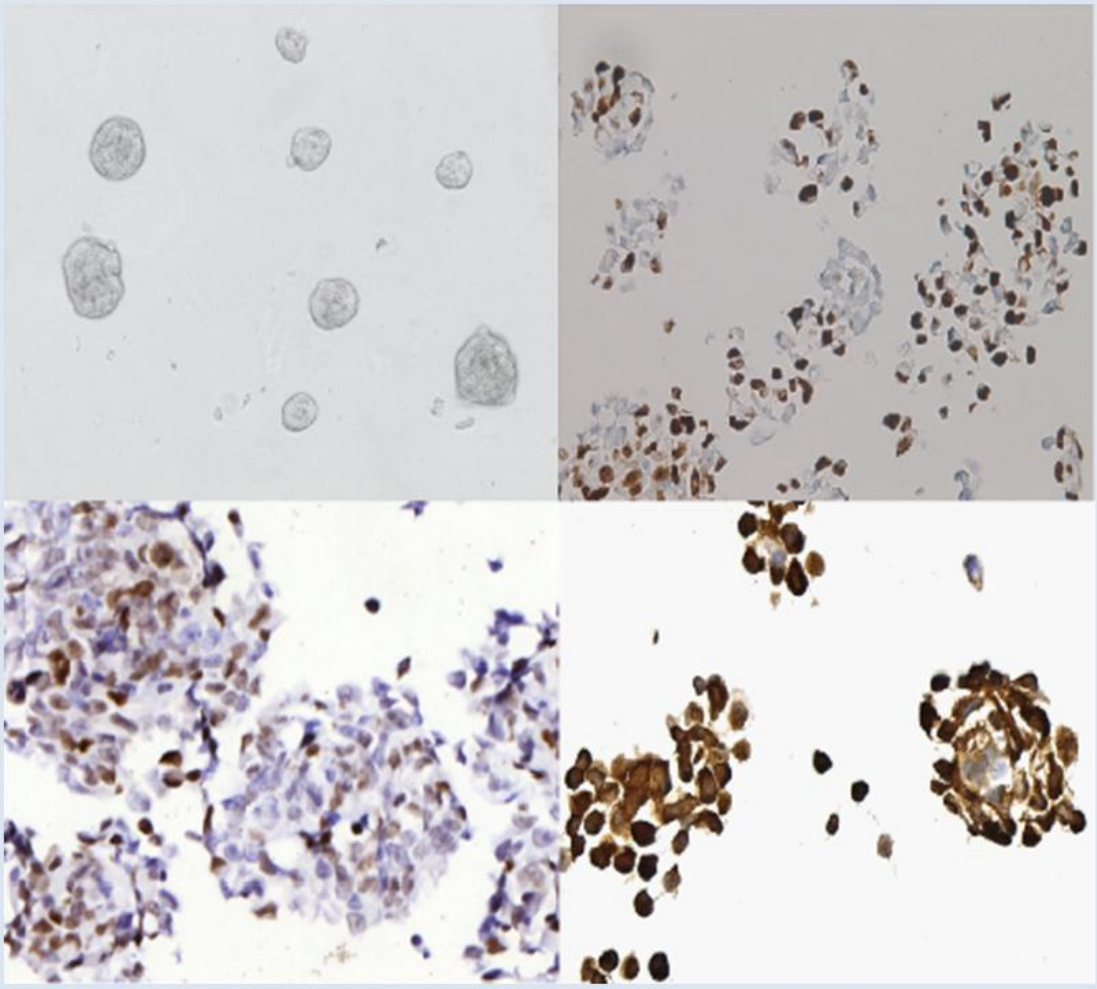

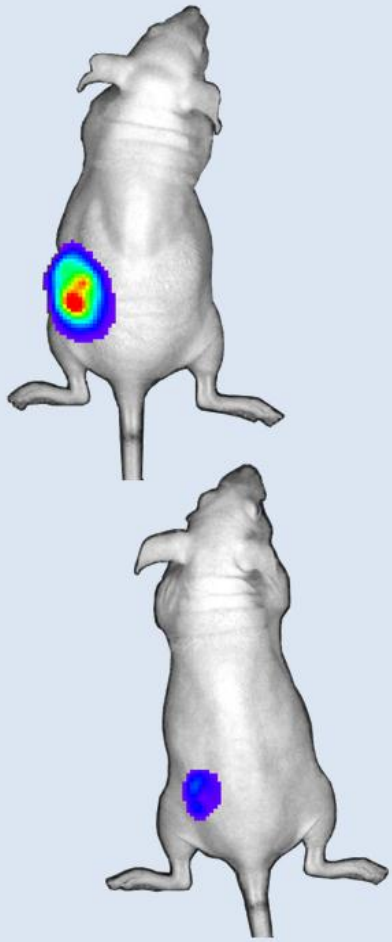

Sara Raquel Martins Neves

Cancer Stem Cells in Human Osteosarcoma: Tracking Stemness to Target Chemoresistance

Doctoral Thesis in Biomedical Engineering,
under supervision of Doctor Célia Maria Freitas Gomes and co-supervision of Doctor Anne-Marie Cleton-Jansen,
presented to the Faculty of Sciences and Technology of the University of Coimbra.

September 2016

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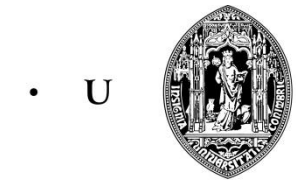
Universidade de Coimbra



UNIVERSIDADE DE COIMBRA

CANCER STEM CELLS IN HUMAN OSTEOSARCOMA: TRACKING STEMNESS TO TARGET CHEMORESISTANCE

CÉLULAS ESTAMINAIS CANCERÍGENAS NO OSTEOSSARCOMA HUMANO: ESTUDO DA ESTAMINALIDADE TENDO COMO ALVO A QUIMIO-RESISTÊNCIA

Doctoral Thesis

Tese de Doutoramento

To obtain the Doctoral degree in Biomedical Engineering
at the Faculty of Sciences and Technology of the University of Coimbra

Para obter o grau de Doutor em Engenharia Biomédica
da Faculdade de Ciências e Tecnologia da Universidade de Coimbra

Sara Raquel Martins Neves

September 2016

SCIENTIFIC PROPOSITIONS

Accompanying the Dissertation

CANCER STEM CELLS IN HUMAN OSTEOSARCOMA: TRACKING STEMNESS TO TARGET CHEMO-RESISTANCE

I. The difficulties in identifying a consistent and representative phenotype for osteosarcoma cancer stem cells can reflect the lack of specific markers for osteosarcoma in general and the absence of exclusively expressed antigens on mesenchymal stem cells, possible osteosarcoma cells-of-origin. (*This thesis*)

II. The activation/inactivation status of Wnt/β-catenin in osteosarcoma has been subject of debate and no definitive causal relationship has been established so far. (*This thesis*)

III. Several studies suggest that chemotherapy can promote or enhance a stem cell-related phenotype in previously differentiated tumor cells. (*This thesis*)

IV. "(…) carcinomas are caricatures of tissue renewal, in that they are composed of a mixture of malignant stem cells, which have a marked capacity for proliferation and a limited capacity for differentiation under normal homeostatic conditions, and of the differentiated, possibly benign, progeny of these malignant cells." (*Pierce and Speers, Cancer Res 1988;48:1996-2004*).

V. "(…) given (that) cancer stem cell phenotypes are unstable and heterogeneous among patients and markers are highly expected to be applied in early-stage diagnosis and prognosis estimation, cancer stem cells defined by markers should be confirmed by functional assays." (*Wang et al., Cancer Lett 2015;357(1):63-68*).

VI. "Successful people never worry about what others are doing!" (*Vijay Sak*).

VII. *In Science, say anything you can support on a scientific basis*.

Cover image by Sara R. Martins-Neves, with collaboration from Dr. Célia Gomes and Eng. Otília C. d'Almeida

Left panel: Osteosarcoma cancer stem cells isolated using the sphere-forming assay. Spheres (upper left) are Ki-67-negative (upper right), overexpress the pluripotency marker Sox2 (lower left) and display activated Wnt/β-catenin signaling, as indicated by nuclear β-catenin (lower right)

Right panel: Representative bioluminescent images of the anti-tumoral effects of IWR-1 (Wnt inhibitor) in a xenografted-osteosarcoma mouse model

Disclosure of financial & competing interests

The authors of the studies described in this thesis have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials herein discussed. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties. No writing assistance was used in the production of this thesis.

PhD Promoting Committee

Supervisor: Dr. Célia Maria Freitas Gomes[1]

Co-supervisor: Dr. Anne-Marie Cleton-Jansen[2]

1. Pharmacology and Experimental Therapeutics, Institute for Biomedical Imaging and Life Sciences (IBILI), Faculty of Medicine, University of Coimbra, Coimbra, Portugal

2. Department of Pathology, Leiden University Medical Centre, Leiden, The Netherlands

The research described in this thesis was performed at the Pharmacology and Experimental Therapeutics Group of the Institute for Biomedical Imaging and Life Sciences (IBILI), Faculty of Medicine, University of Coimbra, Coimbra, Portugal and at the Bone and Soft Tissue Pathology Group at the Department of Pathology, Leiden University Medical Centre, Leiden, The Netherlands.

Grant Support

The work herein presented was financially supported by "Fundação para a Ciência e Tecnologia" (FCT, Portuguese Foundation for Science and Technology), COMPETE, QREN and FEDER – individual doctoral grant to Sara Raquel Martins Neves (SFRH/BD/69603/2010); and by "Núcleo Regional do Centro da Liga Portuguesa Contra o Cancro" and "Centro de Investigação em Meio Ambiente, Genética e Oncobiologia da Faculdade de Medicina da Universidade de Coimbra" (NRC-LPCC/CIMAGO Grant 2014) - individual research grant to Sara Raquel Martins Neves.

Additional financial support was provided by Portuguese Foundation for Science and Technology (FCT) through COMPETE, QREN and FEDER funding in the following grants: Pest-C/SAU/UI3282 (IBILI), FCT reference UID/NEU/04539/2013 and COMPETE 2020 reference POCI-01-0145-FEDER-007440 (CNC.IBILI), CIMAGO research grant 16/09 (to Dr. Célia Maria Freitas Gomes) and institutional funding of Leiden University Medical Centre (to Dr. Anne-Marie Cleton-Jansen).

À minha Leonor linda e

Ao Pai dela e meu Marido, Nuno

Acknowledgments

First of all, a kind word of gratitude to my supervisors Dra. Célia Maria Freitas Gomes and Dra. Anne-Marie Cleton-Jansen, for their support, mentorship and friendship throughout this project.

I am also thankful to Dr. Carlos Fontes-Ribeiro for the acceptance in the Pharmacology and Experimental Therapeutics group in the Faculty of Medicine of the University of Coimbra and to Dr. Pancras Hogendoorn and Dra. Judith Bovée for the acceptance in the Bone and Soft Tissue Tumor group in the Department of Pathology of the Leiden University Medical Centre.

A word of gratitude for the excellent technical help I received from Brendy, Inge, Pauline, Réne, Wim, Hans, Frans and Jaap and assistance in immunohistochemistry, qRT-PCR, cell culture and flow cytometry experiments. I now know that technical perfection is the key to trustable results, even if based on the simplest ideas - many thanks for teaching me that.

