact of OB-EVs on gene expression. Intriguingly, bioinformatic analyses (GO, Reactome, KEGG, Panther) revealed a consistent suppression of the Wnt/ β -Catenin and NOTCH signalling pathways. Conversely, miRNA profiling of OB-EVs identified 13 highly expressed miRNA, some of which were predicted to be involved in the modulation of differentially expressed genes in MNNG/HOS cells. Fluorescence microscopy confirmed the efficient transfer of EV-RNA into recipient osteosarcoma cells. Finally, integration of transcriptomic and miRNA target prediction data indicated that OB-EVs may regulate tumour-associated pathways. **Conclusions:** Taken together, these findings suggest that OB-EVs modulate osteosarcoma cell phenotype, with miRNA shuttling being a potentially important mechanism.

1. Introduction

Among primary bone tumours, osteosarcoma is the most prevalent (20-40%), affecting mainly children and adolescents, with a median age of 15 years old at diagnosis [1] and a worldwide incidence of 2-3/million/year [2]. Osteosarcoma is a high-grade tumour that localises mainly in bone segments with the most extensive longitudinal growth, such as proximal tibia, distal femur and proximal humerus [1]. Osteosarcoma is typically managed in a multidisciplinary fashion, which includes surgical excision, radiotherapy and multiagent chemotherapy, underscoring the need to find better therapeutic options for patients [3]. The most dramatic consequences of this tumour are related to the high rate of recurrence and the poor prognosis of metastatic disease, with lungs being the most commonly affected sites [4]. Osteosarcoma typically arises from multipotent mesenchymal stem cells (MSCs) that have undergone malignant transformation, likely because of defective osteogenic differentiation programme [5], eventually leading to the production of defective bone with osteoid deposition.

It is well known that tumour cells interact with the host tissue to promote their local growth and dissemination, but the cross-communication of osteosarcoma cells with the bone microenvironment is currently an understudied issue. Bone is a complex micro-environment where osteosarcoma cells could liaise with different resident cells,

including osteoclasts, osteoblasts, osteocytes, stromal cells and endothelial cells [6]. Understanding the molecular mechanisms that mediate the interaction between malignant cells and host tissue could be the key to identify more efficacious therapeutic targets. To this point, we previously demonstrated a mutual crosstalk between osteosarcoma cells and osteoblasts by means of extracellular vesicles (EVs). A growing body of evidence demonstrated that EVs are biological nano-scale messengers released by nearly all cell types, which are recognised as an effective communication tool for tumour cells, being involved in tumour development, growth and invasion [7]. EVs shuttle a wide range of biologically active macromolecules, such as proteins, DNA, mRNAs and non-coding RNA, including miRNAs, lncRNAs, circRNAs, tRNAs [8]. Indeed, we found that human MNNG/HOS osteosarcoma-derived EVs are able to reprogramme osteoblast homeostasis, by reducing the expression of cell cycle and pro-osteoblastogenic genes, whilst increasing transcriptional expression and protein release of pro-osteoclastogenic/inflammatory cytokines [9]. Conversely, underscoring the potential bi-directionality of the crosstalk between osteosarcoma and the microenvironment through EVs, we also demonstrated the ability of osteoblast-derived EVs (OB-EVs) to influence MNNG/HOS cells phenotype. Specifically, we found OB-EVs to decrease MNNG/HOS metabolic activity, viability, motility and in vitro invasion, all very important mechanisms in malignancy [10].

In this work, we confirmed the anti-tumoral effect of OB-EVs on Saos-2 and U2OS osteosarcoma cells, then we venture to find whether some of the observed phenotypical changes could be explained by changes in gene expression profile and study the role of miRNAs in the phenomenon. This was achieved through in-depth transcriptomic analyses on OB-EVs-treated osteosarcoma cells, and miRNA profiling in OB-EVs.

2. Materials and Methods

2.1 Materials

Dulbecco's-modified minimum essential medium (DMEM), Dulbecco's Phosphate-Buffered Saline (DPBS), penicillin, streptomycin, and trypsin were from Euroclone (Milan, Italy). Fetal bovine serum (FBS) was from Thermo Fisher Scientific (Waltham, MA, USA). Sterile plasticware was purchased from Falcon Becton–Dickinson (Cowley, Oxford, UK) and Euroclone. Ultracentrifuge tubes were from Beckman Coulter (Brea, CA, USA). The Trizol reagent, primers, and reagents for qRT-PCR were from Invitrogen (Carlsbad, CA, USA). The SensiFAST SYBR no-ROX kit was from Meridian Bioscience (Cincinnati, Ohio, USA). The RNAeasy kit was from Qiagen (Heidelberg, Germany), while the Illumina-based RNAseq was performed by BMR Genomics (Padua, Italy). The SYTOR-NASelect Green Fluorescent Cell Stain and the TaqMan Advanced miRNA Human Serum/Plasma Cards were purchased from Thermo Fisher Scientific. All other reagents were from Sigma-Aldrich (St. Louis, MO, USA).

2.2 Animals

Procedures involving animals were designed and performed in conformity with national and international laws and policies (European Economic Community Council Directive 86/609, OJ L 358, 1, December 12, 1987; Italian Legislative Decree 4.03.2014 n.26, Gazzetta Ufficiale della Repubblica Italiana no. 61, March 4, 2014; National Institutes of Health guide for the Care and Use of Laboratory Animals, National Institutes of Health

Publication no. 85-23, 1985) and the Animal Research: Reporting of in Vivo Experiments (ARRIVE) guidelines. Animal procedures received approval by the Italian Ministry of Health authority (Approval no. 173/2016-PR).

2.3 Cell lines

The human osteosarcoma cell lines MNNG/HOS, Saos-2 and U2OS and the human fetal osteoblastic cell line hFOB1.19 were purchased by the European Collection of Authenticated Cell Cultures (ECACC, Salisbury, UK). Cells were cultured at 37°C, 5% CO₂ in DMEM plus 10% FBS, 100 IU/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine.

2.4 Osteoblast primary cultures

Calvariae from 7-day-old CD1 mice were collected and subjected to three sequential enzymatic digestions with 1 mg/ml Clostridium histolyticum type IV collagenase and 0.25% trypsin, at 37°C for 15, 30 and 45 min, respectively, under agitation. The supernatant from the first digestion was discarded, while those arising from the subsequent digestions were centrifuged at 300 g for 7 min, and cell pellets were resuspended in DMEM supplemented with 10% FBS and cultured. At confluence, cells were detached, pooled together and plated according to the experimental needs.

2.5 Extracellular vesicles isolation

Extracellular vesicles (EVs) from mouse primary osteoblasts (OB) conditioned medium (CM) were isolated according to Ucci et al and Ponzetti et al. [9,10]. At 80% of confluence, OB were washed in DPBS and starved in serum-free DMEM for 24 hours. OB-CM was then subjected to two sequential centrifugations, the first at 300 g, 4°C for 5 min to remove dead cells, and the second at 5000 g, 4°C for 25 min to remove membrane debris. The latter supernatant was collected and ultracentrifuged at 100,000 g, at 9°C for 70 min. The pellet, containing osteoblast-derived EVs (OB-EVs), was resuspended in DPBS. For quantification, OB-EVs were subjected to nanoparticle tracking analysis and protein extraction, the latter giving a yield of 4.9 ± 1.3 µg/12 ml CM.

2.6 MTT assay

Metabolic activity of Saos-2 and U2OS cells was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) bromide reduction assay. Both cell types were plated onto 96-well plates at a density of 9,000 cells/cm², starved overnight (O/N) in serum-free DMEM and then treated with OB-EVs isolated from one 175 cm² flask (12 ml medium) or with DMEM (control). After 48h of treatment, MTT dissolved in DPBS at 5 mg/ml concentration was added at 1:6 (v/v) ratio directly into the cell supernatant. Three hours later, medium was removed and DMSO was added to dissolve the precipitated formazan salts arising from the reaction. Plates were shaken at 160 rpm on an orbital shaker for 10 min, then absorbance at 595 nm was recorded.

2.7 EdU (5-ethynyl-2'-deoxyuridine) cell proliferation assay

Saos-2 and U2OS cells were plated onto 96-well plates at a density of 9,000 cells/cm² and, upon reaching 80% confluence, were starved O/N in serum-free DMEM and treated with OB-EVs isolated from one 175 cm² flask (12 ml medium) or with DMEM (control). After 48 hours, cell proliferation assay was performed by using the EdU HTS Kit 488. Briefly, Saos-2 and U2OS cells were incubated for 5 hours with 5 µM EdU (5-ethynyl-2'-

deoxyuridine) in DMEM, washed in DPBS and fixed in 4% paraformaldehyde (PFA) for 10 min. Then, cells were permeabilised with 0.5% Triton X-100 in PBS, incubated with the click assay cocktail for 30 min at room temperature, protected from light, and then washed twice in 1X rinse solution. Nuclei were counterstained with DAPI (4',6-Diamidino-2-phenylindole dihydrochloride). Cell proliferation was assessed under an epifluorescence microscope and plotted as the percentage of proliferating cells (EdU-positive cells).

2.8 Apoptosis

Annexin Red assay was performed on Saos-2 and U2OS cells treated with OB-EVs, by using the Guava® Annexin Red Kit (#FCCH100108- Luminex). Cells were plated in a cell culture dish (100 mm Ø) at a density of 8000/cm², starved O/N in serum-free DMEM and then treated with OB-EVs (isolated from 13 ml of CM collected from one 175-cm² flask), DMEM (control), or 2.5 µM staurosporine (positive control). After 48h, CM was collected, cells were detached and centrifuged together. Pellet was resuspended in a working solution with recombinant Annexin V conjugated to a red sensitive dye CF®647 for 15 minutes at 37 °C in incubator. Samples were then analysed with cyto-fluorimeter Luminex Easycyte equipped with 642nm laser.

2.9. RNAseq analysis of MNNG/HOS cells

MNNG/HOS were cultured to 75% confluence in 9 cm Petri dishes and starved O/N. Cells were then treated with OB-EVs isolated from one 175 cm² flask (12 ml medium), while untreated cells were used as control (N=3). After 48h of treatment, RNA was isolated using the RNAeasy kit (Qiagen, Heidelberg, Germany), checked for integrity by agarose gel and purity by nanodrop, before being sent for further analyses and Illumina-based RNAseq to BMR Genomics (Padova, Italy). After QC and alignment vs the reference transcriptome (Homo Sapiens), the raw counts were normalised to obtain transcripts per million (TPM). The analysis yielded 227818 transcripts and those with TPM<10 in at least 2 over 3 samples in either one or both the experimental groups were excluded, leaving 11125 transcripts for the bioinformatic analyses.

2.10 SYTORNA staining of osteoblast-derived extracellular vesicles

Isolated OB-EVs were incubated for 20 min at 37°C with the SYTORNASelect Green Fluorescent Cell Stain, then the EVs were washed in PBS, followed by ultracentrifugation at 100,000 g, at 4°C for 70 min. Finally, the OB-EV pellets were resuspended in DMEM and used to treat MNNG/HOS cells previously plated in chamber slides at a density of 18,000 cells/cm². After 1, 6 and 18 hours of treatment, cells were fixed in 4% PFA and slides were mounted with a DAPI-containing medium before microscope qualitative analysis.

2.11 miRNA profiling of osteoblast-derived extracellular vesicles

Total RNA was extracted from OB-EVs and control medium using the Plasma/Serum RNA Purification Mini Kit (Norgen Biotek, Canada) following the manufacturer's protocol. MiRNAs analysis was performed by quantitative Real-Time PCR (qRT-PCR) using TaqMan Advanced miRNA Human Serum/Plasma Cards, which allow the simultaneous assessment of the expression levels of 188 unique miRNAs. In this study, we focused on the 136 miRNAs that showed 100% sequence identity between human and mouse. The analysis was performed by comparing OB-EV preparations (N=3) with CM, to account for any background that may be arising from the medium. Samples were analysed on a ViiA7

instrument (Applied Biosystems), and data were processed by QuantStudio Software v 1.3 and Expression Suite software v 1.3 (Applied Biosystems). Synthetic ath-miR159a was spiked-in during RNA extraction and used as exogenous control for normalization of data. MicroRNAs' expression levels were evaluated by the $\Delta\Delta C_t$ method. Manual quality check on PCR amplification plots was also performed. MiRNAs showing the highest expression levels in OB-EVs compared to medium were considered for further analyses.

2.12 Bioinformatic analyses

The differentially expressed gene (DEGs), clustergram, PCA and gene ontology analyses on the RNAseq set were performed by BMR genomics using standard R packages. For gene-set enrichment analysis, the TPM values provided by BMR genomics were first subjected to ENST-gene name conversion, and transcripts with no gene ID were excluded. After calculating the average LogFC for all transcripts, data were analysed using the online gene set enrichment analysis (GSEA) tool Webgestalt (<https://www.webgestalt.org/>). The top 10 enriched/depleted terms with $p < 0.05$ were plotted based on normalised enrichment score (NES). Kaplan-Meier survival analyses were conducted using the R2 Genomics Analysis and Visualization Platform (https://hgserver1.amc.nl/cgi-bin/r2/main.cgi?option=kaplan_main) and using data from database GSE42352 (Mesenchymal osteosarcoma, N=127 <https://pubmed.ncbi.nlm.nih.gov/22454324/>). The cutoff mode adopted was “scan” in all analyses and the minimum group size was set at N=8. The median was used as the separation point between high and low risk groups.

For the miRNA target analysis, Mienturnet (<http://userver.bio.uniroma1.it/apps/mienturnet/>), TargetScan (https://www.targetscan.org/vert_80/), DIANA (<https://dianalab.e-ce.uth.gr/html/mirpathv3/index.php?r=mirpath>), and miRDB (<https://mirdb.org/>) were used to predict the miRNA targets, and the predicted targets were manually or automatically compared with the significantly downregulated targets in RNAseq to find any alignment between the two datasets. MiRNet (<https://www.mirnet.ca/miRNet/home.xhtml>) was used to predict the Reactome pathways most likely to be affected by the predicted targets of the miRNAs present in OB-EVs, which were then compared with the Reactome terms identified in the RNAseq. The overlapping pathways are the most likely to be affected by miRNAs in OB-EVs-treated MNNG/HOS and were therefore manually identified and presented.

2.13 Statistics

Numerical and statistical analyses were performed using GraphPad Prism version 9.0 (GraphPad Software, San Diego, CA, USA). Data are reported as mean $\pm$ SEM. Statistical significance between two groups was assessed using one sample t-test or paired student's t-test, as specified in the figure legends. A p value ≤ 0.05 was considered statistically significant.

3. Results

3.1 Osteoblast-derived EVs selectively impair osteosarcoma cell aggressiveness without affecting normal osteoblasts

In our previous work, we proved that osteoblast-derived EVs reduced MNNG/HOS osteosarcoma cells' aggressiveness and viability through redox-dependent signalling pathways [10]. Therefore, we investigated whether this effect was also true in two additional human osteosarcoma cell lines. We first characterised osteoblast-derived EVs (OB-EVs) for their size and concentration by nanoparticle tracking analysis (Figure 1A). Moreover, western blot analysis showed that OB-EVs protein lysates expressed the typical EVs markers, such as CD81, CD63 and Annexin II (Anxa2) (Figure 1B), while TEM analysis on isolated particles demonstrated their membrane integrity and vesicular nature (Figure 1C).

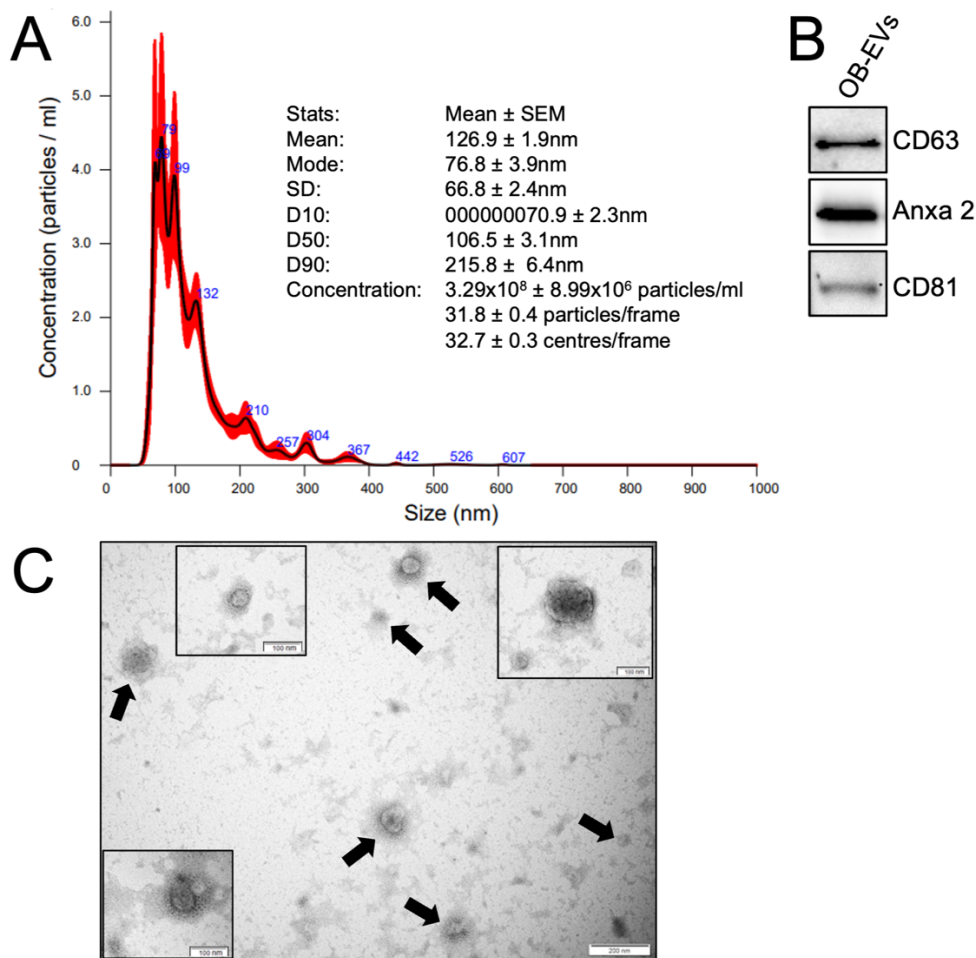


Figure 1. Characterisation of osteoblast-derived EVs. Mouse primary osteoblasts were starved in serum-free DMEM for 24 hours and EVs were isolated from conditioned medium (CM) by ultracentrifugation. (A) Size and concentration evaluation of OB-EVs (isolated from 12 ml of CM collected from one 175 cm² flask, cell density = 3.5x10⁴ cells/cm²) by nanoparticle tracking analysis (NanoSight NS300). (B) Western blot for the EV positive markers CD81, CD63, Annexin II (Anxa2) in protein lysates (8 μ g) extracted from OB-EVs. (C) Transmission Electron Microscopy (TEM) evaluation of OB-EVs (arrows), original magnification 34000x; insets: higher magnification (92000x) in different fields of the same EVs sample. Data are representative of 3 independent preparations.

Next, Saos-2 and U2OS osteosarcoma cell lines were treated with OB-EVs for 48 hours, finding a significant reduction of metabolic activity for both cells compared to untreated cells (Figure 2A,E); a slight but significant increase of proliferation was observed in Saos-2 treated cells (Figure 2B), while this parameter was dramatically impaired in U2OS-treated cells (Figure 2F). Consistent with our previous results [10], treatment of Saos-2 cells with OB-EVs did not affect cell apoptosis (Figure 2C) while it reduced their invasion ability (Figure 2D). Looking at U2OS cells, OB-EVs slightly increased apoptosis (Figure 2G), while it failed to affect invasion (Figure 2H). Taken together, these results show that treatment of different osteosarcoma cell lines with OB-EVs, although acting on different features, impairs tumour cells aggressiveness.

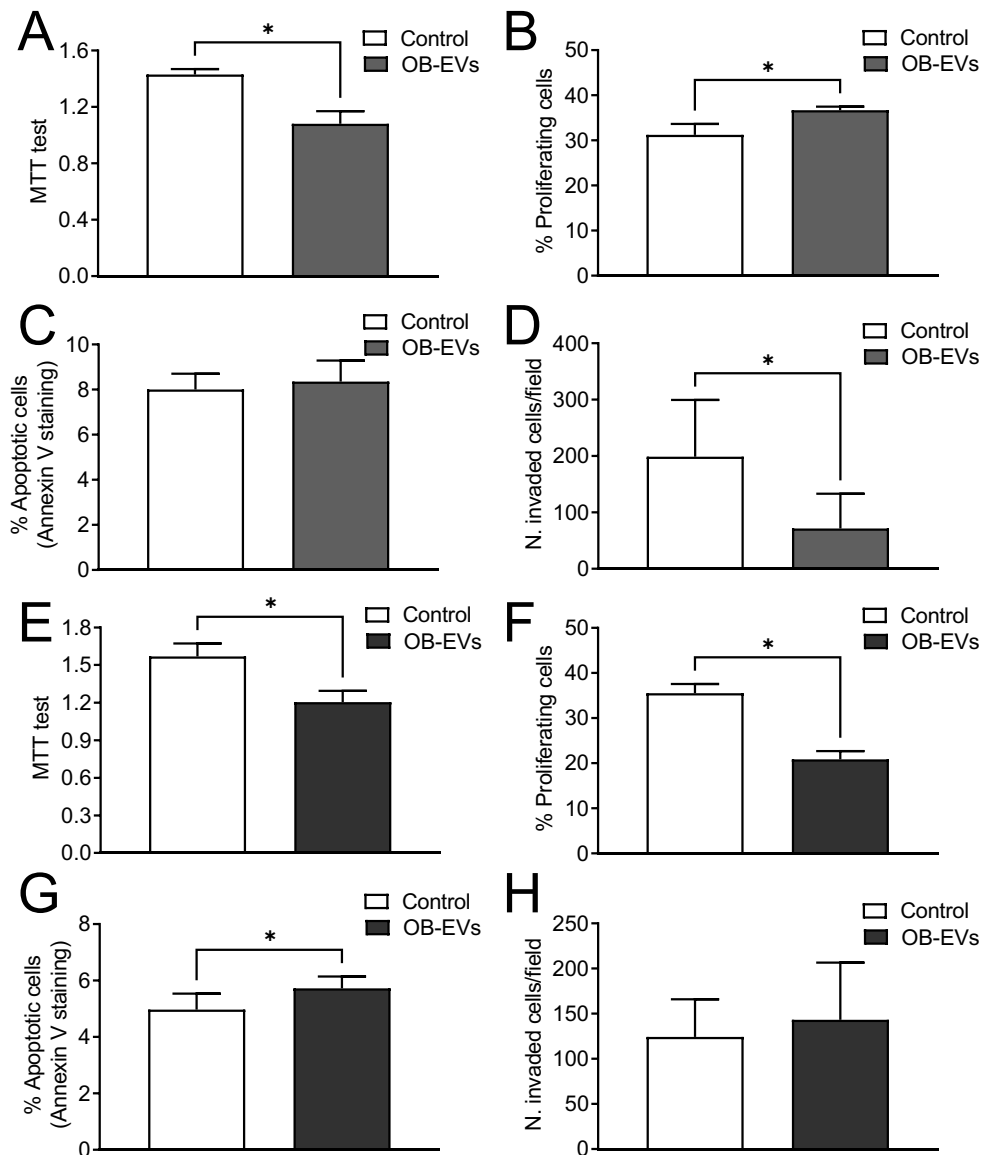


Figure 2. Effect of osteoblast-derived EVs on osteosarcoma cells phenotype. (A-D) Saos-2 and (E-H) U2OS human osteosarcoma cell lines were plated and starved O/N. The next day cells were treated with EVs isolated from mouse primary osteoblasts (OB-EVs) or left in DMEM. Evaluation of (A,E) metabolic activity by MTT assay, (B,F) proliferation by EdU assay and (C,G) apoptosis by Annexin V staining. (D) Saos2 and (H) U2OS cells were pretreated with OB-EVs for 24 hours, then they were detached, resuspended in DMEM without serum and seeded on the upper basket of a matrigel-coated transwell to perform an invasion assay over FBS. Results are the mean $\pm$ SD of three independent experiments; * $p < 0.05$, paired t-test.