I am also thankful to Drs. Ola Myklebost (Radiumhospitalet, Oslo) for providing the MHM and OHS cell lines and Geertje van der Horst for help with the implementation of the Aldefluor™ assay. With Marieke Kuijjer and Jolieke van Oosterwijk (young Drs. and friends) I had valuable scientific discussions during my first year of staying in the Netherlands.

Cell cycle experiments were conducted at the Blood and Transplantation Center of Coimbra, Portuguese Institute of the Blood and Transplantation, Coimbra, Portugal, with special thanks due to Dr. Artur Paiva.

I also cannot forget the helpful contributions of the students of both groups, who lend not only great scientific discussions and input to this project, but also nice break moments.

To all of you, thank you!

Agradeço imenso também à minha inseparável companheira de caminhada académica e Amiga do coração, à Engª. Otília C. d'Almeida ☺, por compreender tão bem todos os altos e baixos de um percurso tão longo e exigente como pode ser um doutoramento.

Agradeço à minha família, especialmente à minha Mãe Fátima Neves, por me dizer várias vezes que "a recompensa chega sempre para quem trabalha com gosto e afinco".

Finalmente, lembrando que os últimos são os primeiros, agradeço do fundo do coração ao meu Marido Nuno Simões, por não ter desistido de mim nestes tempos atribulados, por me incentivar sempre a continuar, mesmo quando isso era o oposto do que ele próprio pretendia para nós; e por me ter dado na recta final deste projecto, o "projecto" mais maravilhoso das nossas vidas: a nossa linda Filha Leonor!

A todos, um muito obrigado!

Sara Neves

CONTENTS

List of Publications

The results presented in this Thesis have been published or submitted for publication in international scientific periodicals with referees as follows:

Research articles presented in the thesis

1. **Sara R. Martins-Neves**, Willem E. Corver, Daniela I. Paiva-Oliveira, Brendy E. W. M. van den Akker, Inge H. Briaire-de-Bruijn, Judith V. M. G. Bovée, Célia M. F. Gomes*, Anne-Marie Cleton-Jansen* (2016) Osteosarcoma Stem Cells Have Active Wnt/β-Catenin and Overexpress SOX2 and KLF4. *Journal of Cellular Physiology* 231(4):876-886. DOI: 10.1002/jcp.25179 (JCR Impact Factor 2015 - 4.155, SCImago 2015 - Q1 Physiology, Clinical Biochemistry, Q2 Cell Biology) (*equal contribution)
2. **Sara R. Martins-Neves**, Daniela I. Paiva-Oliveira, Pauline M. Wijers-Koste, Inge H. Briaire-de-Bruijn, Judith V. M. G. Bovée, Anne-Marie Cleton-Jansen*, Célia M. F. Gomes* (2016) Chemotherapy induces stemness in osteosarcoma cells through activation of Wnt/β-catenin signaling. *Cancer Letters* 370(2):286-295. DOI: 10.1016/j.canlet.2015.11.013. (JCR Impact Factor 2015 - 5.992, SCImago 2015 - Q1 Oncology, Cancer Research) (*equal contribution)
3. **Sara R. Martins-Neves**, Daniela I. Paiva-Oliveira, Carlos Fontes-Ribeiro, Judith V. M. G. Bovée, Anne-Marie Cleton-Jansen*, Célia M. F. Gomes* (2016) Selective eradication of osteosarcoma stem cells by targeting Wnt/β-catenin signaling activity (*equal contribution) (*Submitted for publication*)

The results reported in Chapters 2, 3 and 4 are formatted as requested by the journals where the papers were published or submitted for publication, with minor modifications.

Other articles related to this thesis

4. Cláudia Gonçalves*, **Sara R. Martins-Neves***, Daniela Paiva-Oliveira*, Vitor E. B. Oliveira, Carlos Fontes-Ribeiro, Célia M. F. Gomes (2015) Sensitizing osteosarcoma stem cells to doxorubicin-induced apoptosis through retention of doxorubicin and modulation of apoptotic-related proteins. *Life Sciences* 130:47–56. DOI: 10.1016/j.lfs.2015.03.009. (JCR Impact Factor 2015 - 2.685, SCImago 2015 - Q1 Pharmacology, Toxicology and Pharmaceutics, Q1 Biochemistry, Genetics and Molecular Biology) (*equal contribution)
5. **Sara R Martins-Neves**, Áurio O Lopes, Anália Do Carmo, Artur A Paiva, Paulo C Simões, Antero J Abrunhosa, Célia MF Gomes (2012) Therapeutic implications of an enriched cancer stem-like cell population in a human osteosarcoma cell line. *BMC Cancer* 12:139. DOI: 10.1186/1471-2407-12-139. (JCR Impact Factor 2012 - 3.333, SCImago 2012 - Q1 Oncology, Q2 Cancer Research, Q2 Genetics)

List of Abbreviations

ABC – ATP-binding cassette

ALDH – aldehyde dehydrogenase

ATCC – American Type Culture Collection

ATP – adenosine triphosphate

ATRA – all-trans retinoic acid

BAAA – BODIPY®-aminoacetaldehyde

BCRP (ABCG2) – breast cancer resistance protein

BMP – bone-morphogenetic protein

CDK – cyclin-dependent kinase

CDKN – cyclin-dependent kinase inhibitor

cDNA – complementary deoxyribonucleic acid

CSC – cancer stem cell

DEAB – 4-(diethylamino)benzaldehyde

DNA – deoxyribonucleic acid

EDTA – ethylenediamine tetraacetic acid

EGF – epidermal growth factor

EGFR – epidermal growth factor receptor

EMA – European Medicine Agency

ERK – extracellular signal–regulated kinase

ESC – embryonic stem cell

EURAMOS – European and American Osteosarcoma Study Group

FDA – Food and Drug Administration

FGF – fibroblastic growth factor

GLI – glioma-associated oncogene

HEMA – 2-hydroxyethyl methacrylate

LRP – low-density lipoprotein receptor-related protein

MAPK – mitogen-activated protein kinase

MDR – multidrug resistance

MSC – mesenchymal stem cell

PGP – P-glycoprotein

qRT-PCR – quantitative reverse transcriptase – polymerase chain reaction

RB – retinoblastoma protein

ROS – reactive oxygen species

SEM – standard error of the mean

SFM – sphere-formation media

SFRP – secreted frizzled-related protein

SMO – smoothened

Sox2 – sex-determining region Y-box 2

STR – short tandem repeat

TCF/LEF – T-cell factor/lymphoid enhancer factor

VER – verapamil

WHO – World Health Organization

WIF1 – WNT inhibitory factor 1

WNT – Wingless-type MMTV integration site family

ABSTRACT

Osteosarcoma is the most common primary malignant bone tumor, afflicting mainly young patients. Since the inception of chemotherapy, survival rates augmented significantly, but are stagnated over the past decades, due to the absence of improved therapy. Most patients succumb to metastatic disease, which can occur even after apparent successful histological response to chemotherapy that precedes the surgical removal of the primary tumor.

Recently, the cancer stem cell (CSC) model has been receiving attention and suggests that stem-like cells exist in many tumor types, form the clonogenic core of the tumor and actively contribute to chemoresistance. This theory was originally postulated as a somewhat rigid hierarchical model of tumor development, in which a self-renewing CSC originates differentiated progeny and is responsible for feeding the bulk tumor mass. However, evidence suggests that diverse pools of CSCs might exist, contributing to the intratumoral heterogeneity of solid tumors, including osteosarcoma. Besides, extrinsic factors, e.g. drug exposure, may also constitute a source of stemness within the tumors.

In this thesis we performed a molecular and functional characterization of CSCs in a panel of cell lines representative of two histological subtypes of high-grade osteosarcoma, and investigated the central role of the regulatory Wnt/β-catenin pathway in the stemness properties and survival advantages of CSCs.

In the first part, we demonstrated that different CSCs populations may co-exist in osteosarcoma cell lines exhibiting distinct functional properties. CSCs isolated from fibroblastic tumors are slowly-proliferating populations overexpressing Sox2 and Klf4 pluripotency-related markers, have enhanced tumorigenic potential and specific activation of the self-renewal-related Wnt/β-catenin pathway, as assessed by nuclear β-catenin positivity, *AXIN2* expression and TCF/LEF transcriptional activity. The Aldefluor+ populations detected in the two histological osteosarcoma subtypes are *SOX2*+, but *KLF4*-, whereas the side-population subset phenotype is correlated with *ABCG2* drug-efflux transporter expression. Altogether, these results suggest that distinct functional methods identify CSCs with dissimilar characteristics, which may have implications on the design of CSC-targeted therapies.

In the second part, we evaluated the therapeutic potential of inhibiting Wnt/β-catenin against chemoresistant CSCs. The Wnt signaling antagonist IWR-1 was

selectively cytotoxic for CSCs, by decreasing cell viability, proliferation and cell cycle progression. IWR-1 induced apoptosis of osteosarcoma CSCs and in combination with doxorubicin treatment elicited synergistic cytotoxicity, reversing CSCs intrinsic resistance to this drug. IWR-1 impaired CSC' self-renewal capacity by compromising landmark steps of the canonical Wnt signaling, namely nuclear β-catenin translocation and subsequent TCF/LEF activation and expression of downstream targets. Wnt inhibition also hampered Aldefluor activity and expression of key pluripotency-related genes. We observed a remarkable anti-tumor effect of IWR-1 in osteosarcoma-xenografted models that potentiated the anti-tumor efficacy of doxorubicin, accompanied by down-regulation of TCF/LEF transcriptional activity, Sox2 and *AXIN2* expression and nuclear β-catenin.

In the third part, we explored the striking hypothesis that drugs used in osteosarcoma treatment induce stemness properties in differentiated cells. Doxorubicin, cisplatin and methotrexate induced a phenotypic stem-like cell transition, by increasing Aldefluor activity and ALDH, ABC transporters and pluripotency markers expression. Doxorubicin up-regulated stemness markers via Wnt/β-catenin activation, but co-treatment with IWR-1 prevented the drug-induced phenotype. Altogether, these results are consistent with the ability of doxorubicin to kill rapidly-dividing cancer cells, and of IWR-1 to eliminate chemoresistant CSCs populations and overcome acquired resistance.

Translational significance of this study was conveyed by the pluripotency mRNA signature found in uncultured osteosarcoma patient samples and by the correlation of stemness-related markers expression with a worst prognosis in osteosarcoma patients who responded poorly to chemotherapy (EuroBonet dataset of whole genome expression).

To conclude, our results suggest the existence of phenotypic heterogeneity in osteosarcoma CSCs, and revealed the Wnt/β-catenin as a key determinant of the stemness and chemoresistant profile of osteosarcoma. Targeting the Wnt pathway may simultaneously circumvent chemoresistance and the phenotypic differentiated-to-stem like cell transition induced by chemotherapeutics, and thus contribute to reduce chemotherapy doses and ameliorate the prognostic outcomes of osteosarcoma patients.

Keywords: cancer stem cell, osteosarcoma, Wnt/β-catenin signaling, pluripotency, sphere formation assay, aldehyde dehydrogenase, side-population phenotype, chemoresistance, apoptosis, synergy, doxorubicin, IWR-1, mouse model

RESUMO

O osteossarcoma é o tumor ósseo primário maligno mais comum e afecta sobretudo jovens adolescentes. Com a introdução da quimioterapia, as taxas de sobrevivência aumentaram significativamente, mas estagnaram nas últimas décadas, devido à falta de terapias mais eficazes. Muitos doentes desenvolvem metástases e sucumbem à doença, mesmo após boa resposta histológica à quimioterapia que antecede a remoção cirúrgica do tumor primário.

Recentemente, o modelo das células estaminais cancerígenas (CSCs) sugere a existência de células com propriedades estaminais que formam o núcleo clonogénico tumoral e contribuem para a quimio-resistência. Esta teoria foi inicialmente postulada como um modelo hierárquico rígido, no qual CSCs com capacidade de auto-renovação e de diferenciação sustentam o crescimento do tumor e dão origem à população heterogénea de células diferenciadas. No entanto, há evidências que sugerem a existência de diferentes subpopulações de CSCs, podendo assim contribuir para a marcada heterogeneidade intratumoral típica dos tumores sólidos, incluindo o osteossarcoma. Além disso, factores extrínsecos, como por exemplo a exposição à quimioterapia, podem também contribuir para a aquisição de um fenótipo estaminal nos tumores.

No âmbito desta tese foi realizada uma caracterização molecular e funcional de CSCs num painel de linhas celulares representativas de dois subtipos histológicos de osteossarcoma de alto-grau, tendo-se investigado o papel central da via Wnt/β-catenina na regulação da estaminalidade e na sobrevivência de CSCs.

Numa primeira parte, demonstrámos a existência de diferentes populações de CSCs no osteossarcoma, com propriedades funcionais distintas. As CSCs isoladas a partir de tumores fibroblásticos, caracterizam-se por uma baixa taxa de proliferação, expressão de marcadores de pluripotência (Sox2 e Klf4), elevado potencial tumorigénico e ativação da via Wnt/β-catenina, evidenciada pela localização nuclear de β-catenina, expressão da *AXIN2* e actividade de transcrição do TCF/LEF. As populações Aldefluor+ detectadas nos dois subtipos histológicos de osteossarcoma, são *SOX2+*, mas *KLF4-*, enquanto o fenótipo *side-population* se correlaciona com a expressão do transportador de efluxo *ABCG2*. Estes resultados sugerem que diferentes métodos funcionais identificam CSCs com características distintas, o que pode ter implicações no desenho de terapias dirigidas às CSCs.

Numa segunda parte, avaliámos o potencial terapêutico de inibição da via Wnt/β-catenina em CSCs quimio-resistentes. O inibidor IWR-1 mostrou seletividade citotóxica para as CSCs, como demonstrado pela diminuição na viabilidade, proliferação e

progressão do ciclo celular e indução da apoptose. Demonstrou ainda sinergia em combinação com a doxorrubicina, revertendo a resistência intrínseca das CSCs a este fármaco. A inibição da via Wnt, demonstrada pela diminuição da translocação nuclear da β-catenina e repressão da atividade de transcrição do complexo TCF/LEF, comprometeu a capacidade de auto-renovação das CSCs e diminuiu a actividade do Aldefluor, assim como a expressão de genes de pluripotência. No modelo *in vivo* de osteossarcoma, o tratamento com o inibidor IWR-1 mostrou um efeito anti-tumoral pronunciado, acompanhado por uma diminuição da atividade de transcrição do TCF/LEF e da expressão de *AXIN2,* Sox2 e β-catenina nuclear.

Na terceira parte, avaliámos os efeitos de agentes de quimioterapia utilizados no tratamento de osteossarcoma, nomeadamente a doxorrubicina, cisplatina e o metotrexato na aquisição de um fenótipo estaminal. Observou-se um aumento na atividade e expressão de ALDH, bem como da expressão de transportadores ABC e dos marcadores de pluripotência nas células expostas à quimioterapia, efeito que foi mediado pela ativação da via de sinalização Wnt/β-catenina. A inibição desta via com o IWR-1 preveniu a aquisição de um fenótipo estaminal induzido pela doxorrubicina, o que demonstra a capacidade do IWR-1 em eliminar CSCs e prevenir o desenvolvimento de resistência adquirida.

O potencial translacional desta tese assenta na observação de uma potencial assinatura genética definida pela expressão de marcadores de pluripotência em amostras clínicas de osteossarcoma, e da correlação da expressão destes marcadores com um mau prognóstico, em doentes não-responsivos à quimioterapia, de acordo com a base de dados EuroBonet.

Em conclusão, este trabalho sugere a existência de heterogeneidade fenotípica em CSCs de osteossarcoma e demonstrou que a via da Wnt/β-catenina constitui um fator-chave associado à estaminalidade e à quimio-resistência das CSCs. O desenvolvimento de novas terapias dirigidas às CSCs, tendo como alvo a via da Wnt/β-catenina poderá contribuir para contornar a quimio-resistência intrínseca das CSCs e prevenir a aquisição de um fenótipo estaminal induzido pela exposição a fármacos. Esta abordagem poderá contribuir para reduzir as doses de quimioterapia e melhorar o prognóstico de doentes com osteossarcoma.

Palavra-chave: células estaminais cancerígenas, osteossarcoma, via de sinalização Wnt/β-catenin, pluripotência, ensaio de formação de esferas, aldeído desidrogenase, fenótipo *side-population*, quimio-resistência, apoptose, sinergia, doxorrubicina, IWR-1, modelo de murganho

Outline of the Thesis

In **Chapter 1**, we present a state-of-the-art picture of the pertinent literature with relevance for this thesis, namely key concepts and studies related to human osteosarcoma and biology of CSCs, and also a review of the recent literature reporting the induction of stem cell phenotypes by chemotherapeutics. Rationale and specific objectives related to the results described in subsequent chapters are also described.

In **Chapter 2**, we employed three distinct functional assays to identify and isolate osteosarcoma CSCs in established osteosarcoma cell lines, and unraveled some of their genetic and phenotypic characteristics.

In **Chapter 3**, we explored whether inhibition of Wnt/β-catenin pathway constitutes a strategy to target CSCs and improve the efficacy of chemotherapy in osteosarcoma.

In **Chapter 4**, we investigated whether low concentrations of the chemotherapeutics most used in osteosarcoma had the ability to induce a stem-like phenotype on established cell lines, representative of two histological subtypes of high-grade osteosarcoma.

In **Chapter 5**, results described in Chapters 2 to 4 are discussed and achievements of the study are summarized.

Annexes I and **II** present a detailed description of *in vitro* methodological procedures used to isolate CSCs and Complementary Methods, Results and References, respectively.

"Optimism is the foundation of courage." – Nicholas Murray Butler

I CHAPTER

GENERAL INTRODUCTION

"The ultimate measure of a man is not where he stands in moments of comfort and convenience, but where he stands at a time of challenge and controversy." – Dr. Martin Luther King Jr

I.1 Human Osteosarcoma: Clinical and Molecular Features

I.1.1 Epidemiology of osteosarcoma

Primary tumors of the skeleton are very rare among the overall tumor burden affecting humans. However, osteosarcoma is the most common primary malignant bone tumor and afflicts more children and adolescents than adults (85% *versus* 15%). In fact, osteosarcoma comprises about 20% of all primary bone sarcomas and 3-4% of all childhood malignancies (Ta *et al.*, 2009).

Osteosarcoma is a primary high-grade tumor, particularly incident in children up to 15 years (2.3% of all tumors) and in adolescents (15-25 years, 2.6% of all tumors) (Eyre *et al.*, 2009; Gatta *et al.*, 2009). Overall, the incidence of osteosarcoma worldwide is 2–3/million/year. In Europe, this incidence is higher in adolescents, with an annual incidence peak of 8–11/million/year at 15–19 years (Ritter and Bielack, 2010). For instance, in Europe, between 1978 and 1997, the number of osteosarcoma cases in adolescents aged 15-19 years was 372 per million (Stiller *et al.*, 2006b). Data from the American Surveillance, Epidemiology, and End Results (SEER) Program also clearly shows a triphasic pattern, having a peak during adolescence (8.4-8.6 cases), a plateau in adulthood (1.7 cases) and a second peak during elderly (>60 years, 4.9 cases) (Mirabello *et al.*, 2009). This particular incidence pattern with age suggests that there is a link between osteosarcoma development and rapid bone growth. Incidence of osteosarcoma is different in males and females, varying with age (Stiller *et al.*, 2006a). Below 15 years, the incidence is higher in females, but the ratio reverses to a clear male predominance after 15 years (Stiller *et al.*, 2001; Stiller *et al.*, 2006a; Mirabello *et al.*, 2009).

Osteosarcoma localizes mainly to the long tubular bones, with nearly 75% of all cases being located in the lower extremity (distal femur, proximal tibia) and 10% in the upper extremity (proximal humerus). Osteosarcoma patients normally present symptoms like local pain, swelling and limitations of joint movements (Ritter and Bielack, 2010). Pain may be perceived at first as intermittent and then more persistent over time, and may increase with physical activity and be more intense during the night; swelling in the area around the tumor mass; and symptoms of reduced movement, which may be accompanied by limp development. These symptoms can occur at any stage of the disease and depend on the location and growth rate of the tumor. Pain and swelling

are less common in adults than in active children, which may delay a proper diagnosis and also lead to misdiagnosis (http://www.cancer.org/cancer/osteosarcoma/detailedguide/osteosarcoma-signs-and-symptoms, accessed in September 27, 2016). In fact, misdiagnosis may occur quite often, with tendinitis being among the most common type of confounding diagnosis (Widhe and Widhe, 2000).

1.1.2 Histological classification and clinical features of osteosarcoma

Osteosarcomas are classified according to the World Health Organization (WHO) as osteogenic tumors that produce osteoid or bony matrix, which is defective bone. Radiographic, histopathologic and microscopic criteria are used to classify the different types of osteogenic tumors, and show that osteosarcoma is a complex and multifactorial disease presenting several different cellular forms and histological patterns (Mohseny, 2009; Fletcher *et al.*, 2013). The WHO classification system clusters osteosarcomas in:

Malignant primary central high-grade osteosarcomas

▪ *conventional osteosarcoma* is a high-grade malignant tumor, which includes the three most common subtypes - chondroblastic, osteoblastic and fibroblastic osteosarcoma. This sub-classification is based on the presence or absence of fibrous or cartilage tissue, called matrix, and on its amount and form;

▪ *telangiectatic osteosarcoma* or hemorrhagic osteosarcoma is characterized by the presence of large spaces filled with blood with or without septa (less than 4% of all osteosarcomas);

▪ *small cell osteosarcoma* or osteosarcoma with small cells resembling Ewing sarcoma (1.5% of all osteosarcomas);

Malignant primary central low grade osteosarcomas

▪ *low grade central osteosarcoma* arises from the medullary cavity of bone (1-2% of all osteosarcomas);

Malignant primary peripheral osteosarcomas

▪ *parosteal osteosarcoma* or *juxtacortical low grade peripheral osteosarcoma* arises on the surface of bone (4% of all osteosarcomas);

▪ *periosteal osteosarcoma* is a chondroblastic osteosarcoma of intermediate grade arising also on the surface of bone (less than 2% of all osteosarcomas);

▪ *high-grade surface osteosarcoma* (less than 1% of all osteosarcomas);

Malignant secondary osteosarcomas

▪ *secondary osteosarcoma* occurs in bones that are affected by pre-existing abnormalities such as Paget disease or induced by radiation.

This thesis will focus on primary central high-grade conventional osteosarcoma, which is by far the most common (75%) variant of osteosarcoma and the most challenging tumor type needing novel effective treatment options and improved therapy. In specific, we focus on fibroblastic and osteoblastic osteosarcoma, due to the higher availability of cell lines, which are easy to establish, and also availability of patient samples.