Therefore, we asked whether OB-EVs could also affect normal cells behaviour, by treating the human fetal osteoblast cell line hFOB 1.19 (i.e. hFOB), finding that it was not the case, since neither metabolic activity nor proliferation or apoptosis were altered (Figure 3). These data suggest a specific regulatory effect of OB-EVs on osteosarcoma cells.

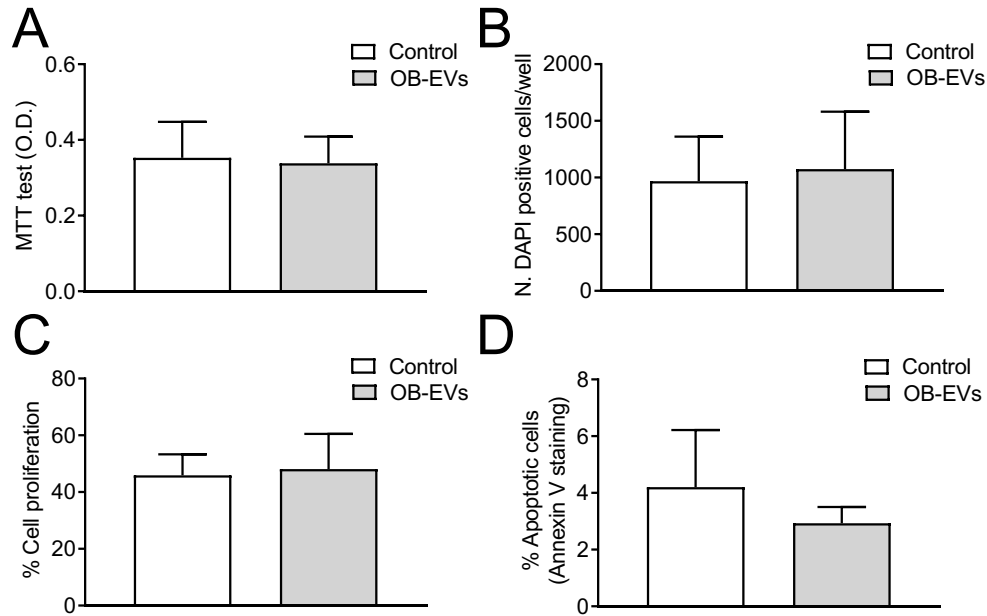


Figure 3. Effect of osteoblast-derived EVs on normal osteoblasts. Human fetal osteoblast-like cells (hFOB) were plated and starved O/N. The next day cells were treated with EVs isolated from mouse primary osteoblasts (OB-EVs) or left in DMEM. Evaluation of (A) metabolic activity by MTT assay, (B) cell number by nuclear staining with DAPI, (C) proliferation by EdU assay and (D) apoptosis by Annexin V staining. Results are the mean $\pm$ SD of three independent experiments.

3.2 Osteoblast-derived EVs modify the transcriptional profile of MNNG/HOS osteosarcoma cells

Based on this finding, we next asked whether OB-EVs could be also determinant in influencing osteosarcoma transcriptomic profile. MNNG/HOS cells were cultured in standard conditions as control or treated with OB-EVs for 48 hours, before extracting their RNA and performing RNAseq on them. Principal component analysis (PCA) of the transcriptomic profiles showed untreated and OB-EVs treated MNNG/HOS cells clustered according to their treatment, indicating that the OB-EVs exerted a similar and detectable effect on all the biological replicates (Figure 4A). Count distribution was similar among all samples (Figure 4B).

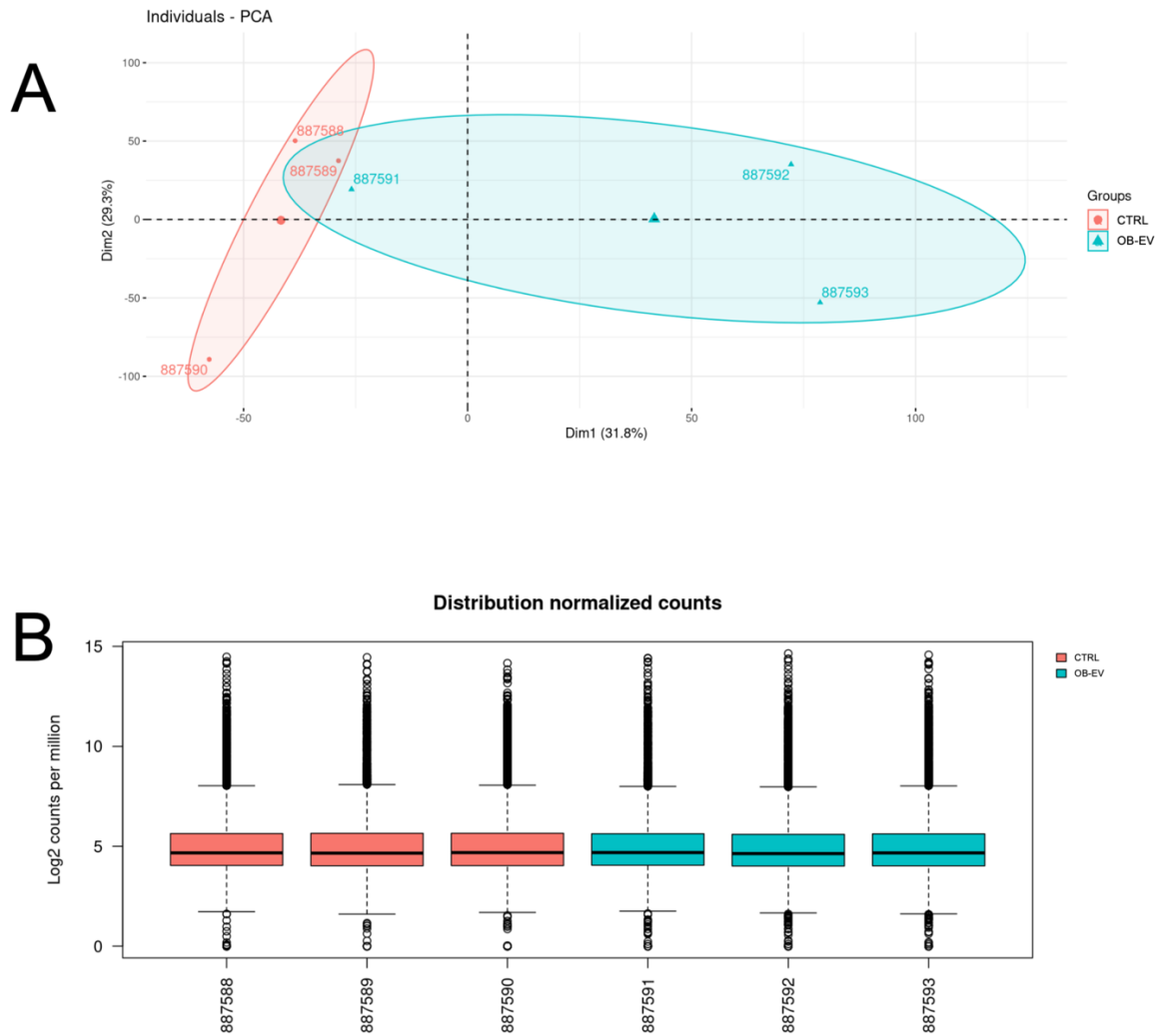


Figure 4. Principal component analysis and distribution of normalized gene expression counts in OB-EVs-treated MNG/HOS. (A) Principal Component Analysis (PCA) plot showing the separation of samples based on gene expression profiles. Samples are grouped into CTRL (red) and OB-EV-treated (blue) conditions, with 95% confidence ellipses indicating variability within each group. The first two principal components (Dim1 and Dim2) explain 31.4% and 29.3% of the total variance, respectively, highlighting distinct clustering between conditions. (B) Boxplot depicting the distribution of log2-transformed normalized gene expression counts across individual samples. Each box represents a sample, with the central line indicating the median expression level. The interquartile range (IQR) reflects sample variability, while whiskers extend to 1.5 times the IQR, with outliers shown as individual points. The consistent distribution across samples confirms effective normalization, ensuring comparability of gene expression levels between conditions.

In these three replicates-based experimental approaches, statistical analysis using $P < 0.01$ and a $\log FC > |0.5|$ as cut-off values, revealed a total of 296 differentially regulated genes (DRGs) (Figure 5A,B), of which 97 were upregulated (top 20 in Table 1), and 199 were downregulated (top 20 in Table 2).

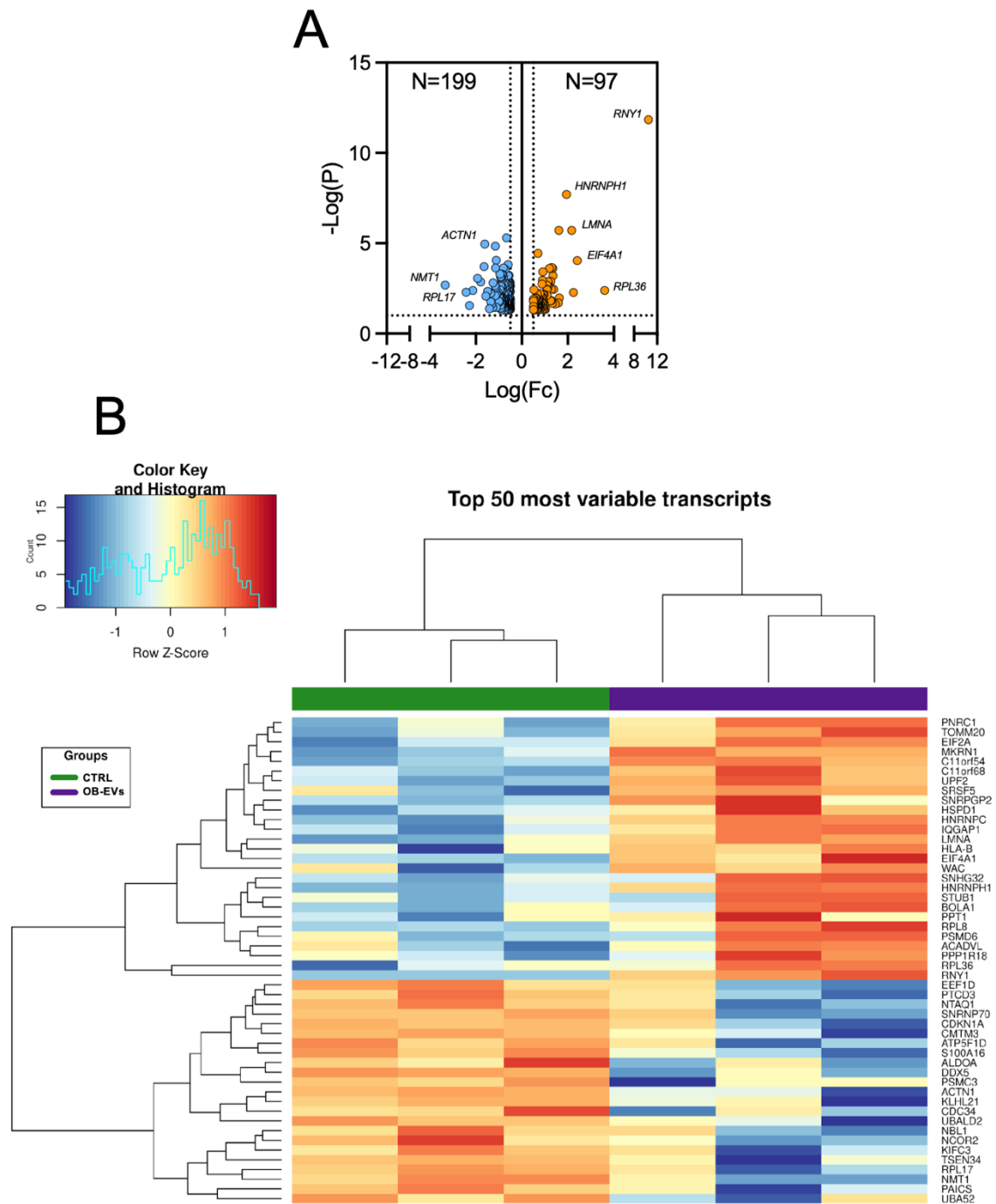


Figure 5. Differentially expressed genes (DEGs) in OB-EVs-treated versus untreated MNNG/HOS cells. (A) Volcano Plot of Differentially Expressed Genes (DEGs). The plot displays the distribution of DEGs between the two experimental conditions. The x-axis represents the Log fold change (LogFc), while the y-axis shows the $-\text{Log}(p\text{-value})$. Upregulated genes are highlighted in orange, downregulated genes in blue. The dotted lines represent the significance threshold. (B) Heatmap of the Top 50 Most Variable Transcripts. Hierarchical clustering of differentially expressed genes across samples, with expression levels represented as Z-score-normalized values (red: high ex-pression; blue: low expression).

Table 1

List of the top 20 upregulated transcripts in OB-EVs-treated MNNG/HOS vs untreated cells.

Gene symbol	EntrezGeneID	Fold Change	logFC	P Value
RNY1	6084	31622776601.68	10.50	$1.42 \cdot 10^{-12}$
RPL36	25873	3981.07	3.60	$4.14 \cdot 10^{-3}$
EIF4A1	1973	257.04	2.41	$9.00 \cdot 10^{-5}$
WAC	51322	177.83	2.25	$5.26 \cdot 10^{-3}$
LMNA	4000	147.91	2.17	$1.95 \cdot 10^{-6}$
HNRNPH1	3187	87.10	1.94	$1.97 \cdot 10^{-8}$
ACADVL	37	42.66	1.63	$1.05 \cdot 10^{-2}$
HNRNPC	3183	40.74	1.61	$1.93 \cdot 10^{-6}$
PPT1	5538	38.90	1.59	$2.02 \cdot 10^{-2}$
STUB1	10273	28.84	1.46	$2.31 \cdot 10^{-2}$
SNHG32	50854	24.55	1.39	$3.59 \cdot 10^{-3}$
MKRN1	23608	22.91	1.36	$6.21 \cdot 10^{-4}$
C11orf68	83638	22.39	1.35	$2.46 \cdot 10^{-4}$
PSMD6	9861	21.38	1.33	$2.12 \cdot 10^{-2}$
EIF2A	83939	19.95	1.30	$2.19 \cdot 10^{-4}$
BOLA1	51027	19.50	1.29	$1.48 \cdot 10^{-2}$
HLA-B	3106	19.50	1.29	$3.49 \cdot 10^{-3}$
UPF2	26019	18.20	1.26	$1.31 \cdot 10^{-3}$
PPP1R18	107987457	17.78	1.25	$1.56 \cdot 10^{-2}$
RPL8	6132	15.85	1.20	$2.45 \cdot 10^{-4}$

OB-EVs=Osteoblast-derived Extracellular Vesicles.

Table 2

List of the top 20 downregulated transcripts in OB-EVs-treated MNNG/HOS versus untreated cells.

Gene symbol.	EntrezGeneID	Fold change	logFC	P Value
NMT1	4836	0.00047	-3.33	$2.07 \cdot 10^{-3}$
RPL17	6139	0.00380	-2.42	$5.17 \cdot 10^{-3}$
UBA52	7311	0.00525	-2.28	$2.85 \cdot 10^{-2}$
PAICS	10606	0.00741	-2.13	$4.15 \cdot 10^{-3}$
UBALD2	283991	0.01202	-1.92	$8.77 \cdot 10^{-4}$
NCOR2	9612	0.01622	-1.79	$1.39 \cdot 10^{-3}$
TSEN34	79042	0.02291	-1.64	$1.99 \cdot 10^{-4}$
ACTN1	87	0.02455	-1.61	$1.12 \cdot 10^{-5}$
EEF1D	1936	0.02754	-1.56	$8.44 \cdot 10^{-3}$
CDC34	997	0.03311	-1.48	$4.61 \cdot 10^{-3}$
SNRNP70	6625	0.03890	-1.41	$4.23 \cdot 10^{-2}$
PSMC3	5702	0.04467	-1.35	$1.71 \cdot 10^{-2}$
PTCD3	55037	0.04467	-1.35	$5.95 \cdot 10^{-3}$
CDKN1A	1026	0.04898	-1.31	$2.31 \cdot 10^{-2}$
KIFC3	3801	0.05495	-1.26	$3.59 \cdot 10^{-2}$
KLHL21	9903	0.05623	-1.25	$1.56 \cdot 10^{-3}$
NTAQ1	55093	0.06918	-1.16	$2.27 \cdot 10^{-3}$
S100A16	140576	0.07079	-1.15	$1.43 \cdot 10^{-5}$
ALDOA	226	0.07079	-1.15	$7.90 \cdot 10^{-3}$
CMTM3	123920	0.07244	-1.14	$2.36 \cdot 10^{-4}$

OB-EVs=Osteoblast-derived Extracellular Vesicles.

We first performed gene ontology (GO) analyses on the regulated genes that met the cut-off values and the quality criteria. GO molecular functions showed an enrichment in transcripts related to cadherin binding, ubiquitin protein (and ubiquitin-like protein) ligase binding, as well as structural constituents of the ribosome (Figure 6A). This suggests that there might be an effect on ubiquitination, cell-cell adhesion and protein translation operated by OB-EVs. Significantly enriched GO biological processes included, among others, mRNA catabolic and ribose phosphate biosynthetic processes, and protein targeting to the mitochondria (Figure 6B). The findings arising from GO-molecular function and GO-biological processes are somewhat corroborated by GO-cellular component analyses, showing enrichment in transcripts related to focal adhesions, mitochondrial inner membrane, ribosomes, lamellipodia, and catalytic step 2 spliceosome (Figure 6C). Taken together, these results show that OB-EVs may regulate many important processes in osteosarcoma, including transcription, translation, metabolism, and motility.

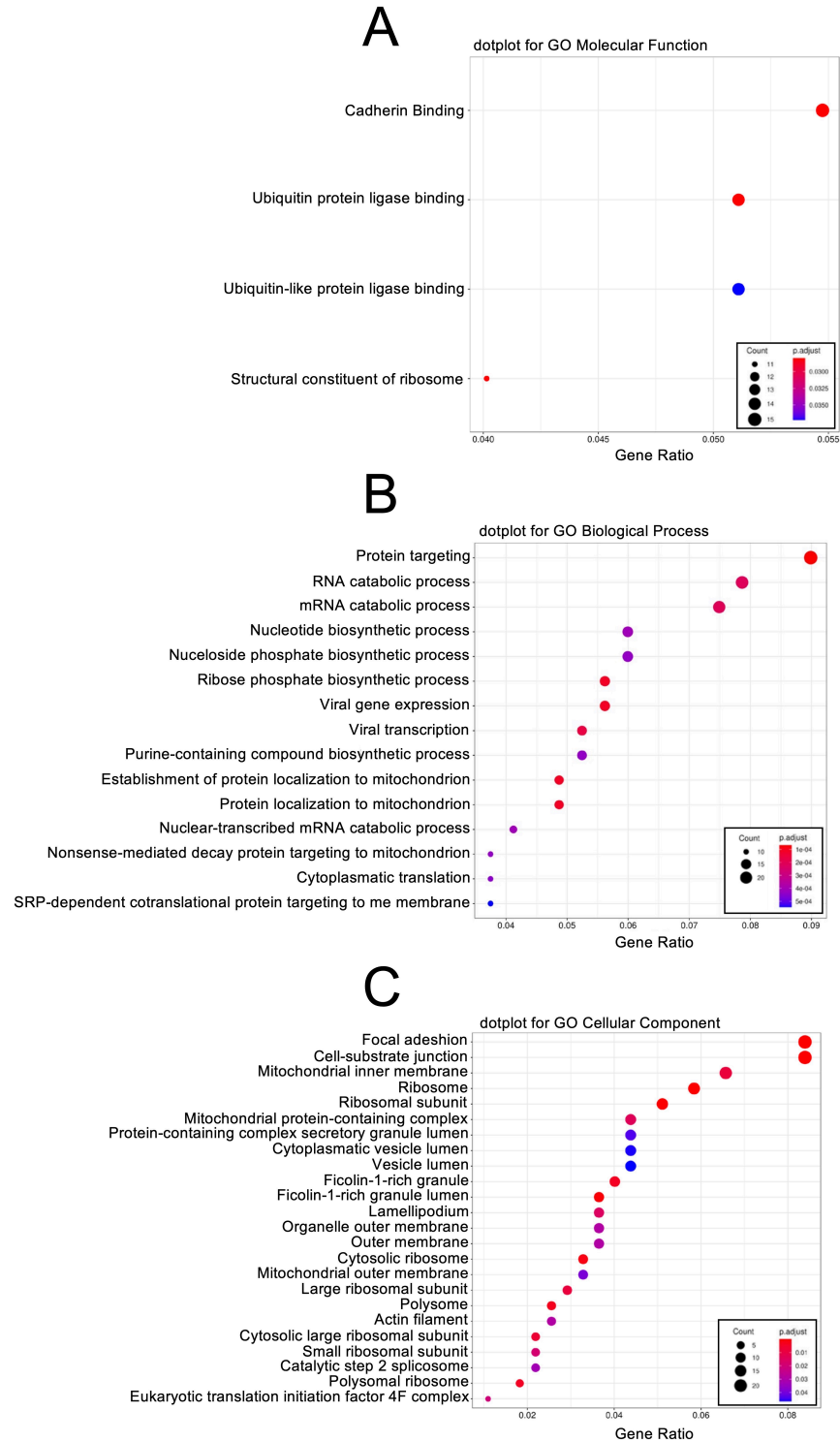


Figure 6. Gene ontology (GO) enrichment analysis of DEGs in OB-EVs-treated MNNG/HOS cells. Representative dot-plots of gene ontology (GO) enriched terms for (A) molecular functions, (B) biological processes and (C) cellular components. The size of the dots represents the number of genes in the significant DEGs list associated with the GO term and the colour of the dots represent the p-adjusted values.

While very powerful, this approach does not give indications on the directionality of the regulations, therefore we decided to complement it with gene set enrichment analyses (GSEA), run on all transcripts that met quality criteria, without applying other cut-offs as is commonly done. To this aim, we employed the WebGestalt tool (<https://www.webgestalt.org/>), to which we provided the gene IDs, previously converted from ENST IDs using gProfiler (<https://biit.cs.ut.ee/gprofiler/convert>) and the regulations as LogFC. KEGG GSEA (Figure 7A) revealed a significant enrichment of pathways related to fatty acid metabolism, protein processing in the endoplasmic reticulum, and mTOR signalling, suggesting a strong modulation of cellular metabolic activity and protein homeostasis in OB-EVs-treated MNNG/HOS cells. Enriched pathways also included lipid and atherosclerosis-related processes and viral infection-associated pathways, which may reflect stress-related or inflammatory-like transcriptional programs.

We then ran similar analysis on Panther as reference database (Figure 7B). The analysis highlighted a consistent depletion of pathways involved in integrin signalling, Wnt signalling, and inflammation mediated by chemokine and cytokine signalling. Additional negatively enriched pathways included CCKR signalling and Huntington disease-associated transcriptional programs, suggesting broad alterations in signalling networks governing cell adhesion, migration and differentiation. No Panther pathways reached significant positive enrichment, reinforcing the predominance of pathway re-expression following OB-EVs treatment.

Intriguingly, Reactome GSEA (Figure 7C) further confirmed a strong enrichment of pathways associated with rRNA processing, RNA polymerase II-mediated transcription, and gene expression, including major nucleolar and cytosolic rRNA processing pathways, pointing to a marked remodelling of transcriptional and ribosomal biogenesis processes. Enrichment was also observed for cytokine signalling in the immune system, ISG15 antiviral mechanisms, and energy metabolism integration, indicating activation of stress-response and immune-related transcriptional programs. In contrast, a large set of pathways was significantly depleted, most notably those involved in cell cycle regulation, including G1/S transition, DNA damage checkpoints, and p53-dependent responses. Importantly, multiple NOTCH1 signalling pathways, including both canonical signalling and cancer-associated NOTCH1 mutant pathways, were consistently downregulated, highlighting a potential suppression of differentiation- and proliferation-related signalling axes.

Taken together, these data indicate that OB-EVs profoundly reprogram osteosarcoma cells at the transcriptional and metabolic level, promoting pathways related to RNA processing, protein homeostasis and lipid metabolism, while suppressing cell cycle progression, cell-matrix interaction signalling, inflammatory pathways, and key oncogenic signalling cascades such as Wnt/ β -Catenin and NOTCH. This coordinated transcriptional shift suggests that OB-EVs may limit osteosarcoma aggressiveness by restraining proliferative and adhesion-related programs while inducing metabolic and stress-adaptive responses.

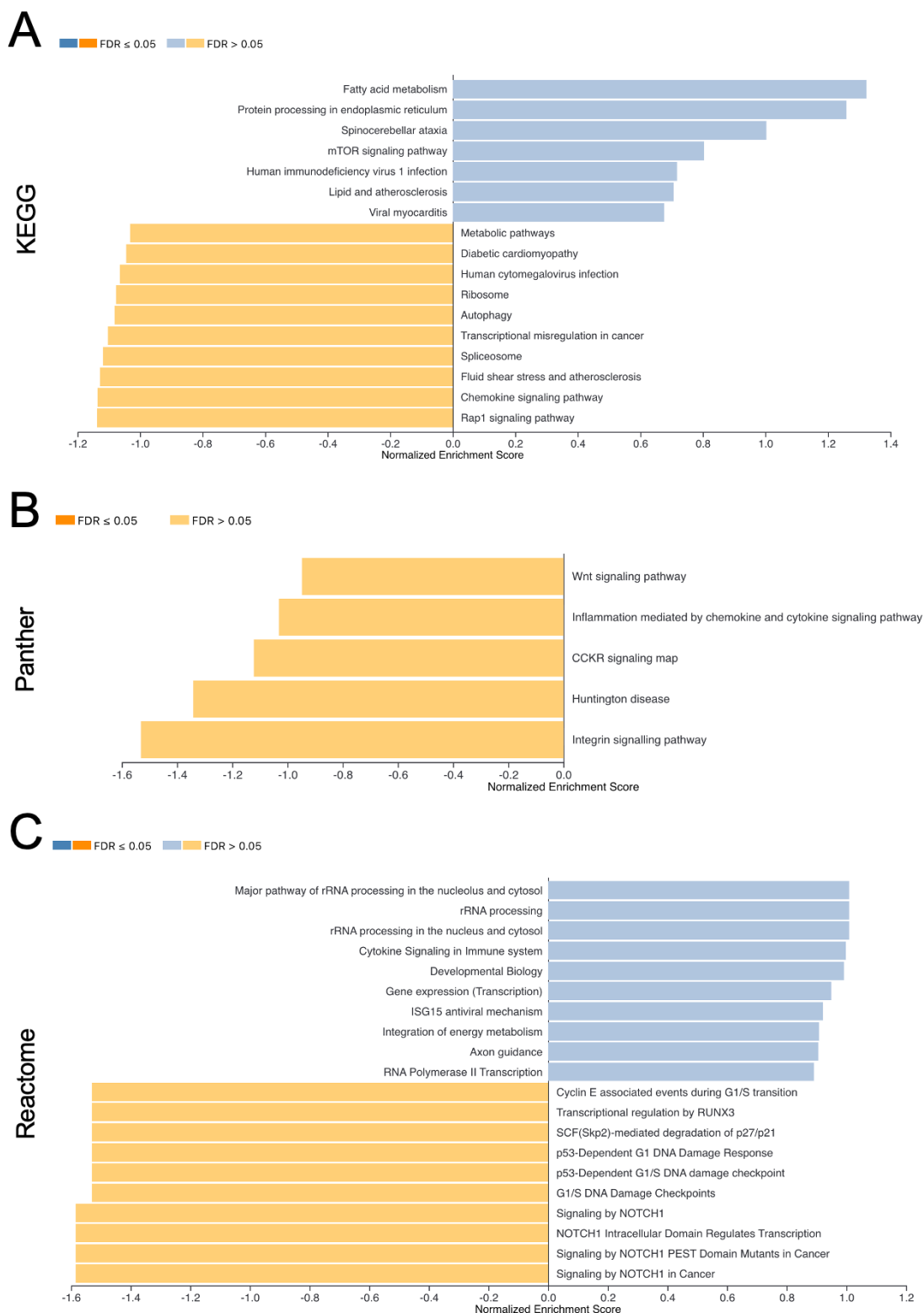


Figure 7. Gene-Set Enrichment Analysis (GSEA) of DEGs in OB-EVs-treated MNNG/HOS cells. Gene-Set Enrichment Analysis (GSEA) using WEB-based GENE SeT Analysis Toolkit (WebGestalt, <http://www.webgestalt.org>). Bar graphs of pathway analysis performed using (A) KEGG, (B) Panther and (C) Reactome databases. The top 10 enriched/depleted terms were plotted based on Normalized Enrichment Score (NES). The NES of top enriched (blue bars) and top depleted (orange bars) pathways in OB-EVs-treated MNNG/HOS compared to untreated MNNG/HOS are listed. Pathways with an FDR < 0.05 are represented with dark blue or orange bars; pathways with an FDR ≥ 0.05 are represented with light blue or orange bars.

While pathway analysis offers valuable insights into the potential mechanisms by which OB-EVs affect osteosarcoma cells, we also sought to explore how the identified differentially expressed genes (DEGs) might influence osteosarcoma cell behaviour. Notably, the top downregulated gene was N-Myristoyltransferase 1 (NMT1), which encodes an enzyme responsible for the co-translational modification of proteins through myristylation. To assess its potential clinical relevance, we interrogated publicly available osteosarcoma patients' databases, containing expression and survival data, through the R2 Genomics Analysis and Visualization Platform (https://hgserver1.amc.nl/cgi-bin/r2/main.cgi?open_page=login), discovering that high expression of NMT1 correlates with higher overall survival (Figure 8A) and metastasis-free survival (Figure 8B). As for the top upregulated transcript in MNNG/HOS treated OB-EVs vs untreated cells, we found RNA, Ro60-Associated Y1 (RNY1), which is not a protein-coding gene, but a small non-coding RNA that forms ribonucleoproteins with a potential supporting role in DNA duplication [12]. RNY1 has never been previously associated with osteosarcoma, but its upregulation is consistent with the prominent enrichment of RNA-related pathways (e.g., rRNA processing and transcriptional programs) identified by Reactome GSEA. Since only protein coding genes are available in the osteosarcoma survival analysis tool mentioned above, we did not run similar analyses with this gene. However, we did so for the next of the most upregulated genes in OB-EVs treated MNNG/HOS, i.e. Ribosomal Protein L36 (RPL36). Interestingly, we found that low expression of RPL36 correlates with higher overall survival (Figure 8C), and metastasis-free survival (Figure 8D).

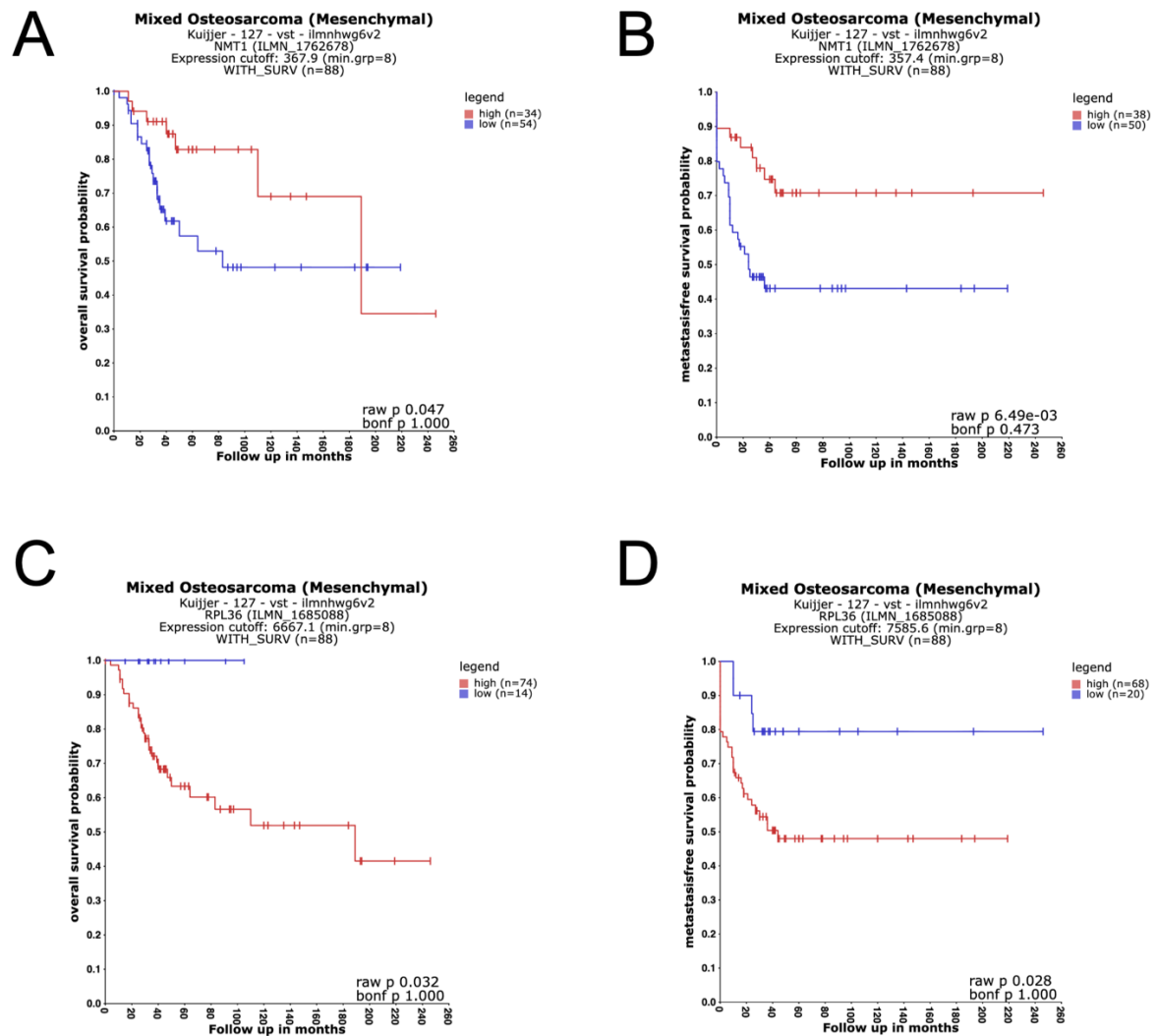


Figure 8. Overall survival and metastasis-free survival analysis of DEGs in OB-EVs-treated MNNG/HOS cells. Kaplan-Meier curves of Overall Survival (OS) and Metastasis-Free Survival (MFS) in osteosarcoma patients based on the expression levels of (A,B) NMT1, the most down-regulated gene, and (C,D) RPL36, the most upregulated one, analysed using the R2: Genomics Analysis and Visualization Platform. In all plots, the x-axis represents follow-up time (months), while the y-axis indicates survival probability. Statistical significance (log-rank p-values) and expression distribution graphs indicate gene expression variability across samples.

3.3 Osteoblast-derived EVs contain micro-RNAs predicted to potentially target MNNG/HOS osteosarcoma cells transcripts

Current knowledge suggests that the most represented class of molecules in EVs are micro-RNAs (miRNAs) which are highly present and have the potential to regulate multiple pathways at once, thus making them possible culprits for the biological regulations observed when treating cells with EVs [13-16]. Therefore, we performed a miRNA profiling in OB-EVs, to find out which were the most highly represented miRNA species and to understand whether they could be mediating some of the effects observed in MNNG/HOS osteosarcoma cells. We found that 27 miRNAs were expressed, which we define as miRNAs with a clean negative control, at least 4 Ct of difference between

medium and OB-EVs, in all 3 OB-EVs preparations assayed (Data S1), while 13 of them were highly expressed in all OB-EVs preparations (Ct<27,5, Table 3).

Table 3
miRNAs expressed in osteoblast-derived EVs.

Target	EVs		Medium	
	CT mean	Ct SE	CT mean	Ct SE
*ath-miR159a	23.852	1.088	19.240	0.073
let-7e-5p	23.738	0.173	–	–
let-7g-5p	25.016	0.390	–	–
miR-140-5p	24.379	0.387	–	–
miR-152-3p	24.662	0.177	–	–
miR-185-5p	27.472	0.189	–	–
miR-195-5p	27.445	0.390	–	–
miR-26a-5p	22.512	0.396	–	–
miR-29b-3p	23.311	0.768	–	–
miR-30b-5p	23.602	0.373	–	–
miR-30c-5p	23.910	0.427	–	–
miR-324-5p	26.354	0.391	–	–
miR-34a-5p	22.985	0.913	–	–
miR-365a/b-3p	24.384	0.463	–	–

*spike-in miRNA used as internal control for miRNA purification.

To have the most reliable data possible, these 13 miRNAs (let-7e-5p, let-7g-5p, miR-140-5p, miR-152-3p, miR-185-5p, miR-195-5p, miR-26a-5p, miR-29b-3p, miR-30b-5p, miR-30c-5p, miR-324-5p, miR-34a-5p, and miR-365-3p) were used for further analyses. In order to assess whether such miRNAs were playing a role in molecular circuitries involving DRGs, we performed a target gene prediction analysis using the 3 different prediction web tools TargetScan release 8 (https://www.targetscan.org/vert_80/) accessed through Mienturnet (<http://userver.bio.uniroma1.it/apps/mienturnet/>), DIANA

(<https://dianalab.e-ce.uth.gr/html/mirpathv3/index.php?r=mirpath>), and miRDB (<https://mirdb.org/>). Next, we checked whether any of the predicted targets matched the DRGs in OB-EVs-treated MNNG/HOS and then considered reliable predictions those that were common to at least 2 of the 3 tested databases (Table 4).

Table 4

OB-EVs-expressed miRNA targets downregulated in OB-EVs-treated MNNG/HOS cells. Evaluation conducted in the databases specified in the header.

miRNA(s)	TargetScan	DIANA	miRDB	Common to 2+
miR-365a-3p/miR-365b-3p	SP1, CMTM3	-	CMTM3	CMTM3
miR-29b-3p	TSPAN9, COL1A1, CD276, DEF8, DCTN1, ETV4, SP1, NCOR2	CD81, PPP2R1A, ATP6V1H, ARL4C, SLC4A2, CDCA4, ILK, PUF60, DHPS, GRK6, CD276, CDC25B, TSPO, SP1, FASN, FN1, TMEM135, NPTN, DDX5, CMTM3, CDKN1A, NCOR2, PAICS, RPL17	COL1A1, CDCA4, CD276, ETV4, SP1, CDKN1A	CDCA4, CD276, SP1, CDKN1A, COL1A1, ETV4, NCOR2
miR-140-5p	HDAC7, CAPN1	PFKL, PLXND1, PLK1, IER3, RAC2, EIF4G1, VASP, LAP3, LRP5, NT5E, DHPS, CKB, GPC1, EFHD2, DEF8, DCTN1, PKM, CS, MIER2, GALE, IGF2BP3, SMARCA4, ETV4, SEMA7A, ATG16L1, SP1, CALD1, UBASH3B, CAPN1, ITGB5, ANXA11, TPBG, NPTN, KIFC3, PTCD3, CDC34	HDAC7, CAPN1	CAPN1, HDAC7
miR-152-3p	CDC25B, ITGA1, U2AF1, UBASH3B, NPTN	TKT, PLXND1, PLK1, IER3, RAC2, BTBD2, VASP, PEMT, LAP3, LRP5, ILK, CKB, GPC1, EFHD2, CDC25B, COMT, PKM, SLC35A4, MIER2, GALE, ETV4, SP1, CALD1, UBASH3B, ITGB5, NT5DC2, CMC1, ANXA11, TPBG, NPTN, ATL2, KIFC3, CDC34, NMT1	BTBD2, TSPO, CS, NPTN	BTBD2, CDC25B, UBASH3B, NPTN
miR-34a-5p	ELOVL6, SLC25A23, U2AF1, ALDOA	CD81, PFKL, PPP2R1A, PLK1, RAC2, EIF4G1, LY6E, TSPO, MCM5, CALD1, PPP1R18, TMEM135, NPTN, ATL2, CMTM3, ALDOA, CDKN1A, PAICS	AGTRAP, SLC25A23, MIER2, LMNA, CMC1, ALDOA	ALDOA, SLC25A23
miR-30b-5p/miR-30c-5p	NT5E, MIER2, PPP1R18, ATL2, ACTN1, NCOR2	CD81, PPP2R1A, ARL4C, PLK1, EIF4G1, ILK, CDC25B, TSPO, SP1, CALD1, PPP1R18, TMEM135, NPTN, ATL2, DDX5, CMTM3, NCOR2, PAICS, RPL17, NMT1	ARL4C, NT5E, GRK6, MIER2, PPP1R18, PRPF19, OSBPL9, ATL2	ARL4C, NT5E, MIER2, PPP1R18, ATL2, NCOR2
miR-26a-5p	ARL4C, HMGA1, REEP4	CD81, ARL4C, IER3, RAC2, IGF2BP3, VASP, ELOVL6, LRP5, SLC25A23, CS, ACSS2, SEMA7A, ATG16L1, REEP4, SP1, MFSD1, TMEM135, OSBPL9, DDX5, PAICS	ARL4C, HMGA1, ITPRID2, REEP4, TMEM135	ARL4C, REEP4, TMEM135, HMGA1
let-7e-5p/let-7g-5p	PLXND1, HMGA1, COL1A1, EFHD2, CD276, GALE, IGF2BP3, ATG16L1, MDFI, RRM2, CDC34	PFKL, PLXND1, PLK1, THOP1, BCAR1, IER3, RAC2, EIF4G1, VASP, LAP3, LRP5, NT5E, DHPS, CKB, GPC1, EFHD2, PKM, CS, MIER2, GALE, IGF2BP3, SMARCA4, ETV4, SEMA7A, ATG16L1, SP1, CALD1, UBASH3B, CAPN1, ITGB5, ANXA11, TPBG, KIFC3, PTCD3, CDC34	PLXND1, HMGA1, EFHD2, GALE, IGF2BP3, ATG16L1, MDFI, RRM2, ATL2, CDC34	PLXND1, EFHD2, GALE, IGF2BP3, ATG16L1, HMGA1, MDFI, RRM2, CDC34
miR-195-5p	GDI2, AGTRAP, PTGES2, ELOVL6, FLYWCH2, NUTF2, KRT81, LRPAP1, WLS, VPS41, DEF8, ACTG1, CD9, ITGA1, SAPCD2, SP1, PPP1R18, NPAS2, TMEM135, NME2, CMC1, TPBG, ALDOA, ACTN1	-	TMEM135, CMC1	TMEM135, CMC1

miR-185-5p	RHOA, CNIH2, TMEM135	TKT, PLXND1, PLK1, IER3, RAC2, BTBD2, EIF4G1, VASP, PEMT, LAP3, LRP5, ILK, CKB, GPC1, EFHD2, CDC25B, COMT, PKM, SLC35A4, MIER2, GALE, ETV4, SP1, CALD1, ITGB5, NT5DC2, FN1, CMC1, ANXA11, TPBG, NPTN, ATL2, KIFC3, CDC34	CLSTN1, HMGA1, KANK2, COL1A1, ACSS2, SAE1, RHOA, FN1, CNIH2	FN1, RHOA, CNIH2
miR-324-5p	SMARCA4, SP1	-	GPC1, ITGA1, SMARCA4, SP1	SMARCA4, SP1

Using this method, by primarily focusing the analysis on down-regulated genes, we identified 37 potential target transcripts among the 199 downregulated, showing that 18% of them can have the potential of being also direct targets of the OB-EV-contained miRNAs. Interestingly, some of the target transcripts were predicted to be targeted by 2 (namely ATL2, CDC34, CMTM3, COL1A1, EFHD2, ETV4, FN1, GALE, HDAC7, HMGA1, NCOR2, NPTN, PLXND1, PPP1R18, REEP4, RHOA, RRM2, SLC25A23, SMARCA4) or 3 miRNAs (namely SP1, TMEM135, ARL4C). Knowing that miRNAs are among the most represented RNA species in EVs [17], we used an RNA-specific fluorescent dye (SYTO RNaselect) to tag RNAs contained in OB-EVs, and we then treated MNNG/HOS cells with the tagged EVs. Fluorescence microscopy analysis showed that OB-EVs RNAs were efficiently shuttled to MNNG/HOS cells (green-fluorescent dotted pattern) already 1 hour after treatment, with a consistent signal achieved at 6 and 18 hours from the treatment (Figure 9A).

As described before for the RNAseq, we decided to also have a more holistic look at the pathways regulated by OB-EVs, using miRNet (<https://www.mirnet.ca/miRNet/home.xhtml>) to understand the pathways that are most likely regulated by the miRNAs present in OB-EVs. We then reasoned that it would be important to understand the pathways that we found to be regulated by OB-EVs, and those that are predicted to be influenced by the miRNAs present in OB-EVs, and Reactome analyses carried out using this method showed that 25 pathways responded to these criteria (Figure 9B). From the analysis, it appeared clear that among the pathways, Wnt/ β -Catenin stood out as the most prominent, with multiple Reactome terms showing significant regulation both in the RNAseq data and the miRNet analysis (Table 5). Other interesting pathways regulated in both analyses were mitotic cell cycle, and oncogene-induced cellular senescence.

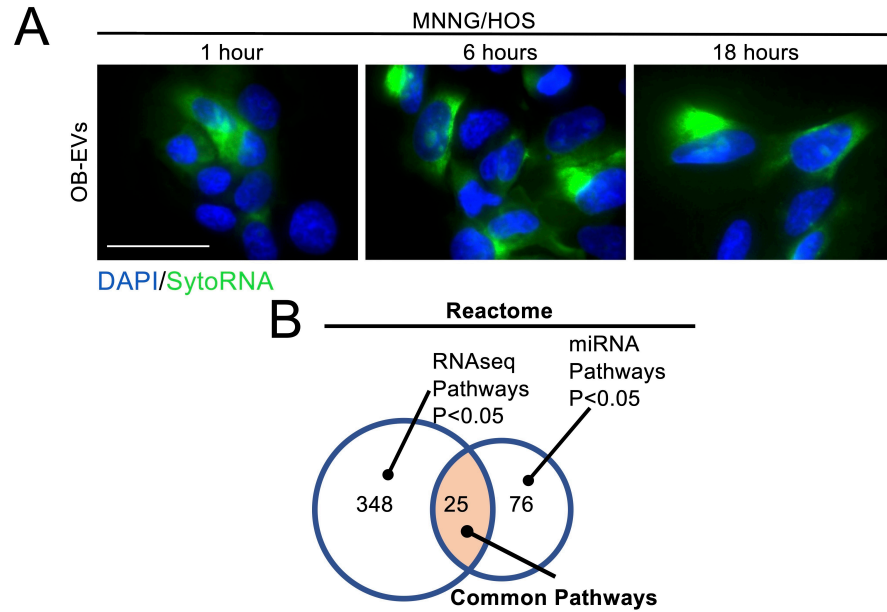


Figure 9. Potential effects of OB-EVs-expressed miRNAs on MNNG/HOS cells. (A) MNNG/HOS cells were treated for 1, 6 and 18 hours with OB-EVs loaded with the RNA-specific green fluorescent dye, SytoRNA Select and analysed, at the same timepoints, by epifluorescence microscopy. Cells nuclei were counterstained with DAPI. Scale bar = 50 μ m. (B) Venn diagram illustrating the overlap between pathways experimentally regulated by OB-EVs (left circle) and those predicted to be influenced by OB-EV miRNAs (right circle), with 25 pathways meeting both criteria (orange). Each pathway was considered significant based on p-value < 0.05.

Table 5

Reactome description in common between the predicted OB-EVs miRNA target genes and the observed RNAseq data on MNNG/HOS treated with OB-EVs.