High-grade conventional osteosarcomas are histologically characterized by the presence of unmineralized bone matrix (osteoid) and also variable amounts of fibrous tissue and/or cartilage, which are used to further sub-classify into osteoblastic, fibroblastic or chondroblastic osteosarcoma, as previously mentioned. Historically, there is no correlation between prognostic significance and sub-classification of osteosarcoma, although some correlation of the distinct histological subtypes to specific clinical outcomes has been observed, especially when contemporary multi-disciplinary therapy is used. Moreover, there is some tendency for chondroblastic osteosarcoma to show more often a poor histological response to chemotherapy, although this resistance trend does not translate into poorer survival rates due to its slightly better long-term survival and prognosis (Hauben *et al.*, 2002; Hauben *et al.*, 2006).

Cytological appearance of osteosarcoma cells may vary, with tumors presenting an anaplastic pleomorphic structure composed of at least two of these cell types: epithelioid, plasmacytoid, fusiform, ovoid, small round cells, clear cells, mono- or multinucleated giant cells, or even spindle cells (Fletcher *et al.*, 2013).

Factors such as large tumor volume, metastatic lesions and axial location account for a negative prognostic for osteosarcoma patients. Moreover, high alkaline phosphatase levels (Ren *et al.*, 2015) and tumors responding poorly to neoadjuvant chemotherapy present a higher risk to metachronous metastasis and recurrence (Carrle and Bielack, 2006). Metastases detected at diagnosis are a particularly poor predictor of survival.

1.1.3 Osteosarcomagenesis and associated molecular markers

High-grade conventional osteosarcoma is characterized by extensive genetic instability, complicating the identification of unique causative gene alterations. The most common genetic alterations include losses in chromosomes 3q, 6q, 9, 10, 13, 17p, and

18q and gains in chromosomes 1p, 1q, 6p, 8q, and 17p. The rarer subtypes of osteosarcoma generally present genetic alterations which are different and less frequent in conventional tumors, and may serve as a basis for differential diagnostic (Martin *et al.*, 2012).

Genetic alterations in the retinoblastoma (*RB1*) (located at chromosome 13q14.2) and *TP53* (located at chromosome 17p13.1) genes lead to inactivation of the RB1 and p53 proteins, respectively, and are perhaps the most common and well described events involved in osteosarcomagenesis, which may also account for development of metastatic disease. RB1 and p53 proteins function as tumor suppressor proteins and are essential regulators of cell cycle progression after DNA damage recognition (Berman *et al.*, 2008; Martin *et al.*, 2012). Other genetic alterations that may play an important role in sporadic osteosarcoma are *CDKNA2*, *CDKN2A*, *CDKN2B*, *MDM2*, *TWIST*, *CMYC*, and *FGFR2*, among others, as previously reviewed (Kansara and Thomas, 2007). Also alterations occurring in the osteogenic differentiation cascade may give an important contribution for osteosarcoma development, as will be outlined later on in this text. However, it should be noted that none of these genetic alterations are specific for osteosarcoma, since they occur in many other tumor types.

- **RB1 protein.** In normal cells, this tumor suppressor protein prevents the transition from G1 to S phase after DNA damage. During the G1 phase, RB proteins bind to and inhibit the transcriptional activity of E2F1-3 proteins, which then regulate the transcription of genes required for cell cycle progression, such as cyclins A, D and E. This process is strictly regulated by cyclin-dependent kinases (CDKs), and cyclin-dependent kinase inhibitors (CDKNs) that properly promote the stability of the cellular genome (Manning and Dyson, 2011). In osteosarcoma, genetic alterations in *RB1* lead to inability of defective RB1 protein to block the G1 to S transition thereby allowing uncontrolled cell cycle progression and proliferation (Kansara and Thomas, 2007).
- **p53 protein.** Deregulation of p53 protein is also relevant in the development of osteosarcoma. p53 is activated after recognition of DNA damage and up-regulates p21 protein that binds to cyclin D/CDK4/6 or cyclin E/CDK2 complexes promoting cell cycle arrest, quiescence (which may enable DNA repair to occur), senescence, or apoptosis, depending on the severity and extension of DNA damage. In osteosarcoma, p53 is commonly inactivated and involved in its etiology, as in many other tumors (Kansara and Thomas, 2007; Levine and Oren, 2009). Recently, Chen and colleagues detected *TP53* gene alterations in almost all osteosarcomas of the discovery cohort tested using

whole genome sequencing, which further emphazises the crucial role of p53 pathway in the genetic landscape of osteosarcoma (Chen *et al.*, 2014).

- **MDM2 protein.** The oncoprotein MDM2 is a well-defined p53 inhibitor and is involved in both p53 degradation and down-regulation of p53 transcription. p53 inactivation by MDM2 prevents cell cycle arrest and apoptosis following DNA damage, thereby enabling uncontrolled cell proliferation. A positive feedback loop also regulates MDM2 expression, which is transcriptionally up-regulated by p53. In addition, MDM2 may also target p53 for proteosomal degradation, by exerting E3 ubiquitin ligase activity, and leading to even further potency of p53 abnormal activity in cancer cells (Kansara and Thomas, 2007). MDM2 amplification at chromosome 12q15 is not a common event in primary osteosarcoma (Duhamel *et al.*, 2012), but occurs more frequently in metastases and recurrences than in primary tumors (Miller *et al.*, 1996; Lonardo *et al.*, 1997).

- **p14, p15, p16 proteins.** Alterations in the genes *CDKN2A/p16/INK4A*, *CDKNA2/p14/ARF*, and *CDKN2B/p15/INK4B*, located at chromosome 9p21, have been implicated in osteosarcomagenesis. In normal cells, p14 and p16 proteins play a crucial regulatory role in G1 phase. In fact, $p14^{ARF}$ can inhibit MDM2 function, resulting in stabilization of p53 and cell cycle arrest or apoptosis. Deletions on chromosome 9p21 have been found in osteosarcomas (Nielsen *et al.*, 1998; Mohseny *et al.*, 2009; Mohseny *et al.*, 2010) and correlated with uncontrolled cell proliferation, tumor progression and particularly poor prognosis (Mohseny *et al.*, 2009).

- **Osteogenic differentiation.** Several studies suggest that osteosarcoma displays a phenotype similar to undifferentiated osteoprogenitors, in what concerns proliferative capacity and expression of typical osteogenic markers, such as alkaline phosphatase, osterix, Runx2 and Wnt signaling, which are markers of early osteogenic differentiation and more expressed in committed osteoprogenitors and early osteoblasts (Thomas *et al.*, 2004). In this process, osteogenic bone-morphogenetic proteins (BMPs) may also give their contribution, by controlling pro-tumorigenic events occurring in osteochondroprogenitors, through the up-regulation of early differentiation target genes (Luo *et al.*, 2008). Overexpression of Runx2 was actually correlated with poor response to chemotherapy in osteosarcoma patients (Sadikovic *et al.*, 2010). The levels of expression of these markers vary according to the degree of cell differentiation – early or late osteogenic differentiation – and also in osteosarcoma this is observed, with the more aggressive tumors displaying a phenotype similar to undifferentiated progenitors and the less aggressive tumors showing similarities with committed

osteogenic cells that progressed further along the differentiation cascade. Moreover, osteosarcoma cells normally fail to undergo terminal differentiation, which contributes to their capacity for uncontrolled proliferation. As depicted later (**Figure 1.**), the degree of dedifferentiation of osteosarcoma cells may correlate with defects on the osteogenic differentiation cascade and also with a worse prognosis (Wagner *et al.*, 2011).

1.1.4 Mesenchymal stem cells and osteoblasts as possible cells-of-origin in osteosarcoma

Osteosarcoma is considered a differentiation disease, derived from multipotent mesenchymal stem cells (MSCs). Recent findings suggest a potential link between a defective osteogenic differentiation of MSCs (**Figure 1.1**) and the development of osteosarcoma (Tang *et al.*, 2008). Moreover, previous studies proposed a model describing the pre-malignant stages of osteosarcoma development, in which transformed MSCs, with complete loss of the *CDKN2A* locus, formed osteosarcomas upon grafting in murine models (Mohseny *et al.*, 2009, Shimizu *et al.*, 2010). These studies elucidated further about the mechanisms by which transformed murine MSCs led to the formation of tumors in nude mice, as reported previously (Tolar *et al.*, 2007).

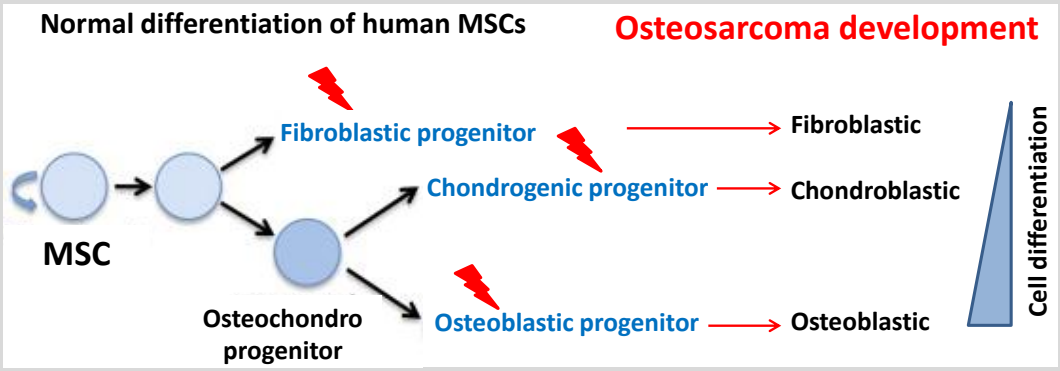


Figure 1.1. Development of osteosarcoma resulting from defects in the normal differentiation of MSCs. Mutations occurring in fibroblastic, chondrogenic or osteoblastic progenitor cells, which are derived from differentiation of normal MSCs, may originate mutated cells that form the basis for osteosarcoma development. Depending on the mutated cell, different degrees of cell differentiation exist corresponding to the different most common subtypes of conventional osteosarcoma.

The functional parallelism as well as the phenotypic behavior observed between normal and transformed MSCs, provides large evidence for a MSC origin of osteosarcoma, as illustrated in **Figure 1.2**. In this model, similar to osteosarcomas, also other bone and soft tissue tumors, such as chondrosarcoma, fibrosarcoma and liposarcoma are likely to originate from the malignant transformation of MSCs.

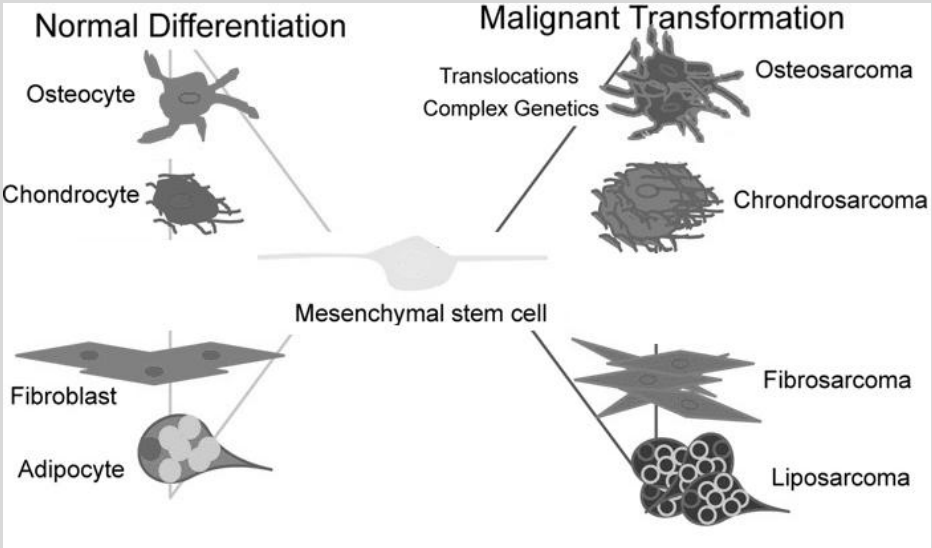


Figure 1.2. Transformed multipotent MSCs may originate osteosarcoma and other sarcomas. Multipotent MSCs, residing in all tissues, are able to differentiate into specific lineages, such as osteogenic, chondrogenic, fibroblastic and adipogenic. Chromosomal translocations or complex genetic alterations may occur and lead to the malignant cell transformation, giving rise to the diverse types of sarcoma. Adapted from (Teicher, 2012).