Reactome description	P value (predicted miRNA targets)	P value (RNAseq)
PIP3 activates AKT signalling	$1.93 \cdot 10^{-7}$	$8.61 \cdot 10^{-6}$
Signalling by WNT	$1.40 \cdot 10^{-15}$	$1.36 \cdot 10^{-4}$
Transcriptional activity of SMAD2/SMAD3:SMAD4 heterotrimer	$1.06 \cdot 10^{-7}$	$1.43 \cdot 10^{-4}$
Regulation of mitotic cell cycle	$3.02 \cdot 10^{-12}$	$2.10 \cdot 10^{-4}$
TCF dependent signalling in response to WNT	$1.06 \cdot 10^{-10}$	$3.74 \cdot 10^{-4}$
Deactivation of the beta-catenin transactivating complex	$7.37 \cdot 10^{-8}$	$9.87 \cdot 10^{-4}$
Beta-catenin independent WNT signalling	$9.08 \cdot 10^{-8}$	$1.50 \cdot 10^{-3}$
Signalling by ERBB2	$3.52 \cdot 10^{-10}$	$2.18 \cdot 10^{-3}$
Signalling by FGFR2	$2.08 \cdot 10^{-9}$	$2.22 \cdot 10^{-3}$
SMAD2/SMAD3:SMAD4 heterotrimer regulates transcription	$2.88 \cdot 10^{-6}$	$2.68 \cdot 10^{-3}$
Oncogene Induced Senescence	$5.16 \cdot 10^{-10}$	$3.01 \cdot 10^{-3}$
G2/M Checkpoints	$1.51 \cdot 10^{-10}$	$3.89 \cdot 10^{-3}$
Downstream signalling events of B Cell Receptor (BCR)	$6.81 \cdot 10^{-9}$	$4.22 \cdot 10^{-3}$
Signalling by FGFR	$2.08 \cdot 10^{-9}$	$5.32 \cdot 10^{-3}$
Programmed Cell Death	$2.55 \cdot 10^{-12}$	$5.50 \cdot 10^{-3}$
Signalling by FGFR3	$2.08 \cdot 10^{-9}$	$6.08 \cdot 10^{-3}$
Apoptosis	$2.47 \cdot 10^{-12}$	$6.09 \cdot 10^{-3}$
Signalling by FGFR4	$2.08 \cdot 10^{-9}$	$6.65 \cdot 10^{-3}$
Cell Cycle Checkpoints	$1.54 \cdot 10^{-9}$	$8.25 \cdot 10^{-3}$
Signalling by EGFR in Cancer	$5.94 \cdot 10^{-12}$	$1.05 \cdot 10^{-2}$
Fc epsilon receptor (FCER1) signalling	$1.92 \cdot 10^{-15}$	$1.19 \cdot 10^{-2}$

Taken together, these results show that miRNAs contained in OB-EVs have the potential of regulating at least some of the pathways that we found influenced by OB-EVs treatment, most interestingly, the Wnt/ β -Catenin pathway.

4. Discussion

Extracellular vesicles are well known mediators of cellular communication, as they carry bioactive molecules that exert a direct effect on target cells, reprogramming their transcriptional and proteomic profile. Osteosarcoma is the most widespread primary bone tumour in childhood and adolescence, derived from a malignant transformation of MSCs and characterised by the ability to communicate with resident cells, which in turn increase its local growth and dissemination. In this study, we aimed to characterise the effect of osteoblast-derived extracellular vesicles (OB-EVs) on osteosarcoma cells, shedding light on the potential mechanisms and pathways influenced by their treatment. By integrating RNA sequencing, bioinformatics analyses, and miRNA profiling, this work provides a comprehensive view of OB-EVs' multiple roles, highlighting their potential as a tool for understanding osteosarcoma biology.

In our previous work, we showed that osteoblast-derived EVs (OB-EVs) reduce the aggressiveness of MNNG/HOS osteosarcoma cells by impairing viability, motility and invasion. To assess whether these effects were reproducible across additional models, we tested OB-EVs in Saos-2 and U2OS cells. OB-EVs significantly reduced metabolic activity in both lines, indicating a consistent negative impact on cellular viability across distinct genetic backgrounds. However, Saos-2 cells showed a modest increase in proliferation without changes in apoptosis but a clear reduction in invasive capacity, suggesting a preferential effect on migration-related malignant traits. In contrast, U2OS cells exhibited a marked decrease in proliferation associated with a mild increase in apoptosis, consistent with a more pronounced growth-inhibitory response, while invasion was largely unaffected. Importantly, when testing non-malignant osteoblast-like cells (hFOB 1.19), OB-EVs did not significantly alter metabolic activity, proliferation, or apoptosis, supporting the notion that OB-EVs exert a selective regulatory effect on osteosarcoma cells rather than broadly impairing osteoblastic cell behaviour.

Having established that OB-EVs consistently modulate osteosarcoma phenotype, although with line-specific outcomes, we next investigated MNNG/HOS cells using RNA sequencing to define the transcriptional programmes underlying the response to OB-EVs. OB-EVs treatment induced a clear transcriptomic reprogramming, as evidenced by PCA clustering and differential expression analyses, identifying 296 differentially regulated genes, with a predominance of downregulated transcripts. Gene Ontology analyses consistently revealed enrichment in processes related to cell–cell interactions, ubiquitination, ribosomal structure and function, RNA metabolism, mitochondrial activity, and focal adhesion dynamics, suggesting that OB-EVs broadly impact protein homeostasis, metabolic regulation and adhesion-related signalling.

Intriguingly, pathway-level analyses further supported this notion. KEGG-based GSEA revealed positive enrichment of pathways associated with fatty acid metabolism, protein processing in the endoplasmic reticulum and mTOR signalling, alongside stress- and inflammation-associated transcriptional programmes. Conversely, pathways involved in

ribosome function, autophagy, spliceosome activity, transcriptional misregulation in cancer and chemokine signalling were negatively enriched, indicating a coordinated suppression of biosynthetic, inflammatory and pro-migratory pathways. These trends were confirmed by Panther GSEA, which showed a consistent depletion of integrin signalling, Wnt signalling and chemokine- and cytokine-mediated inflammatory pathways, with no pathways reaching significant positive enrichment, highlighting the predominance of transcriptional repression following OB-EVs exposure. Reactome-based GSEA provided additional strong positive enrichment of pathways related to rRNA processing, RNA polymerase II-dependent transcription and ribosomal biogenesis, together with immune- and stress-adaptive programmes. In contrast, a large set of negatively enriched pathways converged on cell-cycle regulation, including G1/S transition, DNA damage checkpoints and p53-dependent responses. Notably, multiple NOTCH1-related signalling pathways were consistently depleted, indicating suppression of differentiation- and proliferation-associated transcriptional axes that are central to osteosarcoma progression.

Aberrant activation of the Wnt/ β -catenin pathway has been extensively documented in osteosarcoma and is strongly associated with tumour initiation, progression, metastatic dissemination, and resistance to therapy. Canonical Wnt signalling promotes osteosarcoma cell proliferation, survival, stemness, and invasive behaviour by inducing the transcription of oncogenic targets such as MYC, CCND1, and AXIN2 [18, 19]. Functional evidence further supports a pro-tumoral role for Wnt signalling in osteosarcoma, as genetic or pharmacological inhibition of β -catenin results in reduced proliferation, impaired migration and invasion, and enhanced apoptosis of osteosarcoma cells both in vitro and in vivo [20, 21]. In addition, Wnt signalling is critically involved in maintaining cancer stem cell-like properties in osteosarcoma, thereby contributing to tumour recurrence and chemoresistance [22]. In this context, the negative enrichment of Wnt signalling observed after OB-EVs treatment provides a strong mechanistic link between EV-mediated transcriptional repression and the reduced metabolic activity and invasive behaviour previously observed.

Similarly, the NOTCH signalling pathway, consistently depleted in both Reactome and Panther analyses, plays a well-established pro-tumoral role in osteosarcoma. NOTCH receptors and ligands are frequently overexpressed in osteosarcoma tissues, and elevated NOTCH activity correlates with increased tumour growth, metastatic potential and poor clinical outcome [23, 24, 25]. NOTCH signalling promotes osteosarcoma cell proliferation and survival through crosstalk with PI3K/AKT, mTOR and Wnt/ β -catenin pathways and contributes to the maintenance of an undifferentiated, stem-like phenotype [25, 26]. Accordingly, the coordinated downregulation of multiple NOTCH1-related pathways observed in OB-EVs-treated cells is biologically meaningful and aligns with the depletion of cell-cycle-associated programmes.

Looking at the single genes, the most downregulated of our dataset was N-myristoyltransferase 1 (NMT1), an enzyme that catalyses the co-translational myristoylation of a broad range of substrates involved in signal transduction, membrane association, cytoskeletal dynamics and protein stability. Although NMT1 has not been previously investigated in the context of osteosarcoma, when we explored its potential clinical relevance using publicly available osteosarcoma datasets, we observed that higher NMT1 expression was associated with better overall and metastasis-free survival,

suggesting that reduced NMT1 levels may be linked to a more aggressive disease phenotype. In this context, the observation that OB-EVs markedly suppress NMT1 expression in MNNG/HOS cells raises the possibility that, at least at the level of this single gene, OB-EVs may promote a transcriptional profile associated with unfavourable prognosis. Evidence from other cancer types, however, addressed an anti-tumoral role to NMT1, since in breast cancer its genetic or pharmacological inhibition suppresses cell proliferation and invasion by activating JNK-mediated stress responses and impairing proteostasis, ultimately triggering autophagy and growth arrest [27]. We then focused on the most upregulated gene of our dataset, ribosomal protein L36 (RPL36), which encodes a component of the 60S ribosomal subunit involved in protein synthesis. In our hands, RPL36 expression was markedly increased following OB-EVs treatment but, when we interrogated publicly available osteosarcoma datasets, we found that lower RPL36 expression correlates with improved overall and metastasis-free survival, suggesting that, at least at the level of RPL36 single gene, OB-EVs may promote a transcriptional profile associated with unfavourable prognosis. Similarly, emerging evidence from other tumour types supports a pro-tumoral role for increased RPL36 expression. For instance, Song et al. showed that high RPL36 level in hepatocellular carcinoma (HCC) is associated to poor prognosis and early tumour progression [28]. Several limitations should be considered, however, when comparing our in vitro findings with survival analyses, as the latter are derived from a relatively small patient cohort (n = 88) and rely on baseline gene expression measured in heterogeneous bulk tumour samples, thus reflecting a biological dimension distinct from our experimental model. Nevertheless, the apparent discrepancy between EV-driven gene regulation and prognostic associations highlights the context-dependent roles of NMT1 and RPL36 in osteosarcoma and further underline the need for future studies involving expanded patient cohorts.

The profiling of miRNAs in OB-EVs identified 27 expressed miRNAs, 13 of which were highly expressed and putatively involved in mediating transcriptomic changes in OB-EVs-treated MNNG/HOS cells. Since miRNAs are among the most abundant RNA species in EVs, we labelled OB-EV RNAs with the RNA-specific fluorescent dye SYTO RNASelect and then treated MNNG/HOS cells with these vesicles. Fluorescence microscopy revealed a rapid uptake of OB-EVs RNAs by MNNG/HOS cells, demonstrating efficient RNAs transfer, although this method did not specifically confirm the delivery of the 13 highly expressed miRNAs. Moreover, by integrating predictions from TargetScan, DIANA, and miRDB tools, 37 out of 199 downregulated transcripts were identified as potential direct targets of these miRNAs, indicating that approximately 18% of the downregulated genes could be also regulated by OB-EVs-derived miRNAs. This percentage aligns with the hypothesis that miRNAs play a significant, even partial, role in modulating gene expression following OB-EVs uptake. Interestingly, many of the predicted targets have been implicated in oncogenic processes in other cancer types or in fundamental biological pathways relevant to malignancy. For example, FN1 (fibronectin) and COL1A1 (collagen type I) encode extracellular matrix components critical for cell adhesion and migration, processes that are commonly dysregulated in tumour invasion and metastasis [29]. Similarly, RHOA, a small GTPase, plays a central role in cytoskeletal dynamics and has been linked to enhanced invasiveness in multiple tumour models [30]. A subset of targets, including CDKN1A (p21), CDC25B, CDC34, CDCA4 and RRM2 (ribonucleotide reductase regulatory subunit M2), are key regulators of the cell cycle and DNA synthesis, consistent with the downregulation of cell cycle-related pathways observed in GSEA.

Deregulation of these factors has been associated with uncontrolled proliferation and poor prognosis in various cancers [31, 32]. Several downregulated targets function in transcriptional and epigenetic regulation (e.g., SP1, SMARCA4, NCOR2, HDAC7, HMGA1, MIER2), highlighting a potential impact on global gene expression pathways. Components of the SWI/SNF chromatin remodelling complex such as SMARCA4 have been shown to modulate oncogenic transcriptional networks in diverse tumour contexts [33]. Transcription factor SP1 is similarly implicated in the regulation of proliferation-related gene networks [34]. Additional targets, including ALDOA, GALE, SLC25A23, TMEM135 and ATG16L1, participate in metabolic and stress response pathways, aligning with the enrichment of fatty acid metabolism, protein processing, and mTOR signalling identified in KEGG GSEA. Cancer cells frequently exploit such pathways to support biosynthesis and survival under stress [35, 36]. Lastly, Reactome analyses highlighted 25 pathways influenced by OB-EVs' miRNAs, with the Wnt/ β -Catenin pathway standing out as the most prominently regulated. The consistent depletion of Wnt/ β -Catenin signalling across multiple analyses suggests this pathway's central role in OB-EVs' action.

Looking forward, our data open several avenues for future investigation. The consistent suppression of Wnt/ β -catenin and NOTCH signalling suggests that OB-EVs act through coordinated regulatory mechanisms rather than single dominant effectors, potentially involving both EV-associated miRNAs and protein cargo. Dissecting the relative contribution of these components through targeted gain- and loss-of-function approaches will be essential to define the upstream regulators and downstream consequences of EV-mediated signalling. Moreover, the concerted modulation of genes involved in proteostasis, ribosomal biogenesis and transcriptional control, including NMT1 and RPL36, raises the possibility that OB-EVs reshape osteosarcoma cell behaviour by altering fundamental cellular homeostatic processes. Ultimately, a deeper understanding of the molecular determinants underlying OB-EV activity may uncover novel vulnerabilities in osteosarcoma cells, potentially driving to new therapeutic strategies.

5. Conclusions

Overall, our findings highlight the ability of osteoblast-derived extracellular vesicles to modulate transcription, translation and metabolic processes in osteosarcoma cells, at least in part through their miRNA cargo. The consistent depletion of Wnt/ β -Catenin and NOTCH signalling pathways suggest potential anti-tumoral effects of OB-EVs. Moreover, the strong regulation of genes such as NMT1 and RPL36 identifies new candidates that warrant further investigation to advance our understanding of osteosarcoma biology.

6. Note on the study scope and data availability

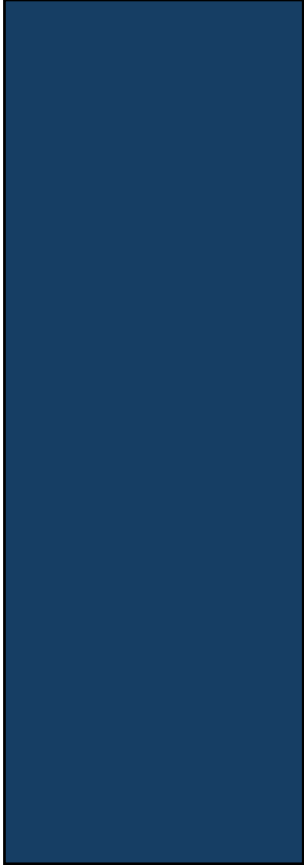
The phenotypic in vitro data on U2OS and Saos-2 cell lines were generated and included during the revision process of the associated manuscript, following to reviewers' comments aimed at extending the experimental validation beyond the MNNG/HOS previously published work. At the time of writing, transcriptomic sequencing and bioinformatic analyses are available only for MNNG/HOS cells, as presented in the original study, while sequencing of U2OS and Saos-2 cells is currently ongoing.

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Chapter 5

“Human osteosarcoma cell secretome impairs neonatal mouse calvarial osteogenic cells functions and modifies the nanoparticles-derived protein profile”

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Abstract

Osteosarcoma is the most common pediatric primary bone tumour, whose growth strictly relies on a complex interplay among tumour cells, resident cells, and the bone matrix. We investigated the effects of secretome collected from the human osteosarcoma cell line MNNG/HOS on mouse primary osteogenic cells, finding that prolonged exposure alters osteoblast phenotype and activity. MNNG/HOS secretome also reduces the production and release of collagen type I, the most abundant constituent of the bone matrix, and hinders osteoblast ability to form nodule of mineralization, compared to osteogenic cells treated with their own secretome. Given the crucial role exerted by secretome on tumour growth, we aimed also to determine whether osteosarcoma cells secretome can influence the osteoblast release of extracellular nanoparticles (NPs) as well as NPs protein cargo. Intriguingly, we found that MNNG/HOS secretome exerts a direct effect on osteoblast-NPs, reprogramming their protein cargo and subsequently influencing extracellular matrix composition and collagen formation, in favor of tumour progression. Overall, our findings indicate the ability of MNNG/HOS cells to fuel their own malignancy by deranging bone matrix composition and stimulating osteoblast-nanoparticles shuttling of osteosarcoma promoting factors.

1. Introduction

Although rare, osteosarcoma is unfortunately the most prevalent malignant bone tumour, occurring in adolescents and young adults [1,2]. The common sites of tumour formation are those with the most extensive longitudinal bone growth, such as distal femur, proximal tibia and proximal humerus [3]. To date, treatment strategies include surgical resection combined with adjuvant chemotherapy [3]; however, osteosarcoma is still characterized by a poor prognosis due to its high aggressiveness, eventually leading to metastasis development, with the lung being the preferential affected organ [4]. Osteosarcoma derives from a malignant transformation of mesenchymal stem cells (MSCs) and is characterized by deposition of an osteoid-like matrix, with varying degrees of mineralization [5]. This osteoid matrix in turn enables a supportive scaffold for tumour progression [6]. Indeed, osteosarcoma cells grow in a complex and dynamic microenvironment, the bone matrix, in which they interact with different types of resident cells, like osteoblasts, osteoclasts, osteocytes, MSCs, endothelial and immune cells. This interplay has a crucial role in tumour progression and invasion [5]. Moreover, the extracellular matrix includes several components such as collagens, fibronectin, osteopontin, bone sialoprotein (BSP) II and γ -carboxyglutamic acid (GLA) proteins, whose deregulation contributes to aberrant signalling, eventually promoting osteosarcoma growth [6]. As an example, it has been demonstrated that matrix-Gla protein (MGP) overexpression in osteosarcoma cells increases lung metastases in an orthotopic mouse model, by a mechanism involving metalloproteinase activity and TGF β -induced Smad2/3 phosphorylation [7]. Fibronectin is an adhesion protein highly expressed in the bone matrix, which is able to interact with collagen, and a significantly higher expression of this glycoprotein has been found in resistant osteosarcoma cell lines [8]. The intercellular

crosstalk is a crucial process for the physiological and pathological tissue homeostasis, mediated by different cellular strategies such as the release of the secretome, a complex cellular product composed by soluble and extracellular vesicles (EVs) bound molecules, such as proteins, lipids and nucleic acid [9]. With regard to the interplay between bone cells and osteosarcoma cells, we recently demonstrated a bidirectional crosstalk by means of EVs as mediators [10,11]. In particular, we found that treatment of bone cells with osteosarcoma cells-derived EVs promotes a series of anti-osteoblastogenic, pro-inflammatory and pro-angiogenic effects, eventually contributing to the tumour expansion [10]. Likewise, we also demonstrated that osteoblast-derived EVs reduced osteosarcoma cells' aggressiveness and viability through redox-dependent signalling pathways [11]. Based on the above background, in this work we aimed at investigating how osteosarcoma cells-secretome alters osteoblast homeostasis and interferes with its autocrine regulation. Given the crucial role exerted by extracellular nanoparticles in the crosstalk between bone cells and tumour cells, our goal was also to determine whether osteosarcoma cells secretome is able to influence the release of osteoblastnanoparticles as well as the nanoparticles protein profile, the latter assessed by proteomics approach through nano liquid chromatographic coupled tandem mass spectrometry (nanoLC-MS/MS). Our finding demonstrated that osteogenic cells educated by osteosarcoma cell secretome show an impaired ability to release collagen I and mineralize; moreover, osteosarcoma cell secretome is able to change the profile of osteoblast-derived nanoparticles towards a phenotype that could promote their growth.

2. Materials and methods

2.1 Materials

Dulbecco's modified Minimum Essential Medium (DMEM), Fetal Bovine Serum (FBS), penicillin, streptomycin and trypsin were from GIBCO (Uxbridge, UK). Sterile plasticware was from Falcon Becton Dickinson (Cowley, Oxford, UK), Costar (Cambridge, MA, USA) or Euroclone (Milan, Italy). The anti-Annexin II (cat #sc-9061), -Matrilin-4 (cat #sc-374652), -CD81 (cat #sc-166029) and β -Actin (cat #sc-81178) antibodies were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA); the anti-CD63 antibody (cat #PA5-92370) was from Thermo Fisher Scientific (Waltham, MA, USA); the anti-Collagen type I (cat #AB82138) was from Immunological Sciences (Rome, Italy). The SuperSignal Chemiluminescent substrate was from Thermo Scientific. The aqueous glutaraldehyde (cat #16210) was from EMS (Hatfield, PA, USA). All other reagents were of the purest grade from Sigma-Aldrich Co. (St. Louis, MO, USA).

2.2 Cell lines

The human osteosarcoma cell line MNNG/HOS (RRID: CVCL_0439) was obtained from the European Collection of Authenticated Cell Cultures (ECACC, Salisbury, UK) and grown at 37 °C, 5 % CO₂ in DMEM supplemented with 10 % FBS, 100 IU/ml penicillin, 100 μ g/ml streptomycin, and 2 mM L-glutamine. Phenotypic characterization of these cells was monitored, as reported in our previous works [10,11].

2.3 Osteoblast primary cultures

Calvariae from 7-day-old mice were explanted, cleaned free of soft tissues and digested three times with 1 mg/ml Clostridium histolyticum type IV collagenase and 0.25 % trypsin, at 37 °C with gentle agitation for 15-, 30- and 45-min, according to well established protocols [12,13]. Cells from the second and third digestions were plated following centrifugation at 300g for 7 min and maintained in culture at 37 °C, 5 % CO₂ in DMEM plus 10 % FBS. At confluence, cells were trypsinized and plated according to the experimental protocol and are considered as neonatal mouse calvarial osteogenic cells.

2.4 Alkaline phosphatase activity assay

Neonatal mouse calvarial osteogenic cells were cultured for 2 days in standard conditions (serum free-DMEM, Control) or in presence of undiluted serum free-conditioned medium collected from osteogenic cells (OB-CM) or from MNNG/HOS cells (MNNG/HOS-CM) after 24 h of conditioning. Parallel experiments were conducted culturing neonatal mouse calvarial osteogenic cells in mineralization medium (DMEM+10 % FBS supplemented with 10 mM β -glycerophosphate and 50 μ g/ml ascorbic acid) and in mineralization medium supplemented with undiluted OBCM or MNNG/HOS-CM for 7, 14 and 21 days of culture; during the timeframe of the experiment the culture medium was changed every 3 days. Osteogenic cells were then fixed in 4 % buffered paraformaldehyde for 10 min and washed twice with Dulbecco's Phosphate Buffered Saline (DPBS). Semi-quantitative histochemical detection of Alkaline phosphatase (Alp) activity (pH 9.3) was evaluated by a cytochemical assay employing the Sigma-Aldrich kit#86C-1KT, following the manufacturer's instruction.

2.5 Mineralization assay

Neonatal mouse calvarial osteogenic cells were cultured (5×10^4 cells in 6 multiwell plate, growth area: 9.6 cm²) for 7, 14 and 21 days in 2 ml of mineralization medium (DMEM+10 % FBS supplemented with 10 mM β -glycerophosphate and 50 μ g/ml ascorbic acid,) or in 2 ml of OB-CM or MNNG/HOS-CM (harvest from 2×10^4 cells in 6 multiwell plate, growth area: 9.6 cm² for 24 h) plus 10 mM β -glycerophosphate and 50 μ g/ml ascorbic acid. During the timeframe of the experiment the culture medium was changed every 3 days, using fresh mineralizing DMEM, OB-CM or MNNG/HOS-CM; at the end, osteogenic cells were fixed in 4 % buffered paraformaldehyde for 10 min and washed twice with distilled water, then the presence of mineralization nodules was detected by von Kossa staining. Briefly, fixed osteogenic cells were incubated with 5 % aqueous solution of Silver Nitrate (AgNO₃) and exposed to ultraviolet light for 1 h; then cells were washed twice with distilled water, incubated with 5 % solution of Sodium Thiosulfate (Na₂S₂O₃) for 2 min, washed twice with distilled water, and air dried in a fume hood. Pictures of mineralization nodules (stained brown to black) were taken under an inverted phase-contrast microscope, and densitometric analysis was performed using NIH ImageJ tool.

2.6 4',6-Diamidino-2-phenylindole dihydrochloride staining

Neonatal mouse calvarial osteogenic cells were cultured for 7, 14 and 21 days in mineralization medium (DMEM+10 % FBS supplemented with 10 mM β -glycerophosphate and 50 μ g/ml ascorbic acid) and in mineralization medium supplemented with undiluted OB-CM or MNNG/ HOS-CM; during the timeframe of the experiment the culture medium was changed every 3 days. Neonatal mouse calvarial osteogenic cells were then fixed in 4 % buffered paraformaldehyde for 10 min, washed twice, and then stained with the nuclear dye 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI) (#D9542, Sigma-Aldrich) for 30 min in the dark at room temperature (RT), and analyzed by fluorescence microscopy.

2.7 Comparative real time PCR

RNA from neonatal mouse calvarial osteogenic cells cultured for 2 days in presence of undiluted conditioned medium collected from osteogenic cells (OB-CM) or from MNNG/HOS cells (MNNG/HOS-CM) after 24 h of conditioning was extracted using TRIzol reagent, then 1 μ g of RNA was reverse-transcribed into cDNA using the Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase and subjected to real time PCR using primer pairs and amplification conditions described in Table 1. All reactions were carried out using a SYBR green based master mix containing ROX as reference dye. Data were analyzed via dedicated software (MxPro, Stratagene, La Jolla, CA) using the $\Delta\Delta C_t$ method and Gapdh was chosen as the housekeeping gene.

Table 1. List of mouse primer sequences (5' to 3'). Primers were designed to anneal at 60°C.

Gene ID	Forward primer	Reverse primer
<i>Akp2</i>	CCAGCAGGTTTCTCTCTTGG	CTGGGAGTCTCATCCTGAGC
<i>Col1a1</i>	CACCCTCAAGAGCCTGAGTC	GTTCCGGGCTGATGTACCAGT
<i>Col1a2</i>	CCGTGCTTCTCAGAACATCA	GAGCAGCCATCGACTAGGAC
<i>Gapdh</i>	TGTGAGGGAGATGCTCAGTG	TGTTCTACCCCCAATGTGT
<i>Cyclin D1</i>	TCAAGTGTGACCCGGACTG	ATGTCCACATCTCGCACGTC
<i>Runx2</i>	CCCAGCCACCTTTACCTACA	TATGGAGTGCTGCTGGTCTG
<i>Osx</i>	TGCTTCCCAATCCTATTTGC	AGAATCCCTTTCCCTCTCCA
<i>Bglap</i>	TTCTGCTCACTCTGCTGACC	GGGACTGAGGCTCCAAGGTA
<i>Sost</i>	ACAAGGATGGGAGGTGACTG	ACCCCGTGTAGACTGGTGAG

2.8 Nanoparticles isolation

Nanoparticles (NPs) from neonatal mouse calvarial osteogenic cells treated with OB-CM or MNNG/HOS-CM were isolated according to well established protocols [10,11,14]. Briefly, upon reaching 80 % confluence, neonatal mouse calvarial osteogenic cells were washed in DPBS and starved in serum free-DMEM to prevent contamination from FBSNPs. Cells were maintained with serum-free DMEM or CM from OBCM or

MNNG/HOS-CM for 24 h. Following 3 times extensive washing to eliminate contaminating NPs, fresh DMEM was added [15,16]. For EVs harvesting, after 24 h the conditioned medium (CM) was collected and sequentially centrifuged at 300g, 4 °C for 5 min to remove dead cells and at 5000g, 4 °C for 25 min to remove membrane debris. The supernatant was collected and transferred to a Beckman L7-65 ultracentrifuge in a Beckman SW41-Ti or SW28 rotor and centrifuged at 100,000g, at 9 °C for 70 min. Supernatant was discarded, while the pellet, containing NPs, was resuspended in different buffers, according to the experimental needs. To quantify OB-NPs, they were subjected to nanoparticle tracking analysis as well as to protein extraction, the latter giving a mean yield of $4.5 \pm 1.2 \mu\text{g}/12 \text{ ml CM}$. Freshly isolated NPs were used for all subsequent experiments.

2.9 Nanoparticles tracking analysis

Nanoparticles were isolated from CM (12 ml collected from one 182 cm² flask, cell density = 3.5×10^4 cells/cm²) of neonatal mouse calvarial osteogenic cells treated for 24 h with OB-CM (CMOB-OB-NPs) or MNNG/HOS-CM (CMHOS-OB-NPs) and resuspended in 100 μl of filtered (0.22 μm) DPBS. NPs were then diluted 1:100 and used for nanoparticle tracking analysis using a nanosight NS300 NTA apparatus. Flow and camera gain were adjusted following the manufacturer's instructions based on the NS300 quality control parameters. Five camera acquisitions of 60 s each were analyzed for every biological replicate.

2.10 Western blot analysis

For cells and NPs protein extraction, samples were lysed in RIPA buffer (50 mM Tris HCl pH 7.5, 150 mM NaCl, 16.6 mM Nonidet P-40, 12.1 mM sodium deoxycholate, 3.47 mM SDS) containing protease inhibitors. Ten micrograms of NPs protein lysates, 50 μg of cell protein lysates and 40 μl of conditioned media were resolved by 10–15 % SDSPAGE under reducing conditions and transferred to nitrocellulose membranes. Blots were incubated 2 h in 5 % nonfat dry milk in TBS-T, probed with the primary antibody in 1 % milk O/N at 4 °C or for 1 h at room temperature (RT), washed and incubated with the appropriate HorseRadish Peroxidase (HRP)-conjugated secondary antibody for 1 h at RT. After washing, protein bands were revealed using the SuperSignal Chemiluminescence substrate, according to the manufacturer's instructions, and acquired using a BioRad Gel Doc XR+ Imaging System. The analysis of band intensities was performed using the NIH ImageJ tool.

2.11 Transmission electron microscopy

Ten microliters of NPs isolated from CM (12 ml collected from one 182 cm² flask, cell density = 3.5×10^4 cells/cm²) of osteogenic cells treated for 24 h with OB-CM (CMOB-OB-NPs) or MNNG/HOS-CM (CMHOS-OB-NPs) were fixed in 10 μl of 2 % glutaraldehyde for 30 min, put onto Formvar/Carbon coated grids and allowed to absorb for 20 min. Grids were then washed in distilled water, contrasted with 1 % phosphotungstic acid (PTA) for 2 min, washed again in distilled water and air-dried overnight. Samples were then observed under a Philips CM100 TEM (Transmission Electron Microscopy), with PHURONA camera (Emsis), at 80 kV.

2.12 Nanoparticles proteomics analysis and data processing

Shotgun proteomics were performed to evaluate the protein outfit of CMHOS-OB-NPs compared to CMOB-OB-NPs. NPs from three independent biological samples from each cell line were lysed by sonication on ice (Sonicator U200S control, IKA Labortechnik, Staufen, Germany) at 70 % amplitude in a lysis buffer (urea 6 M in 100 mM Tris/HCl, pH = 7.5). Bradford assay (Bio-Rad, Hercules, CA, USA) was used to taste the protein concentration by using Bovine Serum Albumin (BSA, SigmaAldrich, St. Louis, MI, USA) standard for the calibration curve in order to digest 20 µg of proteins for each NP sample obtaining two different pools (CMHOS-OB-NPs and CMOB-OB-NPs). Filter Aided Sample Preparation (FASP) protocol was used for overnight tryptic digestion at 37 °C. Each NPs sample was analyzed in triplicate by nanoLC-MS/MS through UltiMate™ 3000 UPLC (Thermo Fisher Scientific, Milan, Italy) chromatographic system coupled to the Orbitrap Fusion™ Tribrid™ (Thermo Fisher Scientific, Milan, Italy) mass spectrometer. All analytical parameters and specifications were detailed in Potenza et al. [17] setting the flow rate at 300nL/min, with a total run time of 65 min using a chromatographic gradient from 2 % to 90 % of phase B (mobile phase A: 0.1 % formic acid in H₂O and mobile phase B: 0.1 % formic acid in acetonitrile). MaxQuant 1.6.10.50 and Perseus 1.6.10.50 (Max-Planck Institute for Biochemistry, Martinsried, Germany) were used for proteomics MS/MS raw data processing against the UniProt database (released 2020_06, taxonomy Homo Sapiens, 20,588 entries) and bioinformatics analysis, respectively. Processing parameters were set as reported in our previous works [17,18] in order to obtain LFQ Intensities, whose values were used to quantify protein abundance in each sample by comparing CMHOS-OB-NPs vs CMOB-OB-NPs (quantification in at least 2 out of 3 samples replicate, as reported also by Wei et al. [19]). SRplot tool (<https://bioinformatics.com.cn/srplot>) was used for graphical representation of proteomic data. Vesiclepedia tool (<http://microvesicles.org>) was used to confirm the vesicular nature of proteomic data. STRING tool (<https://string-db.org>) was used for Protein-Protein Interaction Networks Functional Enrichment Analysis. DAVID tool (<https://david.ncifcrf.gov/tools.jsp>) was used for Gene Ontology (GO) reclassification.

2.13 Statistics

Results are expressed as the mean ± SD of at least 3 independent experiments, as specified in the figure legends. Statistical analyses were performed using the paired Student's t-test, one sample t-test and ANOVA conducted either on raw data or on curves, according to the type of data. The statistical method used is indicated in each figure legend. A p value ≤0.05 was considered statistically significant. GraphPad Prism (version 9.0) was used to design graphs.

3. Results

3.1 Effect of osteosarcoma cells secretome on neonatal mouse calvarial osteoblast phenotype

To investigate whether osteosarcoma cells secretome could interfere with osteoblast phenotype, we treated neonatal mouse calvarial osteogenic cells (hereafter simply referred to as osteogenic cells) with serum free conditioned medium (CM) collected from MNNG/HOS (MNNG/ HOS-CM) or with osteogenic cells-derived CM as control (OB-CM), which also gives an indication of any autocrine effect. Osteogenic cells treated with serum free-DMEM were included as additional control. Although we are aware that 48 h serum-free conditions could reduce cell proliferation, we checked whether this culture condition could induce apoptosis, finding that it was not the case. In fact, WB analysis for caspase-3 expression did not show the cleaved (i.e. active) form in any of our experimental conditions, while it was present in osteogenic cells treated with staurosporine (1 μ M, 6 h) as positive control (Fig. 1).

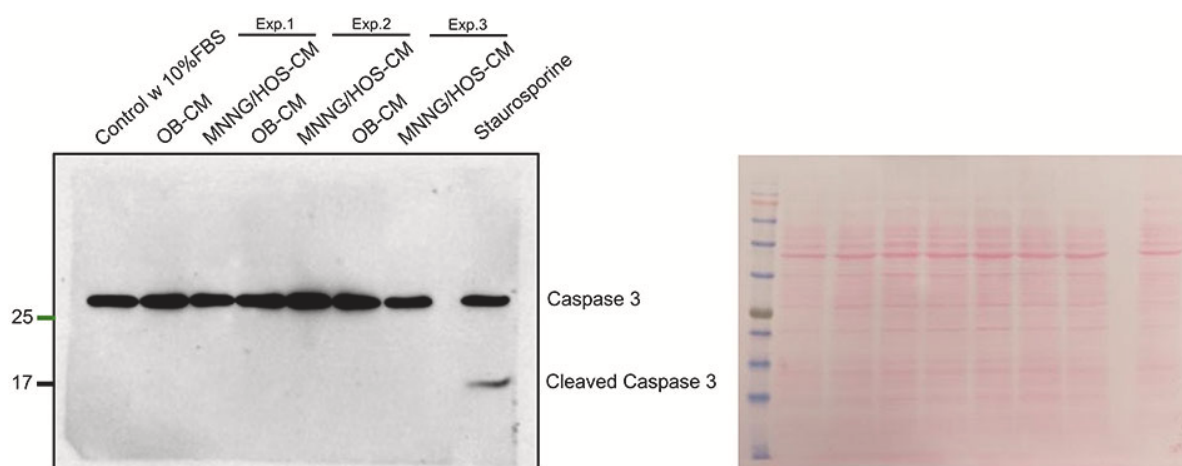


Figure 1. Effect of starvation, OB- and MNNG/HOS-CM on neonatal mouse calvarial osteogenic cell vitality and survival. Left: Neonatal mouse calvarial osteogenic cells were maintained in standard condition (DMEM+ 10 % FBS, n = 1) or starved for 24 h and then incubated (n = 3) with 24 h-conditioned medium (CM) collected from neonatal mouse calvarial osteogenic cells (OB-CM) or from MNNG/HOS cells (MNNG/HOS-CM). Staurosporine (1 μ M, 6 h) is used as apoptotic inducer, highlighted by the appearance of active cleaved caspase 3. Right: Red ponceau staining on the nitrocellulose membrane before incubation with anti-caspase 3 antibody.

Furthermore, these cells typically commit to the osteoblastic lineage upon exposure to mineralization medium, demonstrating a progressive increase in specific osteoblast markers throughout a 21-day culture period (Fig. 2).

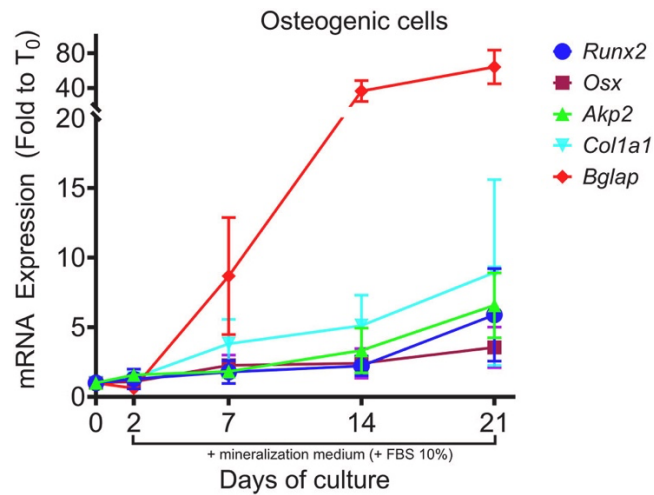


Figure 2. Osteogenic commitment of neonatal mouse calvarial osteogenic cells: Real time RT-PCR analysis to evaluate the transcriptional expression of osteoblastogenic genes Runx2, Osterix (Osx), Akp2 (coding for Alkaline Phosphatase), Collagen type I alpha 1 chain (Col1a1) and Bglap (coding for Osteocalcin) on total RNA isolated from neonatal mouse calvarial osteogenic cells maintained upon starvation for 48 h and then incubated with complete osteogenic medium up to 21 days. Data is the mean $\pm$ SD of 4 independent experiments.

Forty-eight hours of treatment with MNNG/HOS derived secretome did not affect neither Alkaline Phosphatase (Alp) activity, one of the earliest markers of osteogenic cells differentiation (Fig. 3a) nor the transcriptional profile of osteogenic cells differentiating genes, while a trend of reduction ($p = 0.06$) was observed for Cyclin D1 mRNA (Fig. 3b). We next evaluated protein expression of the proosteogenic transcription factors Runt-related transcription factor 2 (Runx2) and Osterix (osx) finding that the former was significantly reduced by treatment with MNNG/HOS-CM (Fig. 3c, d), while the late osteogenic marker osteocalcin (ocn) was present at the same levels in both conditions (Fig. 3e). Also Collagen I protein expression (Col I), which is indicative of osteoblast function, was not affected by treatment (Fig. 3f).

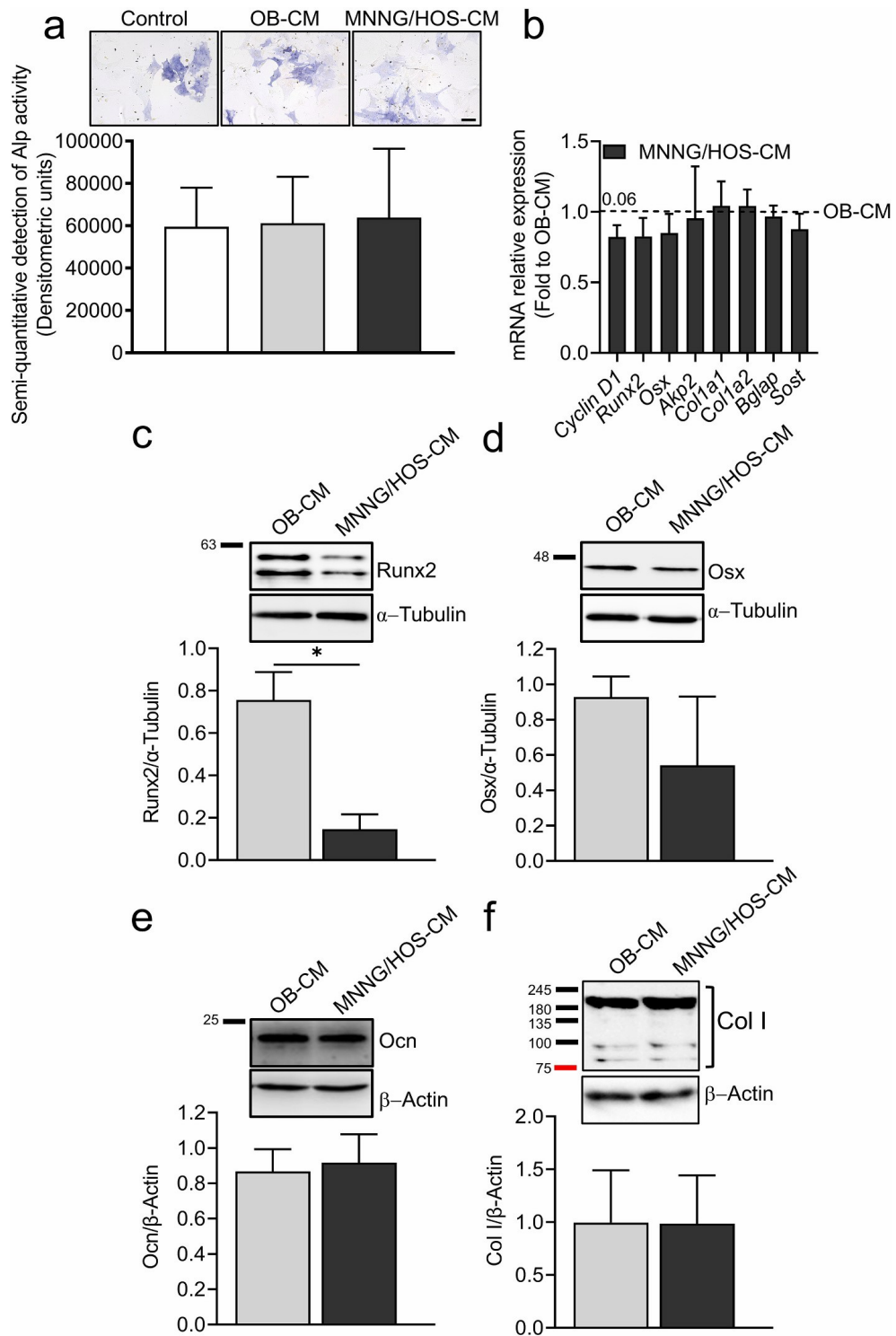


Figure 3. Effect of MNNG/HOS secretome on neonatal mouse calvarial osteogenic cell phenotype: Neonatal mouse calvarial osteogenic cells were starved for 24 h, then medium was replaced with serum free-DMEM (Control) or with 24 h-serum free conditioned medium (CM) collected from neonatal mouse calvarial osteogenic cells (OB-CM) or from MNNG/HOS cells (MNNG/HOS-CM) and cells were cultured for further 48 h. (a) Representative pictures (inset) of cytochemical AlP activity and quantification by densitometric analysis (scale bar = 100 μ m). (b) Real time RT-PCR analysis to evaluate the transcriptional expression of Cyclin D1, and of the osteoblastogenic genes Runx2, Osterix (Osx), Akp2 (coding for Alkaline Phosphatase), Collagen type I alpha 1 chain (Col1a1) and Col1a2, Bglap (coding for Osteocalcin), and Sclerostin (Sost). (c–f) Western blot and densitometric analysis to evaluate protein expression of (c) Runt-related transcription factor 2 (Runx2), (d) osterix (osx), (e) osteocalcin (ocn) and Collagen I (Col I). Alfa-tubulin and β -actin were also evaluated as housekeeping genes, depending on the molecular weight of the

target protein. Data are the mean $\pm$ SD of at least 3 independent experiments; * p = 0.01 vs OB-CM. (a, c-f)) paired t-test, (b) one-sample t-test.

Taken together, these results indicate that treatment for 48 h with MNNG/HOS secretome could affect osteoblast differentiation by a Runx2 dependent mechanism, while it did not alter the main functional markers; the lack of effect by osteoblast-CM treatment also excludes any autocrine conditioning.

We therefore decided to perform long-term treatments, refreshing the medium every 3 days. After 7 days of treatment, we observed no differences in Collagen I expression (Fig. 4a), although a likely immature form (Fig. 4a, bands at 180 kD) of Collagen showed a trend of decrease in osteogenic cells treated with MNNG/HOS-CM, as also demonstrated by densitometric analysis (Fig. 4a, graph). Interestingly, Collagen I release was dramatically reduced in osteogenic cells treated with MNNG/HOS compared to OB-CM treated or untreated osteogenic cells at 7, 14 and 21 days of culture in mineralization medium (Fig. 4b). No differences were found between untreated and OB-CM treated osteogenic cells.