An important component of the osteoblast differentiation program, which may underlie the malignant transformation of MSCs into osteosarcoma (Haydon *et al.*, 2007), is the stem cell niche of the bone marrow and associated secreted factors. In fact, some of the signaling factors that influence the MSC program in osteosarcomas have been described. Mesenchymal stem cells isolated from human osteosarcoma samples were shown to be genetically distinct from tumor cells, suggesting that maintenance of a separate niche might be crucial in maintaining osteosarcoma cells in an undifferentiated state (Brune *et al.*, 2011). Alternatively, it is possible that MSCs preserve osteosarcoma cells in an undifferentiated state through secretion of cytokines such as interleukin-6 (Bian *et al.*, 2010), which has also been shown to promote osteosarcoma proliferation and metastasis through STAT3 activation (Tu *et al.*, 2012). More recently, Bonuccelli and co-workers investigated the mutual effect of normal-cancer cell metabolic programming and found that MSCs can increase their lactate production in response to oxidative

stress induced by osteosarcoma cells, which conversely enhances the migratory ability of tumor cells, an effect likely mediated by exosomes (Bonuccelli *et al.*, 2014).

Altogether, these studies demonstrate the key role of the interactions between tumor cells and their surrounding supportive microenvironment, which is crucial for the survival and proliferation of tumor cells. Still, the mechanisms by which osteosarcoma cells take advantage of normal MSCs have been matter of intense research in the past years, with several groups uncovering information on the signaling pathways that govern MSC programs in osteosarcomas. The involvement of p53 in the context of osteosarcomagenesis and its abnormal function in MSCs has been recently reviewed (Velletri *et al.*, 2016). It is worthy to mention that changes in the p53 status can compromise bone homeostasis since it orchestrates key stages of the osteogenic differentiation program of MSCs, which if compromised may lead to osteosarcoma development, as previously explained. For instance, Rubio *et al.* showed that intrabone or periosteal inoculation of p53-deficient bone marrow- or adipose tissue-derived MSCs originated metastatic osteoblastic osteosarcoma, which when accompanied with the osteo-inductive factor BMP-2 increased the expression of osteogenic markers in a Wnt signaling-dependent manner and further increased the formation of the typical osteoid matrix deposited by osteosarcoma cells (Rubio *et al.*, 2014). An alternative mechanism involves the family of proteins so-called inhibitors of DNA binding (IDs). An elegant study from Williams and colleagues uncovered that deubiquitination and stabilization of ID proteins by the deubiquitinating enzyme USP1 preserved stem cell-like features in osteosarcoma. Moreover, forced expression of USP1 in MSCs also stabilized ID proteins and led to inhibition of osteoblastic differentiation and promoted a more proliferative phenotype. This study implicated deubiquitination as a new mechanism possibly contributing to stem cell states in osteosarcoma and identified new potential therapeutic targets (Williams *et al.*, 2011).

Signaling pathways involved in metastasis formation and epithelial-mesenchymal transition (EMT) also seem to be involved in the crosstalk between MSCs and osteosarcoma cells. Recent findings from Fontanella *et al.*, suggest that bone marrow-derived MSCs cultured with osteosarcoma U2OS cells induced the activation of survival-related AKT and ERK signaling pathways. Also, normal cells triggered an increase in tumor cell migration and invasion that was resultant from increased CXCR4 levels. From a therapeutic perspective, these authors used a new CXCR4 inhibitor, Peptide R, which reduced features of EMT in cancer cells, such as expression of vimentin protein and the

crosstalk between tumor cells and normal stem cells (Fontanella *et al.*, 2016). Another group has shown that conditioned medium from bone marrow-derived stem cells can promote the proliferation and invasion of osteosarcoma cells, effects that possibly involve the stromal cell-derived factor-1/CXCR4 signaling axis (Yu *et al.*, 2015). Together, these data indicate that microenvironment signals from supportive MSCs are crucial to drive osteosarcoma development.

1.1.5 Current therapy for osteosarcoma

Overall survival rates for children and adolescents with osteosarcoma have improved significantly since the 70's, after the inception of conventional chemotherapy (Mirabello *et al.*, 2009). In fact, osteosarcoma treatment follows a multimodal approach constituted by neoadjuvant chemotherapy, used to shrink the tumor mass in order to achieve a maximal safe surgical margin for posterior local surgical resection, and subsequently post-operative or adjuvant chemotherapy (**Figure 1.3**). Neoadjuvant chemotherapy has the advantage that the histological response to chemotherapy can be assessed on the resection specimen. This chemotherapeutic regimen has improved the cure rate and long-term disease-free survival percentage for osteosarcoma patients with localized lesions from 5% to 20% in the pre-chemotherapy era to the 60% to 70% range observed nowadays (Ta *et al.*, 2009; Ritter and Bielack, 2010).

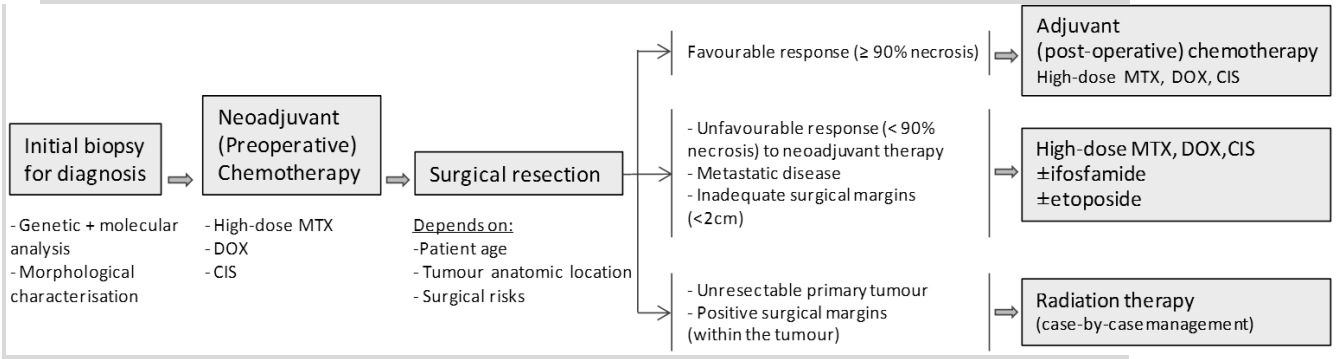


Figure 1.3. Osteosarcoma therapeutic management. Treatment of osteosarcoma patients usually includes a multidrug regimen, where doxorubicin (DOX), cisplatin (CIS) and methotrexate (MTX) are included.

The chemotherapy regimens recommended by the protocol of the **Eur**opean and **Am**erican **Os**teosarcoma Study Group (EURAMOS-1) include doxorubicin, cisplatin and methotrexate. These three drugs, which may be combined with ifosfamide and etoposide after surgery, consist on the chemotherapy regimen most applied in

osteosarcoma patients (Ferrari and Palmerini, 2007; Whelan *et al.*, 2015). Ifosfamide was synthetized from a chemical modification of cyclophosphamide and acts as a DNA alkylating agent (Tascilar *et al.*, 2007). Etoposide inhibits DNA topoisomerase II and acts primarily in the G2 and S phases of the cell cycle, leading to mitotic failure and subsequent cell death (Bromberg *et al.*, 2003). The use of both ifosfamide and etoposide has demonstrated activity in several soft tissue sarcomas and also in osteosarcoma patients (Goorin *et al.*, 2002; Tascilar *et al.*, 2007; Ferrari *et al.*, 2012), but still the addition of a fourth drug to the doxorubicin/cisplatin/methotrexate regimen has been questioned, since clinical outcomes have not improved significantly (Anninga *et al.*, 2011). In fact, recently published results from the EURAMOS-1 clinical trial demonstrate that the addition of ifosfamide and etoposide to postoperative chemotherapy in patients with poorly responding osteosarcoma is not recommended, since it is associated with increased toxicity and adverse secondary effects, while not significantly improving event-free survival (Marina *et al.*, 2016). The authors of this study recommend that the three drug regimen remains the standard of care for osteosarcoma patients, and future trials should incorporate drugs with different mechanisms of action and targeted therapies.

The percentage of necrotic tissue following neoadjuvant chemotherapy is an important prognostic factor in osteosarcoma. Patients with a level of tumor necrosis <90 % are considered poor responders as classified by histological grading (Mintz *et al.*, 2005) and are conducted to further adjuvant chemotherapy (doxorubicin, cisplatin, methotrexate) accompanied or not by additional compounds, or even conducted to radiation treatment depending on the case, as depicted above. The probability of disease-free survival, to date, is essentially determined by the histological response of tumors to neoadjuvant chemotherapy, measured by the extent of tumor necrosis, which is the most important prognostic factor (Bielack *et al.*, 2002; Grimer, 2005; Bacci *et al.*, 2005).

Neoadjuvant, preoperative or induction chemotherapy enables the early treatment of micrometastatic disease and facilitates the surgical resection by shrinking the tumor mass and decreasing vascularity (Rosen *et al.*, 1982). Surgical resection for osteosarcoma patients, usually a limb-salvage surgery, should occur after a defined time-interval, with no advantage being observed with immediate surgery (Goorin *et al.*, 2003) and aims to preserve a functioning limb without increasing the risk of post-operative complications to the patient. Adjuvant chemotherapy after surgical resection is normally performed in order to minimize the likelihood of local recurrence.

Dose-intensification of preoperative chemotherapy in osteosarcoma treatment has been examined in some studies. In 2003, Bacci and colleagues compared the histological response and final outcome of two groups of osteosarcoma patients treated preoperatively with different doses of doxorubicin, cisplatin, methotrexate and ifosfamide. They found no significant differences in terms of good histologic response to chemotherapy or in survival rates (5-year event-free and 5-year overall), which then suggests that the degree of necrosis at the time of definitive surgery reflects the innate biological sensitivity of osteosarcoma cells to chemotherapy, despite the dose intensification (Bacci *et al.*, 2003). Five years later, Lewis *et al.* reported that dose-intensive regimen produced a higher response rate than the standard regimen, as a statistically significant increase in favorable histologic response (more than 90% necrosis) was observed in patients receiving higher dosages of chemotherapy. Unfortunately, this difference did not translate into better overall survival or progression-free survival, which then puts into question the reliance on response rates as an early indicator of treatment benefit (Lewis *et al.*, 2007). Anninga and co-workers have also observed that increasing the dosages of chemotherapy improved the histological response but not outcome (Anninga *et al.*, 2011). Overall, the attempts to increase the proportion of tumor necrosis by means of an intensification of preoperative chemotherapy have not been always beneficial, leaving open the question of how to rescue poor responders to standard therapy.