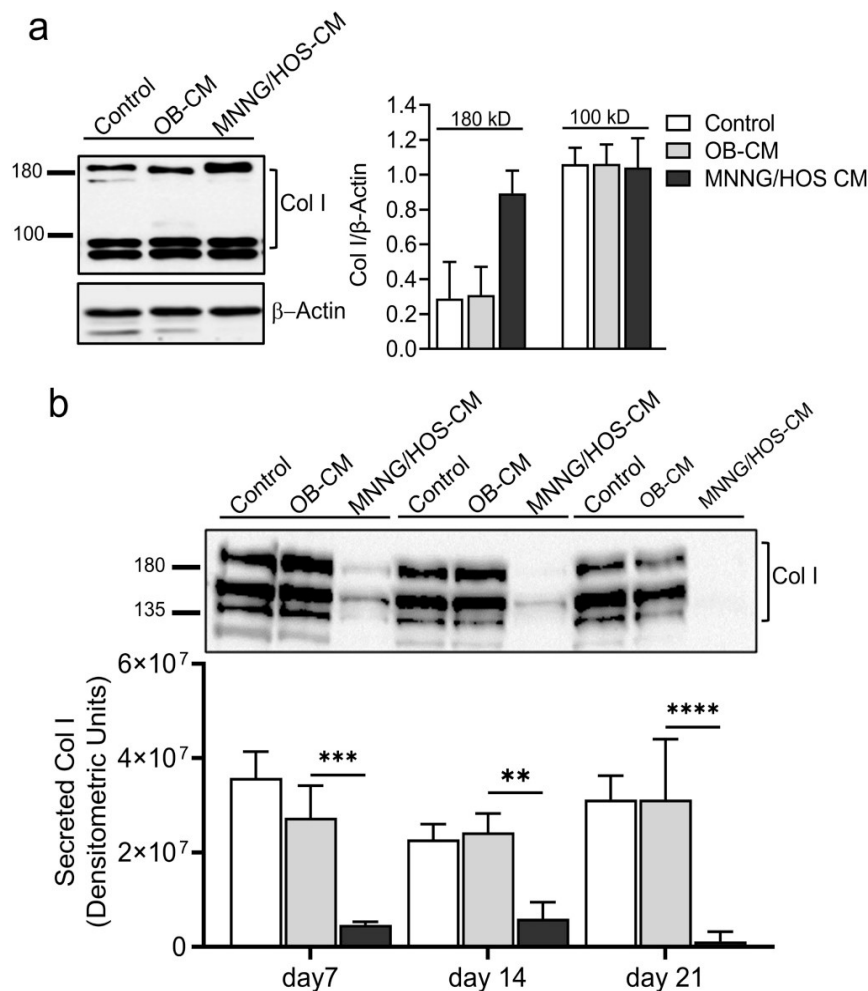


Figure 4. Effect of MNNG/HOS secretome on neonatal mouse calvarial osteogenic cell activity: (a) Neonatal mouse calvarial osteogenic cells were starved for 24 h, then medium was replaced with serum free-DMEM (Control) or with 24 h serum free conditioned medium (CM) collected from neonatal mouse calvarial osteogenic cells (OB-CM) or from MNNG/HOS cells (MNNG/HOS-CM) and cells were maintained in cultures for 7 days, adding 10 % FBS after the first 48 h of treatment. Western blot analysis to evaluate collagen I protein expression and quantification of the higher (180 kD) and lower (100 kD) bands of Collagen I, the former likely an immature form, by densitometric analysis (graph). Betaactin expression was also

evaluated as housekeeping gene. (b) Western blot analysis for Collagen I release assessed in conditioned media (CM) collected from neonatal mouse calvarial osteogenic cells cultured in mineralization medium and treated with OB-CM and MNNG/HOS-CM at the times indicated in the abscissa. Data are the mean $\pm$ SD of at least 3 independent experiments, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ vs OB-CM, two-way ANOVA.

We next asked whether this treatment may affect Alp activity, which was not the case at 7 days of treatment, while at day 14 and 21 a trend of reduction ($p = 0.1$) was observed in osteogenic cells treated with MNNG/HOS-CM compared to untreated or OB-CM treated osteogenic cells (Fig. 5a). Interestingly, osteoblast ability to produce nodules of mineralization, evaluated by von Kossa staining, was significantly impaired after 14 and 21 days of treatment with MNNG/HOS-CM (Fig. 5b). We also checked whether these modulations could be due to a direct effect on osteoblast number, finding that it was the case at 7 days of culture only, while at 14 days we observed a trend of reduction of osteoblast number by MNNG/HOS-CM, which however was unremarkable at 21 days of cultures (Fig. 5c).

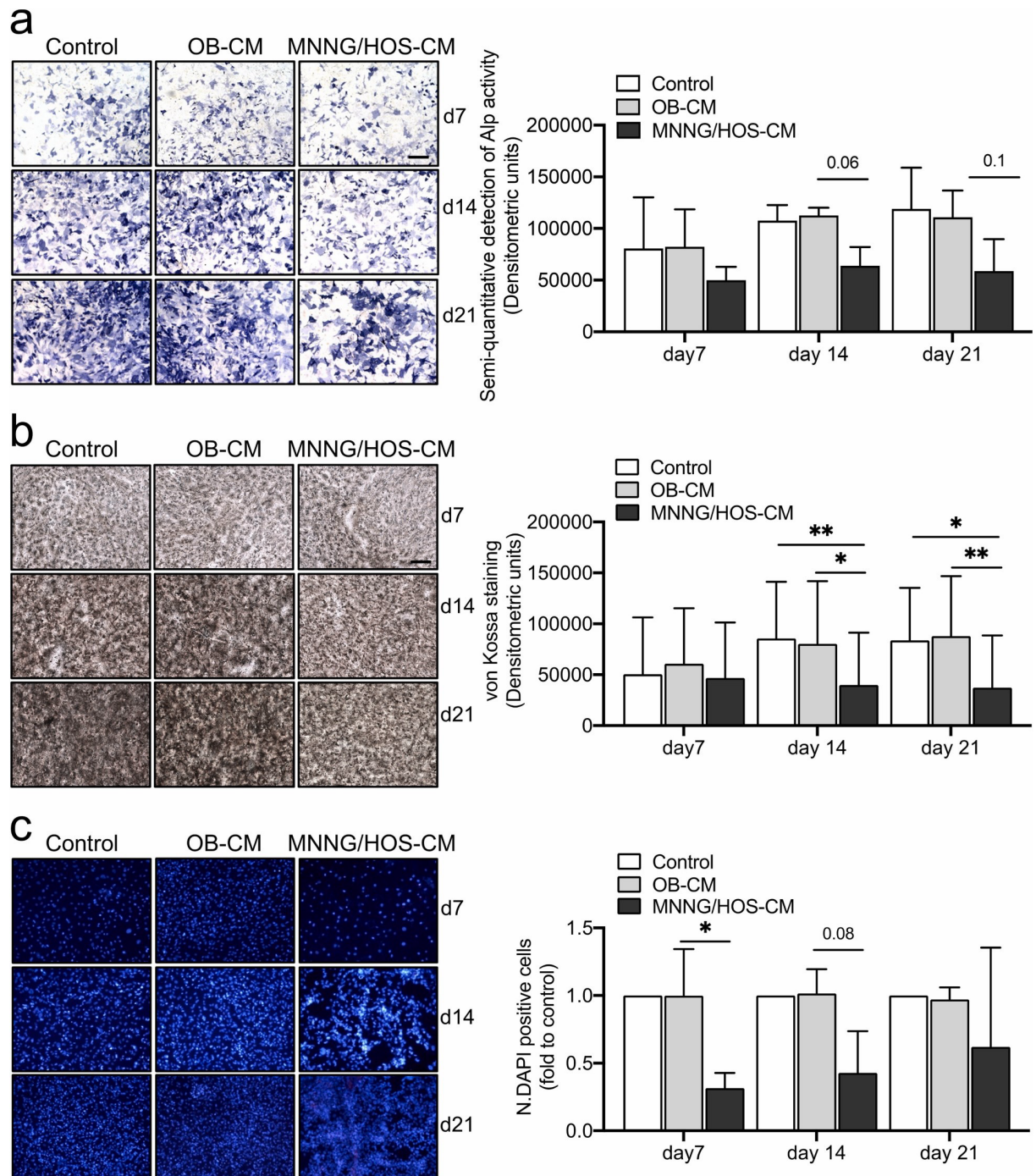


Figure 5. Effect of MNNG/HOS secretome on neonatal mouse calvarial osteogenic cell activity: Neonatal mouse calvarial osteogenic cells were cultured in mineralization medium (Control) and in mineralization medium supplemented with OB-CM or MNNG/HOS-CM for 7, 14 and 21 days. (a) Representative pictures (inset) of osteogenic cells subjected to cytochemical Alp activity and quantification by densitometric analysis (scale bar = 500 μ m). (b) Representative pictures (inset) of Von Kossa staining to evaluate nodules of mineralization and quantification by densitometric analysis (scale bar = 500 μ m). (c) Representative pictures (inset) of osteogenic cells subjected to DAPI staining and quantification of the number of positive nuclei/field (scale bar = 500 μ m). Data are the mean $\pm$ SD of 3 independent experiments, * p < 0.05 vs OB-CM, ** p < 0.01 vs CTRL; (a, b) paired Student's t-test, (c) one-sample t-test.

Taken together, these results indicate that by means of their secretome and after prolonged treatment, osteosarcoma cells significantly reduce collagen production and bone matrix mineralization.

3.2 Effect of osteosarcoma cells secretome on osteoblast release of nanoparticles

Considering the pivotal role exerted by NPs in cell-cell communication, we investigated whether MNNG/HOS-CM could also interfere with the release and the protein cargo of osteoblast-derived NPs (OB-NPs). To this aim, we treated primary murine osteogenic cells with serum-free conditioned medium (CM) of MNNG/HOS cells or osteogenic cells, then we collected osteogenic cells NPs from the CM (CMHOS-OB-NPs and CMOB-OB-NPs, respectively). The vesicular nature of the isolated particles was confirmed by TEM, which also showed the NPs membrane integrity (Fig. 6a). Nanosight analysis showed a similar mean diameter and number/ml of CMOB-OB-NPs and CMHOS-OB-NPs (Fig. 6b). Moreover, Western blot analysis demonstrated the expression of the typical NPs markers, such as Annexin II (Anxa II, Fig. 6c), CD63 (Fig. 6d), CD81 (Fig. 6e) and, interestingly, the latter was significantly upregulated in OB-NPs collected from osteogenic cells educated by MNNG/HOS-CM (CMHOSOB-NPs) compared to CMOB-OB-NPs.

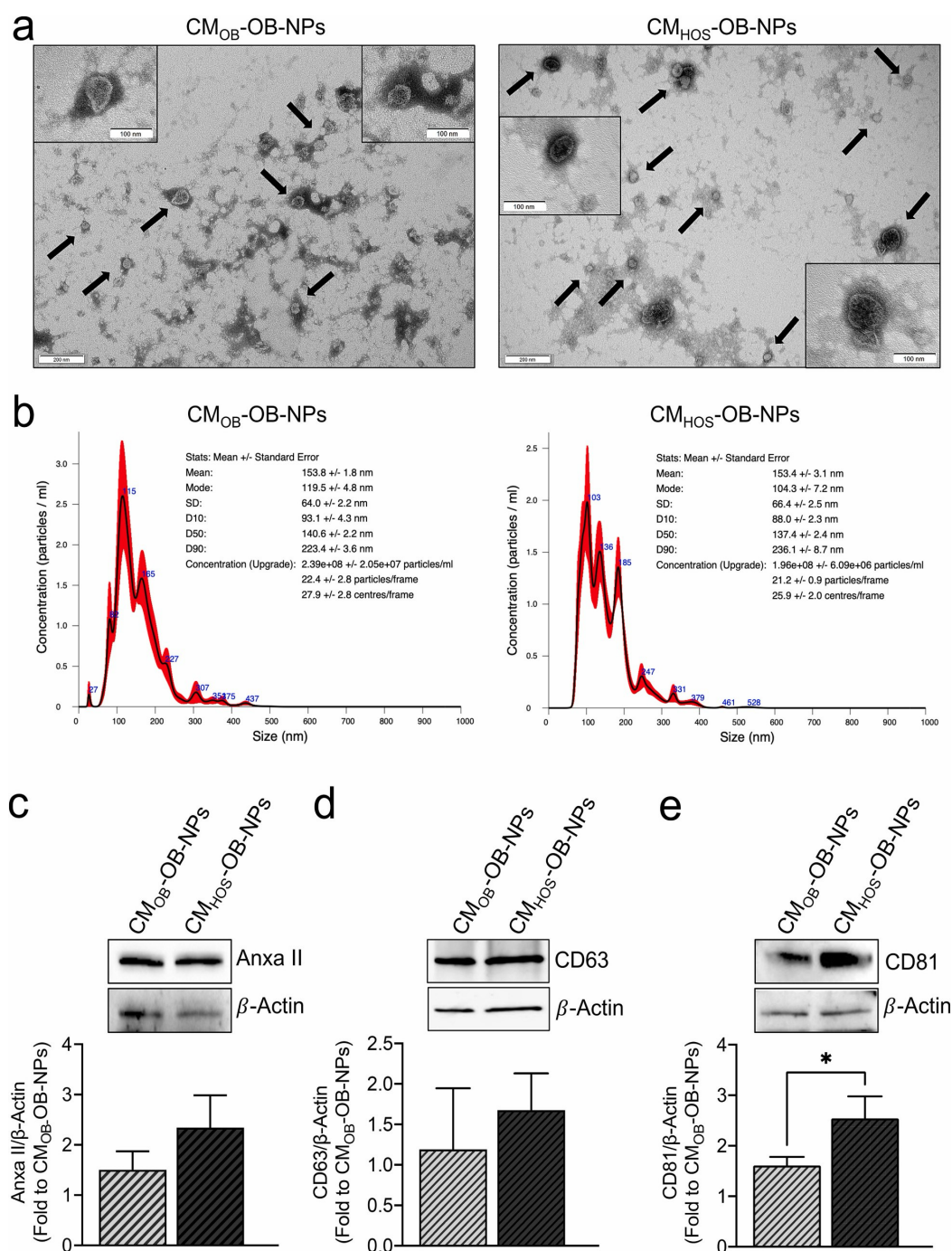


Figure 6. Characterization of nanoparticles (NPs) isolated from neonatal mouse calvarial osteogenic cells treated with OB-CM or MNNG/HOS-CM: Neonatal mouse calvarial osteogenic cells were starved for 24 h, then medium was replaced with serum free conditioned medium (CM) collected from neonatal mouse calvarial osteogenic cells (OB-CM) or from MNNG/HOS cells (MNNG/HOS-CM). After 24 h, neonatal mouse calvarial osteogenic cells were washed and maintained for further 24 h in serum-DMEM, then conditioned media were collected to isolate NPs. (a) Transmission electron microscopy (TEM) evaluation of the morphology of NPs (indicated by arrows) isolated from neonatal mouse calvarial osteogenic cells educated with OB-NPs) or with MNNG/HOS-CM (CM_{HOS} -OB-NPs). Scale bar = 200 μ m. Insets: higher magnification of the same NPs sample (scale bar = 100 μ m). (b) Size and concentration determination of CM_{OB} -OB-NPs and CM_{HOS} -OB-NPs by nanoparticle tracking analysis (NanoSight NS3000). (c–e) Western blot and densitometric analysis to evaluate protein expression of the NP markers (c) Annexin II (Anxa II), (d) CD63 and (e) CD81, and β -Actin as loading control in protein lysates (10 μ g) extracted from CM_{OB} -OB-NPs and CM_{HOS} -OB-NPs. Data are the mean $\pm$ SD of three independent preparations, *p = 0.025 vs paired Student's t-test.

3.3 Proteome profiling of osteoblast-derived nanoparticles preconditioned by osteosarcoma cells

We next investigated whether MNNG/HOS secretome could educate OB-NPs protein cargo by performing shotgun proteomics analysis on NPs protein lysates, using a nano LC-MS/MS system. A total of 286 NP proteins were identified and quantified by this approach and, among them, 36 and 12 proteins were exclusively quantified in CMOB-OB-NPs and CMHOS-OB-NPs, respectively (Fig. 7a and Tables 1 and 2). Moreover, we found that 99 % (284 out of 286) of these proteins were present in the Vesiclepedia database, while 74 out of 286 (i.e. 26 %) were included in the top 100 NP-Proteins reported in the database, suggesting a good enrichment. Using STRING platform, we investigated the ProteinProtein Interaction (PPI) network of the 36 proteins exclusively present in CMOB-OB-NPs, finding an enrichment of proteins involved in cell junction network, such as Protein deglycase DJ-1 (Park/DJ-1), 60S ribosomal protein L18 (RpL18), 40S ribosomal protein S18 (RpS18), RpL30, Proteasome subunit alpha type-7 (PsmA7), Carboxypeptidase E (CpE), WD repeat-containing (Wdr1), Radixin (Rdx), Ptk7, Uba1, Pppr1a, Adam10, Rab18, Rab7, vesicle-associated membrane protein 3 (Vamp3) and Syntaxin-4 (Stx4) was observed (Fig. 7b; blue nodes; $FDR = 3.91 \times 10^{-5}$), while no significant networks have been identified for the 12 proteins exclusively present in CMHOS-OB-NPs (Fig. 7c). We next focused on the 238 common expressed proteins and ran univariate statistical analysis through Perseus tool using a cut-off value of $p < 0.05$, which revealed the presence of 58 differentially expressed proteins (DEPs), among which 26 proteins were downregulated (Table 3) and 32 proteins were upregulated (Table 4) in CMHOS-OB-NPs compared to CMOB-OB-NPs (Fig. 7d).

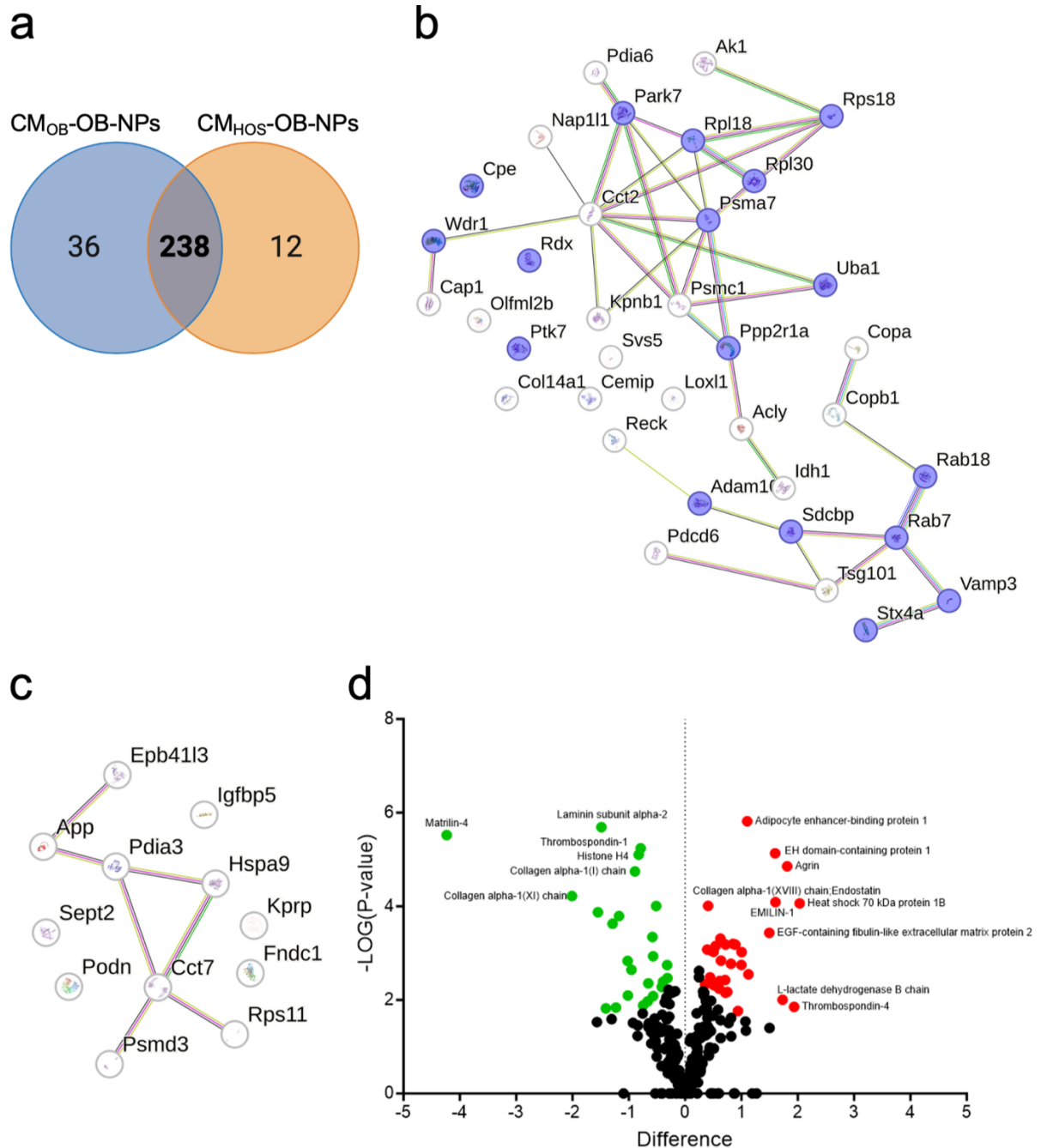


Figure 7. Proteome profiling of nanoparticles (NPs) isolated from osteogenic cells treated with OB-CM or MNNG/HOS-CM: Neonatal mouse calvarial osteogenic cells were starved for 24 h, then medium was replaced with serum free-DMEM (Control) or with 24 h-conditioned medium (CM) collected from neonatal mouse calvarial osteogenic cells (OB-CM) or from MNNG/HOS cells (MNNG/HOS-CM). After 24 h, osteogenic cells were washed and maintained for further 24 h, then conditioned media were collected to isolate NPs and perform proteome analysis. (a) Representative Venn diagram showing the number of unique and commonly shared proteins between CM_{OB}-OB-NPs and CM_{HOS}-OB-NPs groups. (b) Representative Protein-Protein Interaction (PPI) network of proteins uniquely quantified in CM_{OB}-OB-NPs, generated by STRING online tool. Nodes represent proteins (N = 36), and edges indicate the number of interactions (N = 38). Colour saturation of the edges represents the confidence score of a functional association. The number of average interactions per node is indicated by the node degree (N = 2.11). The clustering coefficient specifies the average node density of the map (N = 0.427). Only interactions with a medium confidence score ≥ 0.4 are shown. (c) Representative PPI network of proteins only quantified in CM_{HOS}-OB-NPs, generated by STRING online tool. Nodes represent proteins (N = 12), and edges indicate the number of interactions (N = 7). Colour saturation of the edges represents the confidence score of a

functional association. The number of average interactions per node is indicated by the node degree ($N = 1.17$). The clustering coefficient specifies the average node density of the map ($N = 0.375$). Only interactions with a medium confidence score ≥ 0.4 are shown. (d) Volcano plot representation of differential expressed proteins (DEPs) among CMOB-OB-NPs and CMHOS-OB-NPs groups. Red and green dots highlight significantly upregulated or downregulated proteins in CMHOS-OB-NPs versus CMOB-OB-NPs, respectively (P -value < 0.05). The x-axis shows the difference in proteins expression and the y-axis – LOG(P -Value) rate of proteins being differentially expressed.

Table 1

List of the proteins exclusively expressed by NPs isolated from osteogenic cells educated with OB-CM.