1.1.6 Novel therapeutic options being explored in osteosarcoma

Development of metastatic disease, poor response to standard therapy and relapsed disease still constitute the major clinical problem preventing the cure of high-grade osteosarcoma patients. Of special note, patients presenting metastasis at the time of diagnosis have severely limited therapeutic options (Anninga *et al.*, 2011). It is therefore understandable that new treatment strategies and drugs are warranted to ameliorate the results achievable with the conventional protocols.

Sleijfer and Gelderblom recently reviewed ongoing clinical trials for osteosarcoma and soft tissue sarcomas (Sleijfer and Gelderblom, 2014) and emphasized the importance of designing such studies considering the stratification for tumor subtypes in order to enroll the appropriate patients for the clinical trials that may be most relevant for their specific case. This review summarized the developments in clinical studies and drug classes being explored in osteosarcoma and highlighted compounds such as the immunoregulatory cytokine pegylated interferon α2b and the third-

generation bisphosphonate zoledronate. Pegylated interferon α2b activates the immune response and could therefore be active in osteosarcoma considering its extent of immunogenicity; disappointingly, the EURAMOS-1 investigators working in this study recently reported the lack of statistical significance of adding pegylated interferon to the classic combination doxorubicin/cisplatin/methotrexate (Bielack *et al.*, 2015). Zoledronate showed pre-clinical evidence of activity against osteosarcoma (Goldsby *et al.*, 2013), but also, lack of significant improvement in response was reported, with the addition of zoledronate to chemotherapy having the chance to increase the risk of therapeutic failure and being therefore not recommended (Piperno-Neumann *et al.*, 2016).

Nevertheless, several therapeutic approaches are currently under clinical evaluation in osteosarcoma. Recently, also Hattinger and colleagues elegantly reviewed the emerging drugs for osteosarcoma which are under evaluation in different phases of clinical trials (Hattinger *et al.*, 2015) and for which results are not yet published so far. In summary, these drugs have specific targets and mechanisms of action such as aurora kinase A (alisertib), inhibition of mammalian target of rapamycin (everolimus, CC-115), inhibition of angiogenesis and topoisomerase I (pazopanib, sorafenib), local treatment of pulmonary metastasis (inhaled lipid cisplatin, aerosolized interleukin-2), inhibition of cell proliferation (nab-paclitaxel, eribulin mesylate) and DNA alkylation (*N,N′,N′′*-triethylenethiophosphoramide). Moreover, also muramyl tripeptide (mifamurtide) is recommended to be added to chemotherapy, improving overall survival in some patients (Meyers *et al.*, 2008).

A very recent review from Heymann group also elaborated on the drugs being tested in phase I/II clinical trials, with special emphazises for immunomodulating agents, such as mifamurtide, anti-GD2 therapies and dendritic cell vaccines (Heymann *et al.*, 2016).

1.2 Cancer Stem Cells in Solid Tumors

1.2.1 Stem cells in human cancer – historical perspective

The existence of cancer stem cells was firstly documented in biological literature in 1937 by J. Furth and M. Kahn (Furth *et al.*, 1937). These authors successfully generated a tumor by grafting a single mouse tumor cell into a recipient mouse. Studies from Makino published in 1959 introduced the term "tumor stem cell" defining them as "a small subpopulation of cells that were insensitive to chemotherapy and had chromosomal features different from the bulk of cells" (Makino, 1959). However, the first formal and experimental-based definition of the "tumor stem cell" concept was later on proposed by Pierce and colleagues (1960-1988), owing to a series of landmark experiments in mouse teratocarcinomas and squamous cell carcinoma (Pierce *et al.*, 1960; Pierce and Wallace, 1971; Pierce and Speers, 1988). The presence of both undifferentiated and differentiated cells in tumors, and a possible hierarchical organization were described as "a concept of neoplasms, based upon developmental and oncological principles, states that carcinomas are caricatures of tissue renewal, in that they are composed of a mixture of malignant stem cells, which have a marked capacity for proliferation and a limited capacity for differentiation under normal homeostatic conditions, and of the differentiated, possibly benign, progeny of these malignant cells" (Pierce and Speers, 1988). In the 1970s also Metcalf and Moore referred to the "leukemic stem cells" describing them as "colony-forming cells" that "were shown to be self-replicating" (Metcalf and Moore, 1970). For a comprehensive understanding of the chronological evolution of the notion of cancer stem cells (CSCs) in cancer biology and development, the reader is also referred to reviews of outstanding quality (Clevers, 2011; Nguyen *et al.*, 2012).

1.2.2 Parallelism between Cancer Stem Cells and Normal Stem Cells

Some of the fundaments of the CSC model are based on the similarities between these malignant cancer cells and normal stem cells. Therefore, to understand the fundamental biology of CSCs it is necessary to comprehend some of the properties of normal stem cells.

Normal and cancer tissues have a population of stem cells that function in the maintenance of that individual tissue. Stem cells have unique properties that separate

them from generic cells (Shackleton, 2010), namely the capacity to self-renew and to differentiate into specific cell types (**Figure 1.4**):

- **Self-renewal** refers to the intrinsic capacity of the stem cells to divide into two daughter cells, among which at least one of them possesses a similar developmental potential; in specific, one of the daughter cells is equal to the original stem cell and has the potential to divide indefinitely without acquiring properties of a different cell type.
- **Differentiation** refers to the potential of a stem cell to divide and generate a committed daughter cell that will form another specific cell type, with a more specialized function within the tissues. This committed progenitor cell (also referred to transit-amplifying cell) may therefore give rise to differentiated cells after rapid proliferation and amplification.

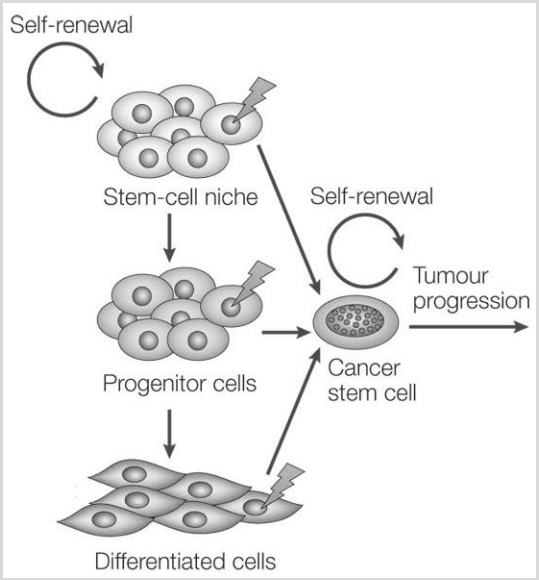


Figure 1.4. Self-renewal and differentiation in normal and cancer stem cells. Self-renewal and differentiation are intrinsic properties of stem cells and of CSCs. Mutations occurring in normal stem cells, progenitor cells or even more differentiated cells might give rise to CSCs and subsequent tumor development and progression. Adapted from (Bjerkvig *et al.*, 2005).

An accurate regulation of stem cell activity is crucial for the maintenance of tissue homeostasis, cell turnover and well-balanced internal steady state of that defined tissue. Therefore, expansion of the stem cell pool has to be restricted in these tissues in order to prevent "uncontrolled" cellular growth and proliferation. This may be accomplished by one of two different types of cell division: asymmetric or symmetric cell division (**Figure 1.5**):

▪ In the process of **asymmetric cell division** one daughter cell remains as a stem cell throughout self-renewal, and the other daughter cell is committed to go into a program of terminal differentiation and specialization. The maintenance of the equilibrium of the stem cell subset is most easily explained by asymmetric cell divisions.

▪ During the **symmetric cell division**, both daughter cells assume the same fate, which may result in the generation of two similar stem cells (symmetric self-renewal) or two similar differentiated cells (symmetric differentiation). In these scenarios, the pool of stem cells is expanded or exhausted, respectively, which may alter the tissue homeostasis in case that uncontrolled cell proliferation occurs.

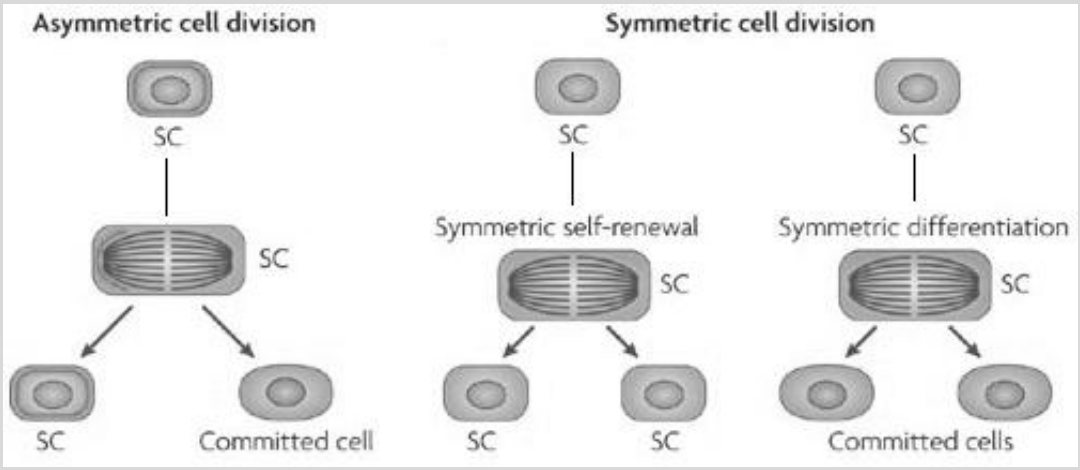


Figure 1.5. Models of cellular division in normal and cancer stem cells. During the asymmetric cell division, two distinct daughter cells (one stem cell (SC) and another committed cell) have dissimilar functions within the tissues; in this process global homeostasis is maintained. During the symmetric cell division, either two stem cells or two committed daughter cells are generated after the mitosis of the original stem cell. Adapted from (Blanpain and Fuchs, 2009).

1.2.3 Theories of cancer development: stochastic clonal evolution model and the hierarchical cancer stem cell (CSC) model

The development of cancer is well-recognized as a disease of uncontrolled cell proliferation. Basic and clinical investigations, conducted all over the world and for many decades, have described theoretical models to unravel the mechanisms underlying cancer progression and development of inter- and intra-tumor heterogeneity. The most well-accepted and best described models are the stochastic model, also known as clonal evolution model, and the hierarchical cancer stem cell (CSC) model (**Figure 1.6**). Despite that initially these two hypotheses were described as opposing, recent investigations suggest that the level of complexity of tumor

development might be bigger than suspected and therefore these models may be, instead, complementary, as will be outlined.

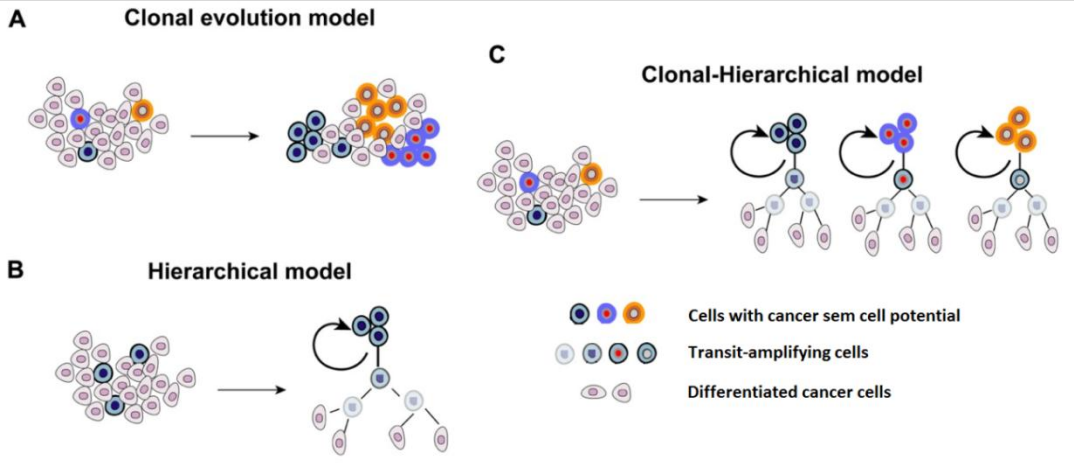


Figure 1.6. Theoretical models for explaining cancer evolution. A. In the stochastic clonal evolution model of tumor proliferation, different clones co-exist in the tumor and retain the ability of cancer initiation, self-renewal and differentiation. Random genetic alterations may confer dominant traits to some of the clones, leading to tumor heterogeneity. **B.** In the CSC hierarchical model, only a minority of tumor cells has the ability of generating the whole tumor population. This subset of stem-like cells possesses long-term self-renewal and gives rise to transit-amplifying cells and progressively more differentiated cells. **C.** In the combined clonal-stem cell model new mutations occurring in CSCs may lead to clonal evolution and multiple CSC clones co-exist within the tumor, further increasing tumor heterogeneity. Adapted from (Maugeri-Saccà *et al.*, 2013).

1.2.3.1 Stochastic clonal evolution model

The **stochastic clonal evolution model** (**Figure 1.6A**) postulates that tumor development follows a Darwinian process, in which the accumulation of random genetic mutations leads to the emergence of dominant cell clones (Nowell, 1976; Merlo *et al.*, 2006). These dominant clones acquired a survival advantage over other cells and therefore possess enhanced capacity to survive in a hostile microenvironment. These selected cell populations are best suited for survival and proliferation and will then dominate the tumor. This stochastic model assumes that virtually every cell within the tumor may act as a cancer-propagating cell and their different activities may be explained by intrinsic factors, which will determine the overall tumor heterogeneity owing to the existence of genetically diverse sub-clones responsive to micro-environmental stimuli (Shackleton *et al.*, 2009; Nguyen *et al.*, 2012; Maugeri-Saccà *et al.*, 2013). One of the best examples in which the clonal evolution model is applicable is

acute myeloid leukemia, as demonstrated by studies from several groups using whole-genome sequencing techniques (Ding *et al.*, 2012; Li *et al.*, 2016). These reports demonstrated that therapeutic failures and relapses are associated with the acquisition of new mutations and subjected to clonal evolution, processes that are shaped by chemotherapy. Moreover, tumor heterogeneity is linked to dynamic genetic and epigenetic events which may affect the biological and clinical features of tumors, as evidenced in osteosarcoma from data derived from whole-exome sequencing analysis (Bousquet *et al.*, 2016).

Nevertheless, histological evidence derived from the analysis of histopathological specimens reveals that some tumors are organized in a hierarchical manner, a feature that the stochastic model cannot explain (van Neerven *et al.*, 2016).

1.2.3.2 Cancer stem cell (CSC) model

In recent years, a novel model of tumorigenesis was proposed (**Figure 1.6B**), based on the identification of a biologically distinct and rare tumor cell fraction within the total malignant cell population, displaying characteristics similar to those of normal stem cells – the **cancer stem cell** hypothesis. The existence of these CSCs was proposed by several authors (Sell and Pierce, 1994; Tu *et al.*, 2002) and several experimental-based studies in fact identified CSCs in acute myeloid leukemia (Bonnet and Dick, 1997), breast cancer (Al-Hajj *et al.*, 2003), brain tumors (Singh *et al.*, 2003) and bone sarcomas (Gibbs CP *et al.*, 2005), among many others as previously reviewed (Visvader and Lindeman, 2008).

Basic principles of the CSC theory. This hierarchical model postulates that a cellular hierarchy exists within the tumors, and only CSCs possess long-term self-renewal and differentiation potential into different lineages. CSCs, as the founders of the entire tumor cell population, reside at the apex of such hierarchy and give rise to committed progenitor cells with progressively higher levels of differentiation, and lower proliferative capacity and restricted renewal potential (Clarke *et al.*, 2006; Visvader and Lindeman, 2012). However, the model does not accommodate the possibility of reversal between cellular states with intrinsic unrestricted or limited proliferative capacities, that is, a committed progenitor cell shall not display proliferative capacity similar to the undifferentiated stem-like cells (Nguyen *et al.*, 2012).

CSCs display intrinsic heterogeneity. The CSC model does necessarily require that CSCs are homogeneous cell subsets at the genetic or functional levels. Indeed, several studies, indicate that different CSC sub-populations that are genetically distinct may co-

exist (Visvader and Lindeman, 2012), which explains the distinct roles that CSCs possess, namely in tumor initiation, chemoresistance or self-renew abilities (Maugeri-Saccà *et al.*, 2013). A comprehensible explanation of the most used *in vitro* techniques available for CSC identification and isolation is given in **Annex I**. Principles, advantages and limitations of the sphere, side-population and Aldefluor™ assays, as well as CD marker sorting techniques, are explained in that section of this thesis.

CSCs have important therapeutic implications. CSCs are relatively resistant to common chemotherapeutic agents, in comparison to their more sensitive and differentiated progeny (Diehn *et al.*, 2009a). In this regard, CSCs may account for cases of relapses post-treatment and also contribute for the development of metastatic disease (Cheng *et al.*, 2011). Therefore, an important clinical implication from this model is the possibility of eradicating tumor growth if CSCs are properly eliminated. Intense research has been conducted in the past years aiming to specifically target chemoresistant CSCs; signaling pathways involved in self-renewal and also depletion of the activity of detoxification systems seem to hold potential for a successive eradication of this chemoresistant cell subset within the tumors, as will be outlined in later sections of this thesis.

1.2.3.3 The clonal and CSC models of cancer development are complementary – the plastic CSC model

The clonal evolution model and the hierarchical CSC model of tumorigenesis are not mutually exclusive, and attempts to better describe tumor heterogeneity suggest a new type of model – the **dynamic or plastic CSC model**, which combines principles of both theories (**Figure 1.6C**). In this model, tumor heterogeneity results from hierarchical organization of different phenotypic cell clones, which may convert from non-tumorigenic to tumorigenic cell fractions. Moreover, cancer cell populations behave dynamically due to their intrinsic cell plasticity, since not only CSC can generate mature progeny, but also differentiated cells are able to switch phenotypically into stem-like cells, in the presence of the appropriate oncogenic insults (Vicente-Duenas *et al.*, 2009; Cabrera *et al.*, 2015). Collectively, these two mechanisms have also been referred to as phenotypic switching (Kemper *et al.*, 2014) or more recently, as bidirectional interconversion (van Neerven *et al.*, 2016). Indeed, several studies characterizing embryonic stem cells (ESCs), adult somatic stem cells and CSCs seem to exclude the scenario in which the clonal evolution model and the CSC model are of antithetic nature:

▪ Takahashi and Yamanaka in 2006 demonstrated that fibroblasts, differentiated mature cells, could be converted in induced pluripotent stem cells by acquiring a stemness state due to forced expression of the core of embryonic transcription reprogramming factors (Oct3/4, Sox2, c-Myc, Klf4), so-called Yamanaka factors (Takahashi and Yamanaka, 2006);

▪ Several studies in different cancer types also demonstrate that stemness in cancer is a cell state, which is affected by multiple conditions such as hypoxia, pH, paracrine signals and activation of the EMT. These conditions promote the expression of stem cell markers and clonogenic ability in differentiated cancer cells, which suggests that reprogramming of stemness may also occur in cancer (Mani *et al.*, 2008; Li *et al.*, 2009; Vermeulen *et al.*, 2010; Hjelmeland *et al.*, 2011);

▪ External influences derived from the microenvironmental conditions surrounding cancer cells, which is a key mainstay of the principles of the clonal evolution model, are also involved in the biology of CSCs, namely by imprinting dynamics to the process of acquisition and retention of stem-like features (Vermeulen *et al.*, 2010; Hjelmeland *et al.*, 2011);

▪ Not only CSCs, but also early progenitors derived from CSCs division, seem to be able to propagate the tumors, although with different temporal patterns, as exemplified in colon cancer (Dieter *et al.*, 2011). This fact is strengthened by other studies demonstrating that CD133-positivity is not exclusive of tumor-initiating cells and CD133-negative cells are also tumorigenic (Shmelkov *et al.*, 2008);

▪ The genetic heterogeneity observed among cancer-propagating cell populations suggests a clonal evolution within the stem cell pool, as exemplified in acute lymphoblastic leukemia, in which sub-clones have variegated genetics and branched evolutionary origins (Anderson *et al.*, 2011).

The clear distinction of the clonal evolution model from the CSC model requires that researchers employ complex methods of clonal tracking to determine whether there exists stochastic causality on CSC activity and propagation. Moreover, a better functional characterization of CSCs is important to identify these cells, since genetic and phenotypic features may differ from the stem-like cell behavior that is detected at the functional levels (Nguyen *et al.*, 2012).

1.2.4 Cancer stem cells in osteosarcoma

The identification of osteosarcoma CSCs has gained increasing attention over the last decade. In this section we highlight some of the studies that already reported the existence of these cells in osteosarcoma cell lines and patient samples, and explored their leading role in osteosarcoma genesis and resistance to therapies.

Spheres. The sphere-formation assay has been widely used to isolate CSCs from diverse solid tumors. Gibbs and co-workers reported for the first time results derived from the application of this assay to osteosarcoma cell lines (Gibbs CP *et al.*, 2005). Since then a growing number of groups also employed the simple and practical principles of the sphere assay to enrich and expand osteosarcoma CSCs, which were subsequently characterized by, for instance, the expression of pluripotency-related markers (Di Fiore *et al.*, 2009; Fujji *et al.*, 2009; Wang *et al.*, 2009; Saini *et al.*, 2012; Martins-Neves *et al.*, 2012) and resistance to chemotherapy (Martins-Neves *et al.*, 2012; Zhang *et al.*, 2013; Salerno *et al.*, 2013; Yu *et al.*, 2016).

Aldefluor™. Activity of aldehyde dehydrogenases (ALDH) using the commercial Aldefluor™ assay kit has been increasingly used in the identification and isolation of CSCs in several tumors types. Honoki and colleagues in 2010 firstly reported the identification of ALDH-positive osteosarcoma cells (Honoki *et al.*, 2010). Later on, studies from Wang and co-workers also revealed the presence of ALDH-positive cells in mouse xenografts and correlated with stem cell marker expression and high tumorigenicity (Wang *et al.*, 2011). Also in murine osteosarcoma cells ALDH seems to cooperate with DKK-1 to enhance tumorigenic properties (Krause *et al.*, 2014).