Protein name	Gene name	Intensity sample #1	Intensity sample #2	Intensity sample #3	Mean intensity
Syntenin-1	<i>Sdcbp</i>	28,922,000	17,143,000	18,599,000	21,554,667
Adenylyl cyclase-associated protein 1	<i>Cap1</i>	7,061,700	6,065,200	6,190,000	6,438,967
Semenogelin-1	<i>Semg1</i>	7,759,400	5,472,500	5,504,900	6,245,600
Lysyl oxidase homolog 1	<i>Loxl1</i>	5,220,200	4,545,800	5,583,700	5,116,567
T-complex protein 1 subunit beta	<i>Cct2</i>	4,841,900	5,793,700	4,579,500	5,071,700
Proteasome subunit alpha type-7	<i>PsmA7</i>	4,042,900	3,937,900	5,004,100	4,328,300
Ras-related protein Rab-7a	<i>Rab7A</i>	2,906,100	2,920,600	2,884,200	2,903,633
Inactive tyrosine-protein kinase 7	<i>Ptk7</i>	2,599,100		2,698,200	2,648,650
Olfactomedin-like protein 2B	<i>Olfml2B</i>	3,009,000	2,217,800	2,480,900	2,569,233
Protein deglycase DJ-1	<i>Park7</i>	2,583,800	2,425,300	1,975,500	2,328,200
40S ribosomal protein S18	<i>RpS18</i>	2,246,800	2,402,500	2,123,300	2,257,533
Ubiquitin-like modifier-activating enzyme 1	<i>Uba1</i>	2,261,400	2,145,900	2,267,700	2,225,000
ATP-citrate synthase	<i>Acly</i>	2,003,400	2,345,400	2,305,600	2,218,133
Syntaxin-4	<i>Stx4</i>	2,268,500	2,284,600	2,082,700	2,211,934
Vesicle-associated membrane protein 3	<i>Vamp3</i>	1,521,400	1,911,800	2,524,500	1,985,900
Adenylate kinase isoenzyme 1	<i>Ak1</i>	1,603,000	1,559,500	1,738,800	1,633,767
Importin subunit beta-1	<i>KpnB1</i>	1,265,600	1,839,900	1,447,900	1,517,800
WD repeat-containing protein 1	<i>Wdr1</i>	1,184,500	1,784,100	1,548,200	1,505,600
Serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A alpha isoform	<i>Pppr1A</i>	1,096,400	1,665,500	1,713,600	1,491,833
60S ribosomal protein L18	<i>RpL18</i>	1,692,700	1,326,900	1,388,800	1,469,467
Nucleosome assembly protein 1-like 1	<i>Nap1l1</i>	1,205,800	1,711,500	1,444,700	1,454,000
60S ribosomal protein L30	<i>RpL30</i>	1,001,600	1,512,600	1,707,200	1,407,133
Ras-related protein Rab-18	<i>Rab18</i>		1,211,100	1,540,500	1,375,800
Collagen alpha-1(XIV) chain	<i>Col14A1</i>	1,452,500	1,259,400	1,351,800	1,354,567
Cell migration-inducing and hyaluronan-binding protein	<i>Cemip</i>	917,060	1,811,900	1,039,600	1,256,187
26S protease regulatory subunit 4	<i>Psmc1</i>	1,363,900		914,240	1,139,070
Programmed cell death protein 6	<i>Pdcd6</i>	873,120		1,318,700	1,095,910
Carboxypeptidase E	<i>Cpe</i>	996,570	1,124,000		1,060,285
Disintegrin and metalloproteinase domain-containing protein 10	<i>Adam10</i>		967,480	1,013,700	990,590
Coatomer subunit alpha	<i>CopA</i>	798,280	1,145,100	990,780	978,053
Protein disulfide-isomerase A6	<i>PdiA6</i>	659,190	899,590	924,310	827,697
Isocitrate dehydrogenase [NADP] cytoplasmic	<i>Idh1</i>		966,210	670,640	818,425
Radixin	<i>Rdx</i>		552,560	814,381	683,470
Reversion-inducing cysteine-rich protein with Kazal motifs	<i>Reck</i>	683,490	611,110	677,050	657,217
Tumour susceptibility gene 101 protein	<i>Tsg101</i>	570,180	635,600		602,890
Coatomer subunit beta	<i>CopB1</i>		507,350	687,550	597,450

Table 2

List of the proteins exclusively expressed by NPs isolated from osteogenic cells educated with MNNG/HOS-CM.

Protein name	Gene name	Intensity sample #1	Intensity sample #2	Intensity sample #3	Mean intensity
Insulin-like growth factor-binding protein 5	<i>Igfbp5</i>	5,505,900	5,388,900		5,447,400

Fibronectin type III domain-containing protein 1	<i>Fndc1</i>	6,111,400	4,069,000	4,703,000	4,961,133
Amyloid beta A4 protein	<i>App</i>	5,787,600	3,728,200	4,053,100	4,522,967
T-complex protein 1 subunit eta	<i>Cct7</i>	3,900,400	4,451,800	3,844,000	4,065,400
Septin-2	<i>Sept2</i>	2,710,900		3,569,100	3,140,000
Podocan	<i>Podn</i>	3,345,000	2,961,900	2,795,000	3,033,967
Protein disulfide-isomerase A3	<i>Pdia3</i>	2,485,100		2,602,700	2,543,900
40S ribosomal protein S11	<i>RpS11</i>	2,472,100	2,406,100		2,439,100
Keratinocyte proline-rich protein	<i>Kprp</i>		2,751,100	2,112,700	2,431,900
Stress-70 protein, mitochondrial	<i>Hspa9</i>	1,804,900	2,051,300	2,846,100	2,234,100
Band 4.1-like protein 3	<i>Epb41l3</i>		1,434,600	2,302,600	1,868,600
26S proteasome non-ATPase regulatory subunit 3	<i>Psmc3</i>	1,464,800	1,384,700	1,619,900	1,489,800

Table 3

List of the downregulated proteins expressed by NPs from osteogenic cells educated with MNNG/HOS-CM compared to OB-CM.

Protein names	Gene names	Intensity	- LOG(P-value)	Difference
Matrilin-4	<i>Matn4</i>	176,420,000	5.52070607	-
Collagen alpha-1(XI) chain	<i>Col11a1</i>	39,247,000	4.21926429	-
Collagen alpha-1(II) chain;Collagen alpha-1(II) chain;Chondrocalcin	<i>Col2a1</i>	427,360,000	3.87160898	-
Laminin subunit alpha-2	<i>Lama2</i>	55,176,000	5.69126321	-
Pappalysin-2	<i>Pappa2</i>	10,666,000	1.8199685	-
EGF-like repeat and discoidin I-like domain-containing protein 3	<i>Edil3</i>	415,360,000	3.62636911	-
Collagen alpha-1(V) chain	<i>Col5a1</i>	93,524,000	1.83667725	-
Clathrin heavy chain 1	<i>Cltc</i>	143,950,000	3.79122046	-
Glypican-1;Secreted glypican-1	<i>Gpc1</i>	9,405,000	2.83164664	-
Proteasome subunit alpha type-3	<i>Psmc3</i>	25,011,000	2.09326969	-
Histone H2B type 1-L;Histone H2B type 1-M;Histone H2B type 1-N;Histone H2B type 1-H;Histone H2B type 2-F;Histone H2B type 1-C/E/F/G/I;Histone H2B type 1-D;Histone H2B type F-S;Histone H2B type 1-K;Histone H2B type 1-A	<i>Hist1h2bl;Hist1h2bm;Hist1h2bn;Hist1h2bh;Hist1h2bf;Hist1h2bc;Hist1h2bd;H2bfs;Hist1h2bk;Hist1h2ba</i>	717,220,000	2.6438692	-
Collagen alpha-1(I) chain	<i>Col1a1</i>	2.1285E+10	4.74550161	-
Histone H4	<i>Hist1h4a</i>	879,440,000	5.10249649	-
Thrombospondin-1	<i>Thbs1</i>	364,600,000	5.23887335	-
Annexin A4	<i>Anxa4</i>	51,814,000	1.88090213	-
Histone H3.2;Histone H3.1t;Histone H3.3;Histone H3.1;Histone H3.3C	<i>Hist2h3a;Hist3h3;H3f3a;Hist1h3a;H3f3c</i>	230,280,000	1.96855332	-
Matrilin-2	<i>Matn2</i>	17,788,000	2.35433429	-
Collagen alpha-1(VI) chain	<i>Col6a1</i>	205,760,000	3.34345265	-
Collagen alpha-2(VI) chain	<i>Col6a2</i>	78,024,000	2.93384691	-
Histone H2A type 1-J;Histone H2A type 1-H;Histone H2A.J;Histone H2A type 2- C;Histone H2A type 2-A;Histone H2A type 1-D;Histone H2A type 1	<i>Hist1h2aj;Hist1h2ah;H2aff;Hist2h2ac;Hist2h2aa3;Hist1h2ad;Hist1h2ag</i>	432,340,000	2.08188306	-
Nidogen-2	<i>Nid2</i>	224,190,000	4.0055587	-
Annexin A2;Putative annexin A2-like protein	<i>Anxa2;Anxa2p2</i>	645,260,000	2.2776614	-
Collagen alpha-2(I) chain	<i>Col1a2</i>	3,187,900,000	2.27678111	-
Dickkopf-related protein 3	<i>Dkk3</i>	10,543,000	2.38900264	-

Tubulin alpha-1B chain;Tubulin alpha-1C chain;Tubulin alpha-1A chain;Tubulin alpha-4A chain;Tubulin alpha-8 chain;Tubulin alpha-3E chain	<i>Tuba1b;Tuba1c;Tuba1a;Tuba4a;Tuba8;Tuba3e</i>	158,480,000	2.74436085	– 0.3184191
Vimentin	<i>Vim</i>	670,390,000	2.46657485	– 0.3152637

Table 4

List of the upregulated proteins expressed by NPs from osteogenic cells educated with MNNG/HOS-CM compared to OB-CM.

Protein names	Gene names	Intensity	-LOG(P-value)	Difference
Heat shock 70 kDa protein 1B;Heat shock 70 kDa protein 1A	<i>Hspa1b;Hspa1a</i>	54339000	4.06088885	2.03280258
Thrombospondin-4	<i>Thbs4</i>	132570000	1.85001867	1.93524551
Agrin;Agrin N-terminal 110 kDa subunit;Agrin C-terminal 110 kDa subunit;Agrin C-terminal 90 kDa fragment;Agrin C-terminal 22 kDa fragment	<i>Agrn</i>	73875000	4.85331711	1.81249746
L-lactate dehydrogenase B chain	<i>Ldhb</i>	66257000	2.00185451	1.73106257
Emilin-1	<i>Emilin1</i>	145570000	4.08815299	1.60500654
EH domain-containing protein 1	<i>Ehd1</i>	42768000	5.13206462	1.59648577
EGF-containing fibulin-like extracellular matrix protein 2	<i>Efemp2</i>	170720000	3.43182328	1.49389267
Angiopoietin-related protein 2; Angiopoietin-like protein 2	<i>Angptl2</i>	6454600	2.54736274	1.12344233
Adipocyte enhancer-binding protein 1	<i>Aebp1</i>	196600000	5.81770225	1.10289383
Cytoplasmic FMR1-interacting protein 1	<i>Cytip1</i>	9914700	3.02297916	1.00271924
Heat shock protein HSP 90-alpha	<i>Hsp90aa1</i>	26915000	2.74798171	0.99891027
MARCKS-related protein	<i>Marcksl1</i>	9977700	1.76310088	0.93705591
Collagen alpha-1(III) chain	<i>Col3a1</i>	1442200000	3.18121429	0.89073181
Follistatin-related protein 1	<i>Fstl1</i>	169500000	3.19555273	0.85017331
Ras-related protein Rap-1b;Ras-related protein Rap-1b-like protein	<i>Rap1b</i>	67189000	2.76982559	0.81293933
SPARC-related modular calcium-binding protein 1	<i>Smoc1</i>	18267000	2.17013636	0.7474378
EH domain-containing protein 4	<i>Ehd4</i>	21564000	3.18070672	0.71789932
ADP-ribosylation factor 1;ADP-ribosylation factor 3	<i>Arf1;Arf3</i>	14561000	2.15182204	0.71670151
Peroxiredoxin-6	<i>Prdx6</i>	6392000	2.42390695	0.7093722
Myosin-9	<i>Myh9</i>	119610000	2.83773712	0.63712756
Triosephosphate isomerase	<i>Tpi1</i>	61900000	3.30872312	0.62577184
14-3-3 protein gamma;14-3-3 protein gamma, N-terminally processed	<i>Ywhag</i>	88577000	2.4032687	0.61179479
T-complex protein 1 subunit theta	<i>Cct8</i>	31896000	2.23734932	0.60697492
Bone morphogenetic protein 1	<i>Bmp1</i>	29794000	3.15288937	0.53964996
14-3-3 protein epsilon	<i>Ywhae</i>	56843000	2.31091587	0.51879374
Collagen alpha-2(IV) chain;Canstatin	<i>Col4a2</i>	41082000	3.02652277	0.50358772
Fructose-bisphosphate aldolase A	<i>Aldoa</i>	113270000	2.33841695	0.4782842
Peroxiredoxin-1	<i>Prdx1</i>	47022000	3.05399173	0.46403821
GTP-binding nuclear protein Ran	<i>Ran</i>	23875000	2.48040243	0.44186783
Collagen alpha-1(XVIII) chain;Endostatin	<i>Col18a1</i>	26430000	4.00811379	0.40743128

Pyruvate kinase PKM	<i>Pkm</i>	115230000	3.07514333	0.39375242
Collagen alpha-2(V) chain	<i>Col5a2</i>	472230000	2.33175076	0.3542188

Hence, to deeply characterize the pathways and processes in which these DEPs could be involved, we performed gene ontology (GO) analysis using DAVID tool and a cut-off value of FDR < 0.05. GO of biological processes revealed that most of the proteins were significantly enriched in elastic fiber assembly, microtubule-based processes, and collagen fibril organization (Fig. 8a). Moreover, GO analysis of cellular components identified proteins significantly enriched in collagen type I trimer, collagen type XI trimer, and basement membrane components (Fig. 8b), while GO enriched molecular functions included, among all, extracellular matrix structural constituents conferring tensile strength, proteoglycan binding, and collagen binding (Fig. 8c). Finally, Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway analysis revealed that proteins were significantly enriched in ECM-receptor interaction, and in protein digestion and absorption (Fig. 8d).

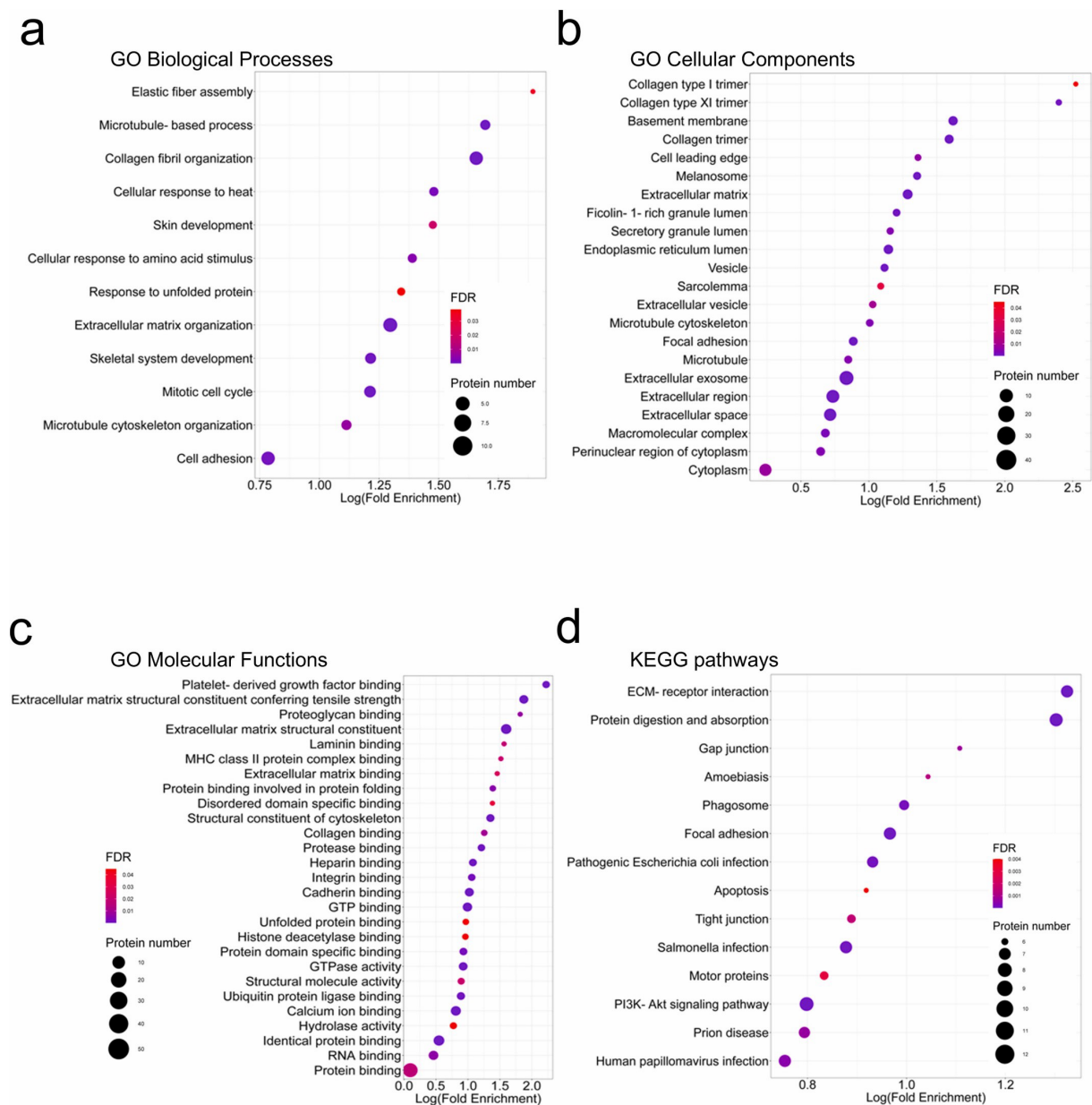


Figure 8. Gene ontology (GO) enrichment analysis of DEPs between CMOB-OB-NPs and CMHOS-OB-NPs. Neonatal mouse calvarial osteogenic cells were starved for 24 h, then medium was replaced with serum free-DMEM (Control) or with 24 h-conditioned medium (CM) collected from neonatal mouse calvarial osteogenic cells (OBCM) or from MNNG/HOS cells (MNNG/HOS-CM). After 24 h, osteogenic cells were washed and maintained for further 24 h, then conditioned media were collected to isolate NPs and perform proteome analysis. Representative dot-plots of gene ontology (GO) enriched terms for (a) biological processes, (b) cellular components and (c) molecular functions. GO processes with the largest LOG (Fold Enrichment) are plotted in order of the LOG (Fold Enrichment). The size of the dots represents the number of proteins in the significant DEPs list associated with the GO term and the colour of the dots represent the False Discovery Rate (FDR) values. (d) Representative dot-plot of KEGG enriched pathways. Pathways with the largest LOG (Fold Enrichment) are plotted in order of the LOG (Fold Enrichment). The size of the dots represents the number of proteins in the significant DEPs list associated with the GO term and the colour of the dots represent the False Discovery Rate (FDR) values.

Taken together, these findings suggest that MNNG/HOS secretome exerts a direct effect on osteoblast-NPs, reprogramming their protein profile and subsequently influencing extracellular matrix composition and collagen formation, as corroborated by in vitro data.

Since GO and KEGG analyses on the 58 DEPs gave us a global view of the processes and pathways influenced by MNNG/HOS cells secretome, we used STRING platform to perform a functional enrichment analysis of the Protein-Protein Interaction network (PPI). As described in Fig. 9a, among the downregulated NP proteins identified in OB-NPs from MNNG/HOS-educated osteogenic cells, we found: i) proteins involved in the extracellular matrix organization, such as matrillin (Matn)4 and Matn2, and collagens, such as Col Ia1, Col Ia2, Col IIa1, Col Va1, Col Xla1 (green nodes; FDR = 5.04×10^{-5}), ii) extracellular matrix structural constituents (red nodes; FDR = 1.71×10^{-7}), such as Laminin (Lama) 2 and collagens, and iii) collagen-containing extracellular matrix (Anxa4, Matn2, Matn4, Gpc1, Thbs1, Nid2, Lama2, Col Ia1, Col Ia2, Col IIa1, Col Va1, Col Vla1, Col Vla2, Col Xla1, blue nodes; FDR = 1.17×10^{-16}). We then focused on the most downregulated protein, that is Matn4 (Fold Change = - 4.23, $p = 3.32 \times 10^{-6}$), due to its role as a downstream orchestrator of the collagens network and confirmed, by Western blot, that pretreatment with MNNG/HOS-CM significantly reduced its content in the OB-NPs (Fig. 9b). Similarly, among the NP proteins isolated from MNNG/HOS educated osteogenic cells, the PPI analysis showed an increased content of: i) proteins involved in the regulation of collagen fibril organization (Fig. 9c; green nodes; FDR = 0.0016), such as EGF-containing fibulin-like extracellular matrix protein 2 (Efemp2), Adipocyte enhancer-binding protein-1 (Aebp1) and Emilin1, ii) extracellular matrix structural constituents (Fig. 7c; red nodes; FDR = 0.00023), such as Emilin-1 and collagens (Col IIIa1, Col IVa2, Col Va2, Col XVIIIa1), and iii) collagen-containing extracellular matrix (Fig. 9c; blue nodes; FDR = 2.44×10^{-11}), such as Pyruvate kinase PKM (Pkm), SPARC-related modular calcium-binding protein 1 (Smoc1), Angiopoietin-related protein 2 (Angptl2), Hsp90a, Agrin (Agrn), Bmp1, thrombospondin 4 (Thbs4), Efemp2 and collagens (Col IIIa1, Col IVa2, Col Va2, Col XVIIIa1).

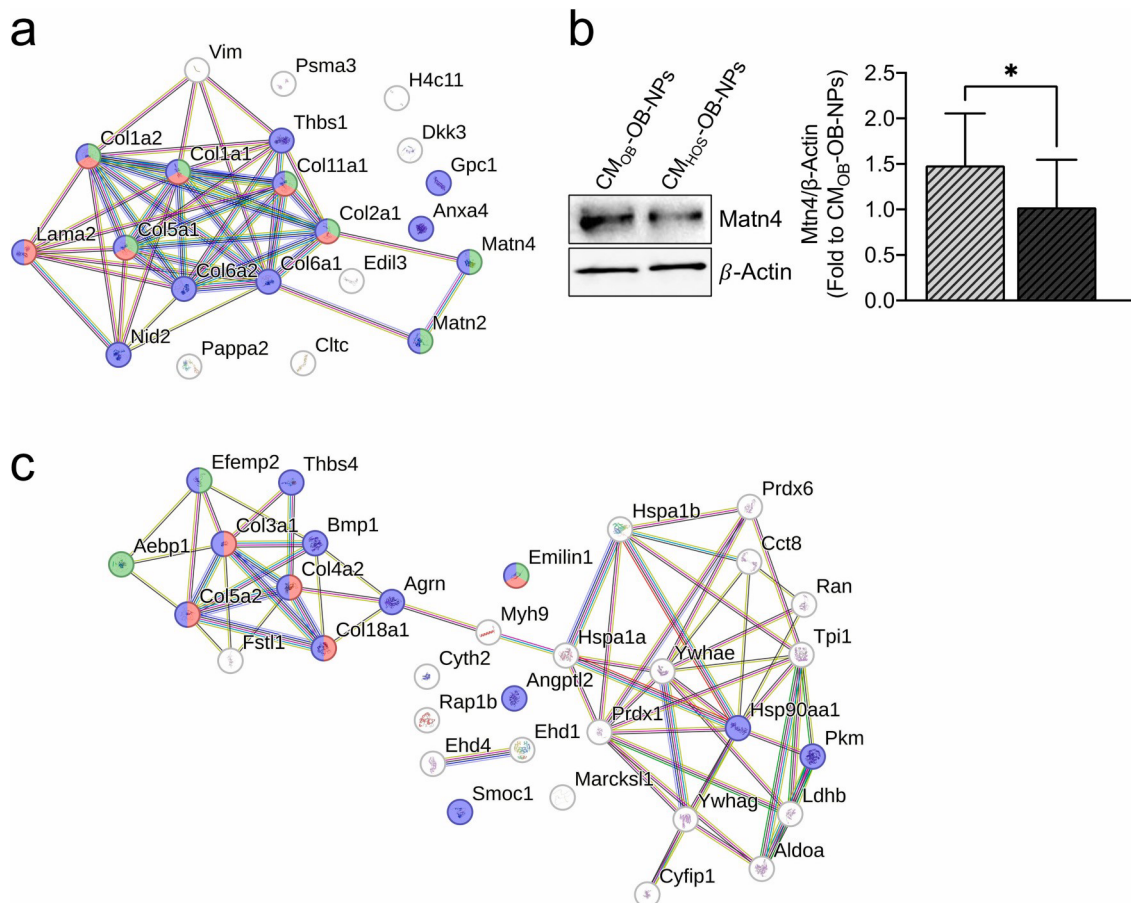


Figure 9. Protein-Protein Interaction (PPI) network analysis of DEPs between CMOB-OB-NPs and CMHOS-OB-NPs (a) Representative PPI network of the downregulated proteins in CMHOS-OB-NPs, generated by STRING online tool. Nodes represent proteins (N = 21), and edges indicate the number of interactions (N = 128). Colour saturation of the edges represents the confidence score of a functional association. The number of average interactions per node is indicated by the node degree (N = 4.38). The clustering coefficient specifies the average node density of the map (N = 0.439). Only interactions with a medium confidence score ≥ 0.4 are shown. (b) Western blot and densitometric analysis to evaluate protein expression of Matn4 and β -actin as loading control in protein lysates (10 μ g) extracted from CMOB-OB-NPs and CMHOS-OB-NPs. (c) Representative PPI network of the upregulated proteins in CMHOS-OB-NPs, generated by STRING online tool. Nodes represent proteins (N = 33), and edges indicate the number of interactions (N = 65). Colour saturation of the edges represents the confidence score of a functional association. The number of average interactions per node is indicated by the node degree (N = 3.94). The clustering coefficient specifies the average node density of the map (N = 0.535). Only interactions with a medium confidence score ≥ 0.4 are shown.

4. Discussion and conclusion

Osteosarcoma is a high-grade tumour of bone deriving from a malignant transformation of MSCs, leading to the production of an osteoid-like matrix with different degrees of mineralization [5]. Osteosarcoma growth strictly relies on a complex crosstalk among tumour cells, resident cells, and the bone matrix [5,6]. With this in mind, we treated osteogenic cells with MNNG/HOS derived secretome for a short period of time (i.e. 2 days), which, apart from a reduction of the master gene Runx2, failed to influence the transcriptional and protein expression of genes related to osteoblast differentiation and activity. The specific downregulation of Runx2 without observable effects on osteoblast

activity markers, at the time points examined, suggests that the tumoral secretome inhibits osteogenic differentiation at its earliest stages by targeting the upstream master regulator Runx2, requiring downstream differentiation markers additional days to reflect this inhibition, consistent with protein turnover rates. Alternatively, the tumour secretome might selectively uncouple osteogenic differentiation from osteoblast function. However, this observation aligns with previously reported mechanisms in tumour cell-osteoblast crosstalk, making it a plausible biological event [20,21]. Since it is known that tumour cells are able to deregulate the extracellular matrix components, we wonder whether osteosarcoma-derived secretome could educate osteogenic cells production and release of the main constituent of the bone matrix, that is Collagen type I, as well as osteocalcin, which is also a late marker of osteoblast differentiation, however this was not the case. Nevertheless, we asked whether a prolonged exposure to MNNG/HOS secretome could influence osteoblast phenotype, finding a trend of reduction of ALP activity, one of the early markers of osteoblast differentiation, after 14 and 21 days of treatment, along with a reduction, at least after 7 and 14 days of culture of osteoblast number. This effect is observed in osteogenic cells cultured in the presence of a mineralization medium, which also has osteogenic properties. Interestingly, education of osteogenic cells with MNNG/HOS secretome significantly reduced Collagen type I release and deposition already at 7 days of treatment, and this reduction is more consistent after 21 days of treatment. Again, no differences are observed between untreated osteogenic cells and osteogenic cells treated with osteoblast-derived secretome. Consistent with a lesser content of Collagen type I, MNNG/HOS secretome-educated osteogenic cells showed a lower ability to form nodule of mineralization. This interesting result is in line with Cmoche et al., who found that stimulation of mineralization by treating osteosarcoma cells with ascorbic acid and B-glycerophosphate impaired their invasive properties by inhibiting invadopodia formation [22]. Taken together, this data demonstrates that osteosarcoma-derived secretome primarily affects neonatal mouse calvarial osteoblast's function and number, eventually leading to a bone matrix whose composition in terms of quantity of collagen fibres and nodule of mineralization is impaired, thus concurring to the production of a pathogenic osteoid matrix. It is well known the crucial role exerted by NPs in the crosstalk between osteogenic cells and tumour cells. Therefore, we next investigated whether osteosarcoma cells secretome is able to influence the release of osteoblast-NPs as well as NPs protein profile. We found no significant difference in terms of NPs content and size released by neonatal mouse calvarial osteogenic cells subjected to an autocrine regulation versus osteogenic cells educated by MNNG/HOS-CM, however in the latter we observed a higher expression of the NPs marker CD81. Besides the role of this tetraspanin as a NPs marker, it is noteworthy that CD81 has also been described as a pro-tumoral factor in osteosarcoma. Indeed, Li et al. found CD81 to be one of the genes aberrantly regulated in human osteoblast FOB1.19 cells transformed by treatment with the carcinogenic promoting agent 12-O-tetradecanoyl phorbol-13-acetate (TPA) [23]. Consistently, Mizishori et al. [24] found that inhibition of CD81 reduces tumourigenesis and lung metastasis in osteosarcoma cells. Regarding proteomic analysis, we identified 238 proteins commonly present in both types of OB-NPs, while 36 and 12 proteins were uniquely detected in

CMOB-OB-NPs and CMHOS-OB-NPs, respectively. While current technological limitations make it challenging to effectively distinguish between proteins intrinsically constituting NPs and those present in the microenvironment and subsequently docked to their surface, which form the so-called “corona coating”, the biological meaning of these proteins remains undiminished, playing a crucial role in the biological context of NPs functions. Looking at the shared proteins, and in agreement with the *in vitro* data, we found that treatment with osteosarcoma secretome downregulates Col Ia1 and Col Ia2 expression in NPs, along with other collagens (Col XIa1, Col IIa1, Col Va1, Col VIa1 and α 2) as well as bone matrix proteins like Laminin α 2 subunits, Nidogen-2, Thrombospondin-1 and the proteoglycan Glypican-1. However, also among the upregulated proteins we found some collagens, such as Col IIIa1, Col IVa2, Col XVIIIa1, and Col Va2, as well as Thrombospondin-4. Therefore, osteosarcoma secretome downregulates the expression of Collagen type I, which is the most abundant component (>90 %) of the organic part of the bone matrix and deregulates the expression of other collagens and non-collagen proteins of the bone matrix, eventually leading to an aberrant expression of the bone matrix constituents. Of note, and consistent with the above results, the most downregulated protein resulted to be Matn4 [24,25], and downregulation of Matn2 was also observed. These proteins are members of the matrilin family, which includes four oligomeric extracellular proteins that are involved in the development and homeostasis of cartilage and bone [26]. While Matn1 (also known as cartilage matrix protein or CMP) and Matn3 are mainly expressed in cartilage and have a role in cartilage and bone development, Matn2 and 4 are present in a wide variety of extracellular matrices [27], where they exert a prominent role in stabilizing the extracellular matrix structure, by self-associating into supramolecular structures, resulting in the formation of filamentous networks [27]. Therefore, by downregulating these proteins, MNNG/HOS secretome likely impairs the bone matrix structure. Going to analyse the upregulated proteins found in OB-NPs isolated from osteogenic cells educated by MNNG/HOS secretome, noteworthy is Angiopoietin-like protein 2 (Angptl2, alias Angiopoietin-related protein 2) which has been identified as a strong promoter of osteosarcoma malignancy and lung pre-metastatic niche formation [28–31], as well as L-lactate-dehydrogenase B chain (LdhB), a glycolytic enzyme highly expressed in osteosarcoma cells and associated with poor prognosis [32,33]. Other upregulated proteins, such as Fructose-bisphosphate aldolase A (Aldo A) [34–36], EH domain-containing protein 1 (EHD1) [37,38], follistatin-related protein 1 (Fst-1) [39] and BMP-1 [40] have been implicated in osteosarcoma progression and metastasis and have been proposed as new targets for osteosarcoma treatment [40]. Therefore, this finding suggests the ability of MNNG/HOS cells to fuel their own malignancy by stimulating OB-NPs shuttling of osteosarcoma promoting factors. Finally, looking at the proteins exclusively expressed in OB-NPs, we obtained some controversial results. In fact, CMOB-OB-NPs protein profile includes Protein deglycase DJ-1, which has been shown to be upregulated in osteosarcoma cells, while its inhibition reduces proliferation and migration and increases apoptosis [41,42]. Nevertheless, Kim et al. demonstrated a physiological role of DJ-1 in osteogenic cells, finding that this protein promotes angiogenesis and osteogenesis by activating FGF receptor-1 signalling [43]. ATP-citrate

synthase, another protein uniquely presents in CMOB-OB-NPs, seems to promote osteosarcoma growth and metastasis [44,45]. With regards to the proteins exclusively expressed by OB-NPs educated by MNNG/HOS-CM, again some of them could play a role in osteosarcoma and in osteoblast homeostasis. As an example, it has been found that IGFBP5 restrains osteoblast differentiation by c-Src activation [46], while miR-515-5p/IGF-1R/IGFBP5 axis promotes osteosarcoma cells stemness [47]; Podocan (Podn) has been proposed as a prognostic biomarker correlating with immune infiltrates in osteosarcoma [48]. Septin 2 has been identified in NPs from SAOS-2 osteosarcoma cells and is associated to therapy-related acute myeloid leukemia, a rare complication of osteosarcoma treatment [49]. Fibronectin type III domain-containing protein 1 (Fndc1) promotes bone loss in a postmenopausal osteoporosis mouse model by inhibiting differentiation of bone marrow mesenchymal stem cells [50]. As far as we know, other proteins identified in our proteomic analysis do not seem to have a direct effect on bone homeostasis and/or on bone tumour development, however they could deserve a deeper investigation to identify a possible role in this context. The analysis reveals the absence of alkaline phosphatase among the proteins identified in both types of OB-NPs. This finding may indicate that certain nanoparticle groups, such as matrix vesicles, are not present in our samples. Alternatively, it could suggest that the sensitivity of the untargeted analysis employed is not adequate for covering all proteins. Interestingly, recent reports highlight a biological role for NPs in ECM homeostasis and a protein profile reflecting the ECM one. Indeed, Xiao et al. [51] performed a proteomic analysis revealing 83 proteins commonly identified in NPs and in the ECM, including type I collagen, vinculin, α -actinins, thrombospondin-2, vascular cell adhesion protein 1 (V-CAM 1), and periostin. Thouverey et al. [52] found 262 shared proteins between NPs and bone-secreting apical membrane of mineralizing osteoblast-like Saos-2 cells, such as Sphingomyelin phosphodiesterase 3, Inorganic pyrophosphatase 1, Annexin A6, Annexin A2, Integrin β 5. Moreover, they observed an enrichment of osteonectin and collagens in EVs, likely playing a role in ECM organization and mineral matrix integrity. Accordingly, selective depletion of annexin A5 or tissue nonspecific alkaline phosphatase in NPs impairs calcium deposition and ECM mineralization [53]. It has been also demonstrated that collagens type VI and XI are electively packaged into NPs, eventually contributing to ECM assembly [54]. Schmidt et al. [55] demonstrated that glycosaminoglycans (GAGs) strongly regulated NPs proteome much more than the cellular proteome, sorting in NPs thrombospondin-1 and -2, fibrillin1, latent transforming growth factor β -binding protein 2 and cysteine-rich protein 6. Sulphated GAGs presence increases protein composition and sorting in NPs, leading to stronger extracellular matrix formation and mineralization. Finally, Martins et al. [56] reported that osteopontin shuttled in EVs derived from mesenchymal stem cells impact on periodontal ECM by affecting osteoblast and osteoclast function. In conclusion, our results put one more piece in the intricate puzzle of the vicious cycle between osteosarcoma and bone, dissecting the role of the osteosarcoma secretome and the modification exerted on osteoblast homeostasis and bone matrix organization, eventually promoting osteosarcoma progression and metastasis.

5. References

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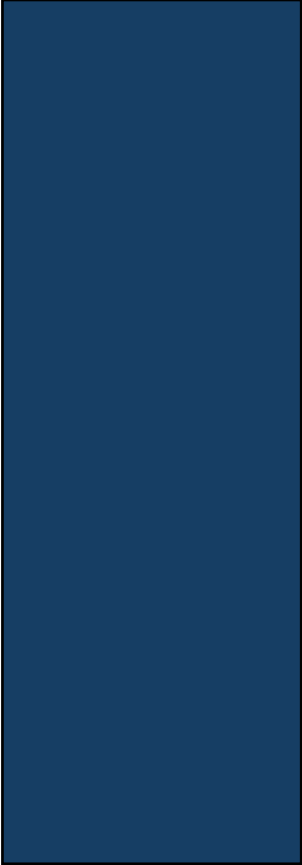
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Chapter 6

“Conclusions”

Conclusions

This PhD thesis was built around a simple but important idea: in osteosarcoma, what happens in the bone microenvironment is not just a consequence of tumour growth, but part of the disease mechanism itself. Osteoblasts, their extracellular vesicles, and the broader secretory output of both tumour and bone cells participate in a continuous exchange of signals that can either restrain or fuel tumour aggressiveness. The studies collected in this thesis were therefore designed to look at this dialogue from both sides—asking, on one hand, whether bone-derived EVs can be used in a therapeutically useful way, and, on the other, how osteosarcoma-derived factors progressively derail osteogenic function and reshape the matrix niche.

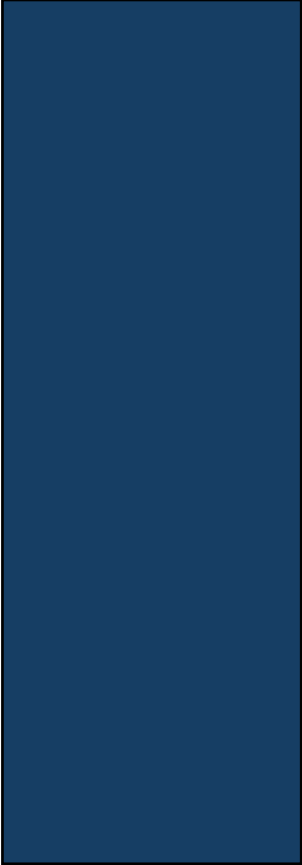
In **Chapter 3**, we demonstrated that extracellular vesicles released by the human fetal osteoblast cell line hFOB can be employed as effective and safe nanocarriers for three conventional osteosarcoma chemotherapeutics, methotrexate (MTX), doxorubicin (DXR) and cisplatin (CPT). By systematically comparing different loading strategies, we showed that loading efficiency and functional activity strongly depend on the specific drug–EV source combination: passive incubation proved suitable for MTX, whereas DXR and CPT required indirect loading through donor cell preconditioning to generate functionally active drug-loaded EVs. Under optimized conditions, encapsulated drug concentrations reached 0.7 µg/mL for MTX, 2.9 µg/mL for CPT and 20.4 µg/mL for DXR. Drug-loaded hFOB-EVs significantly reduced viability and proliferation of MNNG/HOS osteosarcoma cells and increased apoptosis *in vitro*, while failing to impair normal osteoblast homeostasis, indicating a selective anti-tumour effect. Most notably, in an orthotopic *in vivo* model based on paratibial injection of MNNG/HOS cells in nude mice, MTX-loaded hFOB-EVs markedly inhibited tumour growth and showed higher efficacy than free MTX administered at 5 mg/kg (0.03 µg of MTX-loaded EVs *versus* 80 µg of free MTX), supporting a substantial improvement in therapeutic window and effective delivery to the bone tumour niche.

In **Chapter 4**, we investigated the biological activity of osteoblast-derived extracellular vesicles (OB-EVs) on osteosarcoma cells, moving from phenotypic observations to the molecular mechanisms underlying the response. We first confirmed that OB-EVs consistently reduced metabolic activity across additional osteosarcoma models, including Saos-2 and U2OS cells, while producing cell line–specific downstream effects, such as reduced invasive potential in Saos-2 cells and reduced proliferation with a mild increase in apoptosis in U2OS cells. This variability reflects the intrinsic heterogeneity of osteosarcoma and suggests that OB-EVs interfere with core cellular programs rather than imposing a single uniform phenotype. At the molecular level, RNA sequencing performed in MNNG/HOS cells revealed a broad transcriptional reprogramming following OB-EV treatment, with 296 differentially regulated genes. Pathway-level analyses consistently showed suppression of signalling programs associated with tumour aggressiveness, including cell-cycle progression, cell–matrix and integrin-related signalling, inflammatory and chemokine pathways, and major oncogenic cascades such as Wnt/β-catenin and NOTCH signalling, together with modulation of pathways involved in RNA processing, protein homeostasis and cellular metabolism. At the single-gene

level, NMT1 emerged as the most downregulated transcript, while RPL36 was among the most upregulated, pointing to a possible involvement of protein modification and ribosomal dynamics in the OB-EV-mediated response. In parallel, miRNA profiling showed efficient transfer of EV-associated RNAs to tumour cells, identified 27 expressed miRNAs (13 highly expressed), and suggested that a fraction of the downregulated transcriptome could be directly targeted by OB-EV miRNAs, with Wnt/ β -catenin again emerging as a central regulated pathway.

In **Chapter 5**, we addressed the complementary question of how osteosarcoma-derived secreted factors affect osteogenic cells and the bone matrix niche. Using conditioned medium from MNNG/HOS cells, we showed that short-term exposure induced an early and selective molecular effect, characterized by downregulation of the master osteogenic regulator Runx2, suggesting interference with osteogenic commitment at an upstream level. While classical markers of osteoblast differentiation were not immediately affected, prolonged exposure revealed clear functional consequences. In particular, osteosarcoma secretome markedly reduced collagen type I release and deposition over time and significantly impaired the ability of osteogenic cells to form mineralized nodules at 14 and 21 days, with only a limited contribution of changes in cell number. These findings support the idea that osteosarcoma promotes the formation of a pathogenic osteoid-like matrix by weakening the main organic scaffold and mineralization capacity of bone. Extending this analysis to osteoblast-derived nanoparticles (OB-NPs), we found that tumour education did not substantially alter NP size or number but increased expression of the NP marker CD81 and reshaped the NP-associated protein profile, including downregulation of Col Ia1 and Col Ia2 together with broader alterations in collagen and matrix-related proteins. This indicates that osteosarcoma secretome not only affects osteoblast function at the cellular level but also modifies the vesicular and nanoparticle-associated extracellular matrix signature exported by osteogenic cells, potentially amplifying niche dysregulation.

Taken together, the results presented in this thesis demonstrate that extracellular vesicles and secretome-mediated signalling are central components of osteosarcoma biology rather than secondary phenomena. From a translational perspective, they support two complementary strategies: exploiting bone-derived EVs as naturally osteotropic drug-delivery systems to improve efficacy and reduce systemic toxicity of existing chemotherapeutics and targeting tumour-driven reprogramming of osteogenic cells and their vesicular outputs to limit microenvironmental corruption. Overall, this work provides mechanistic and experimental evidence that EV-mediated communication represents a concrete entry point for the development of next-generation therapeutic approaches in osteosarcoma.



Chapter 7

“Achievements”

CURRICULUM VITAE

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PRESENT POSITION

2025 PhD student in Experimental Medicine, University of L'Aquila, Italy (End date, 31 Oct 2025).

ACADEMIC DEGREES

07/2022 Master's Degree in Medical Biotechnology, 110/110 cum laude, University of L'Aquila, Italy.

12/2020 Bachelor's Degree in Biotechnology, 110/110 cum laude, University of L'Aquila, Italy.

PROFESSIONAL EXPERIENCES

2021-2022 Research Fellowship (funded by AIRC) titled "Study of the crosstalk between osteoblasts and osteotropic tumour cells mediated by extracellular vesicles and Identification of new therapeutic targets", Skeletal disease laboratory (Prof. Nadia Rucci).

2019-2021 Internship in the "Skeletal disease laboratory" (Prof. Nadia Rucci).

SKILLS

- Molecular biology (CRISPR/Cas9, nucleic acid extraction, electrophoresis, quantification by spectrophotometer, cDNA, PCR, real-time PCR, genotyping, sequencing).
- Proteomics (protein extraction, western blot, zymography quantification by Bradford and BCA method, ELISA assays).
- Histopathology (inclusion of samples, use of microtome and cryostat, hematoxylin/eosin, Gomori's trichrome, Masson's trichrome).
- IHC and IF on cells and tissues.
- Primary (osteoblasts, osteoclasts, myoblasts) cell cultures and cancer cell line handling (breast cancer and osteosarcoma).
- Metabolic activity, proliferation, migration, invasion assays on cells.
- Cells transfection with plasmids and iRNAs.
- Spheroids formation from primary cultures and cell lines.
- Cytochemical analysis of alkaline phosphatase for the identification of osteoblasts.
- Cytochemical analysis of acid resistant tartrate phosphatase for the identification of osteoclasts.
- Isolation of extracellular vesicles by ultracentrifugation and characterization with NanoSight300 and TEM.
- Chemotherapeutic drugs (methotrexate, doxorubicin, cisplatin) and siRNAs loading procedures for extracellular vesicles (diffusion, electroporation, endogenous loading).
- Use and analysis with optical, epifluorescence and confocal microscope.
- Animal handling and experimental procedures (metastatic and orthotopic tumour models).
- Use of IVIS spectrum and Hamamatsu Photonics 2D bioimaging systems.
- Use of SkyScan 1174 Burkert μ CT, NRecon (reconstruction) and CTAn (3D analysis) software.
- Use of GraphPad Prism, ImageJ and Office (for statistical analysis, data handling and data presenting).
- Ability to design, conduct and optimize in vivo and in vitro experiments.

PUBLICATIONS

- Ponzetti M, Ucci A, Maurizi A, **Giacchi L**, Teti A, Rucci N. Lipocalin 2 Influences Bone and Muscle Phenotype in the MDX Mouse Model of Duchenne Muscular Dystrophy. *International Journal of Molecular Sciences*. 2022;23(2). doi:10.3390/IJMS23020958.
- Ponzetti M, Ucci A, Puri C, **Giacchi L**, Flati I, Capece D, Zazzeroni F, Cappariello A, Rucci N, Falone S. Effects of osteoblast-derived extracellular vesicles on aggressiveness, redox status and mitochondrial bioenergetics of MNNG/HOS osteosarcoma cells. *Front Oncol*. 2022 Dec 5;12:983254. doi: 10.3389/fonc.2022.983254. PMID: 36544705; PMCID: PMC9762506.
- Ucci A, **Giacchi L**, Rucci N. Primary Bone Tumours and Breast Cancer-Induced Bone Metastases: In Vivo Animal Models and New Alternative Approaches. *Biomedicines*. 2024 Oct 25;12(11):2451. doi: 10.3390/biomedicines12112451. PMID: 39595017; PMCID: PMC11591690.

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- Ucci A, **Giacchi L**, Cufaro MC, Puri C, Ciocca M, Di Ferdinando F, Del Boccio P, Cappariello A, Rucci N. Human osteosarcoma cell secretome impairs neonatal mouse calvarial osteogenic cells functions and modifies the nanoparticles-derived protein profile. Life Sci. 2025 Jul 9;379:123837. doi: 10.1016/j.lfs.2025.123837. Epub ahead of print. PMID: 40645405.

CERTIFICATES

- 2024 - FELASA certificate for animal handling and experimental procedures (CORSO DI FORMAZIONE E AGGIORNAMENTO IN MATERIA DI IMPIEGO DEGLI ANIMALI A FINI SCIENTIFICI ED EDUCATIVI Ed. 2024 IZS Teramo, Italy)

INTERNATIONAL AND NATIONAL CONGRESS

- 2021 - III International AICC Exosome Meeting - online meeting.
- 2023 - ECTS PhD training course - Copenhagen, Denmark.
- 2023 - 35th AICC INTERNATIONAL MEETING: "Translational and Precision Medicine: From Patient to cell and back - L'Aquila, Italy.
- 2024 - EuSARC2024 - Le Pouliguen, France.
- 2024 - AIRC IFOM from biological mechanism to new therapies - online meeting.
- 2024 - Tech4EVIta Advanced Technologies for Extracellular Vesicles - Trieste, Italy.
- 2025 - ISEV2025 - Vienna, Austria.
- 2025 - EuSARC2025 - Venice, Italy.

PUBLIC SPEAKING AND CONFERENCE PRESENTATIONS

- 2023 - Oral communication and poster, ECTS PhD training course - Copenhagen, Denmark.
- 2024 - Flash oral communication and poster, EuSARC2024 - Le Pouliguen, France.
- 2024 - Flash Oral communication and poster, Tech4EVIta Advanced Technologies for Extracellular Vesicles - Trieste, Italy.
- 2025 - Poster presentation, ISEV2025 - Vienna, Austria.
- 2025 - Oral communication, EuSARC2025 - Venice, Italy.

AWARDS

- 2024 - Travel Grant of 450£ to attend ISEV2025 congress in Vienna, Austria.
- 2025 - Travel Grant of 500£ to attend EuSARC2025 congress in Venice, Italy.

OTHER EXPERIENCES AND INITIATIVES

- 2024/2025 - AIRC nelle scuole.
- 2025 - AIRC arance della salute.