Side-population. Although the side-population assay is more questionable than the Aldefluor™ assay due to its less accurate technical implementation (Golebiewska *et al.*, 2011), several groups have published data using this assay to identify and isolate osteosarcoma CSCs. Wu *et al.* reported in 2007 the existence of a distinct cell population extruding Hoechst-33342 in osteosarcoma samples, consistent with the side-population phenotype (Wu *et al.*, 2007). The protocol optimized by this group has since then been used by other researchers to identify the side-population in osteosarcoma cell lines (Tirino *et al.*, 2008; Tang *et al.*, 2011a; Rouleau *et al.*, 2012). Isolated side-population cells have been associated with tumorigenic capacity, expression of stemness-related markers including Wnt signaling activation (Yi *et al.*, 2015), sphere-forming capacity, clonogenicity and drug resistance (Wang and Teng, 2016).

Surface markers. Despite that the identification of stem-like cell populations, based on surface marker expression, has been more elusive in mesenchymal tumors than in other types of liquid and solid tumors, several groups have successfully characterized these cell subsets in osteosarcomas. Tirino and co-workers in 2008 reported for the first time the existence of CD133-positive cell subset within osteosarcoma cell lines that formed spheres, had a side-population profile and expressed *ABCG2* (Tirino *et al.*, 2008). CD133 has since then been validated by other independent groups (Di Fiore *et al.*, 2009; He *et al.*, 2012; Fujiwara *et al.*, 2014; Wang *et al.*, 2015a; Zhou *et al.*, 2015), being associated with poor prognosis and chemoresistance. Other groups have also identified surface markers such as CD29, CD90, CD105, CD44, ICAM-1, CD56, identified as a mesenchymal signature (Levings *et al.*, 2009), CD117 (Adhikari *et al.*, 2010; Pu *et al.*, 2016; Yu *et al.*, 2016), CBX3 and ABCA5 (Saini *et al.*, 2012), CD248 (Rouleau *et al.*, 2012; Sun *et al.*, 2015) and CD271 (Tian *et al.*, 2014). In general, expression of these surface markers correlates with stemness signatures in osteosarcoma and aggressive cellular phenotypes.

microRNAs. Recently, increasing attention has been devoted to microRNAs in the biology of cancer cells. Di Fiori and colleagues firstly suggested that microRNAs such as let-7/98 and miR-29a,b,c involved in carcinogenesis and stemness could be new markers for osteosarcoma (Di Fiore *et al.*, 2013). Later on, the same group reported that overexpression of miR-29b-1 reduced proliferation, stemness features and chemoresistance of 3AB-OS cells (Di Fiore *et al.*, 2014). Since then, other authors also reported the involvement of other microRNAs, such as mir-148a (Chang Y *et al.*, 2015), miR-143 (Zhou *et al.*, 2015), Let-7d (Di Fiore *et al.*, 2016), miR-34a-5p (Pu *et al.*, 2016) and miR-26a (Lu *et al.*, 2016), which are involved in osteosarcoma stemness, regulation of cell survival and resistance to therapies. However, caution should be taken when considering microRNAs as therapeutic targets or as diagnostic and prognostic markers, since they may present diverse functional roles and molecular mechanisms within the tumor cells, playing oncogenic but also tumor suppressive roles depending on the specific microRNA, and the tumor type and context.

1.2.5 Pluripotency and self-renewal-related signaling pathways involved in the biology of cancer stem cells

The presence of CSCs in several types of malignancies is now well established. These cells display many characteristics of embryonic and adult stem cells. An increasing number of studies have demonstrated that CSCs typically have tenacious activation or

expression of components of the highly conserved signal transduction pathways involved in development, differentiation and tissue homeostasis, such as the Notch, Hedgehog and Wnt pathways. Abnormal activity of these pathways that control stem-cell self-renewal seems crucial for the tumorigenicity of CSCs, cells in which these embryonic pathways may also interact with many other signaling pathways controlling cellular division, survival and invasion. Therefore, therapeutic targeting of Notch, Hedgehog and Wnt pathways in CSCs may be a leading strategy for the blockade of CSC's self-renewal and proliferation (Takebe *et al.*, 2015).

- **Pluripotency in human embryonic stem cells (ESC)**

The transcriptional regulatory networks in ESCs are governed by the pluripotency-associated factors Oct4, Sox2 and Nanog. Studies employing chromatin immunoprecipitation-based technologies revealed that these transcription factors have extensive co-binding and their gene targets have also been mapped. Oct4 and Nanog are key regulators of the ESC pluripotency due to their relatively unique expression pattern in these cells (Boyer *et al.*, 2005). These studies uncovered the existence of an Oct4-centric module, which includes Oct4, Sox2 and Nanog, as well Klf4 and Tcf3 (Jiang *et al.*, 2008; Cole *et al.*, 2008). Oct4 is also known to interact biochemically with some of these factors, including Sox2 (van den Berg *et al.*, 2010). The auto-regulatory loop formed by Oct4, Sox2 and Nanog generates a stable state for ESCs to maintain their pluripotentiality by keeping them in a controlled gene expression program and by regulating the expression levels of transcription factors, safeguarding the entrance into differentiation programs, which occurs when their expression and function is no longer present (Young, 2011).

Extracellular signals from diverse signaling pathways, such as BMP, FGF, Wnt and Activin/Nodal also regulate human ESC transcriptional regulation that is required for the maintenance of self-renewal and pluripotency (Yeo and Ng, 2013) (**Figure 1.7**). For instance, FGF-signaling via MEK/ERK activation is necessary to prevent extra-embryonic differentiation (Li *et al.*, 2007). Activin signaling, through SMAD2/3 transcription factors, is known to up-regulate Nanog expression (Vallier *et al.*, 2009). Also endogenous activity of Wnt signaling controls the differentiation fate of ESCs, as shown by studies from the Nusse group. These authors demonstrated that human ESC lines had heterogeneous Wnt activation, with the individual levels of cellular activity correlating with the diverse commitment towards lineage specification and clonogenic potential (Blauwkamp *et al.*, 2012).

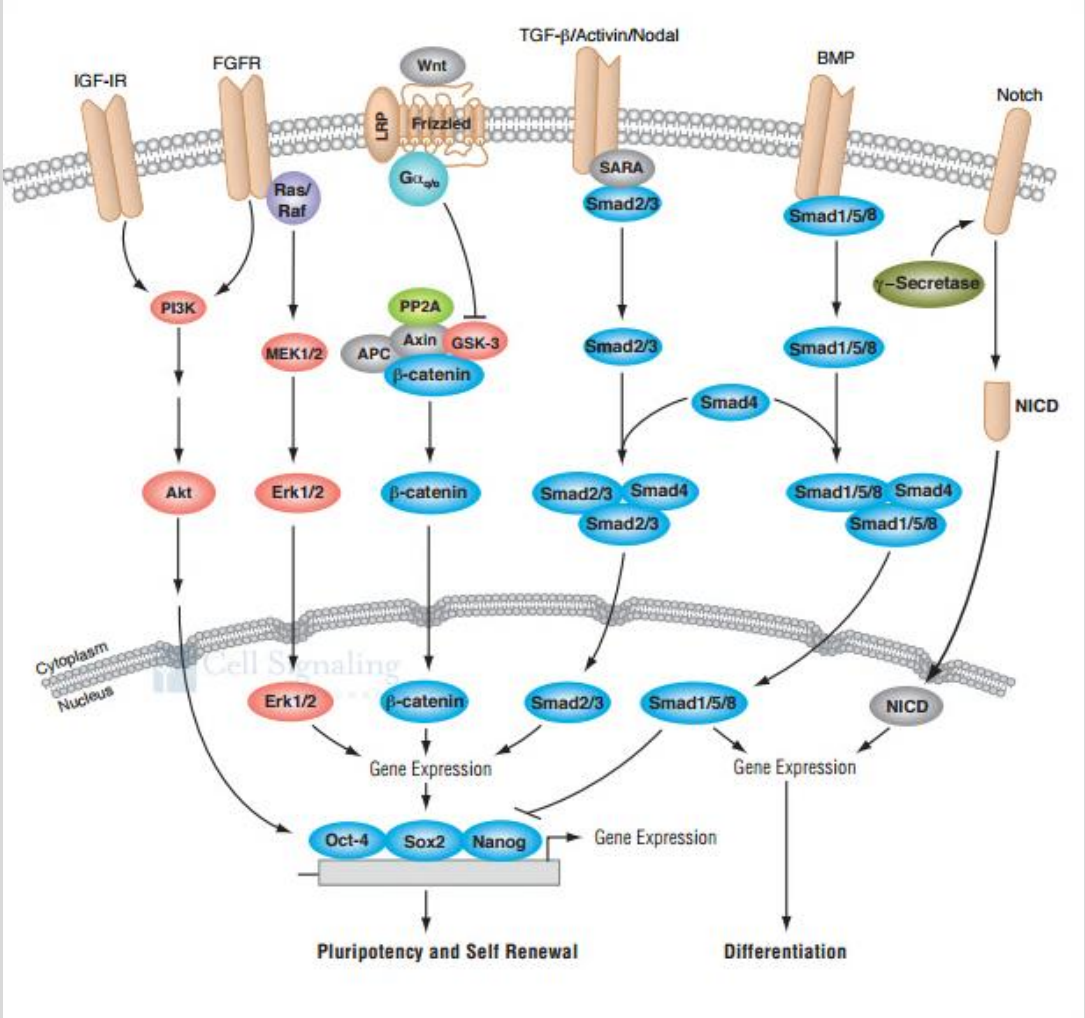


Figure 1.7. Interplay between pluripotency networks and self-renewal-related signaling pathways in human ESCs. Pluripotency in ESCs is maintained by the core transcriptional regulatory network composed of Oct4, Sox2 and Nanog. Activity of these transcription factors is influenced by diverse external clues derived from signaling pathways such as Wnt, FGF, Activin/Nodal and BMPs. Adapted from https://media.cellsignal.com/www/pdfs/science/pathways/esc_pluripotency.pdf (assessed in September, 27 2016).

Although these stem cells express transcription factors (Oct4, Sox2 and Nanog) that are classically associated with pluripotency, there are substantial differences in the features of the transcriptional regulatory networks that characterize them in ESCs and in cancer cells, which are widely known for their deregulation on the more diverse signaling pathways. Deciphering these pluripotent networks in cancer cells may provide new mechanistic insights into how these networks contribute to tumorigenesis and even resistance to therapies (Ng and Surani, 2011; Kim and Orkin, 2011).

- **Pluripotency networks in cancer cells**

The existence of embryonic-related features in tumor cells was firstly proposed by Rudolf Virchow, who hypothesized that tumors arise from embryo-like cells, after having verified that tumors display a high degree of cellular heterogeneity following pathological examination (Virchow, 1855). Later on, other studies proposed that tumors may arise from dormant embryonic remnants that exist in adult tissues, which once reactivated in certain conditions may evolve to tumors (Durante, 1874; Cohnheim, 1875).

Back in 2008, studies from the Weinberg group, demonstrated that histologically poorly differentiated tumors had an expression profile of markers associated with ESC identity and also Nanog, Oct4, Sox2 and c-Myc target genes were more frequently overexpressed in poorly differentiated tumors than in well-differentiated tumors (Ben-Porath *et al.*, 2008). Schoenhals and colleagues compared the expression of Oct4, Sox2, Klf4 and c-Myc in a series of 40 human tumors with that of their normal tissue counterparts using publicly available gene expression data and found significant overexpression of at least 1 out of 4 pluripotency factors Oct4, Sox2, Klf4 or c-Myc in 18 out of the 40 tumors. In some cases the expression of these genes was associated with tumor progression or bad prognosis, which reinforces the key role these transcription factors might have in the pathological self-renewal characteristics of cancer cells (Schoenhals *et al.*, 2009).

Several other groups have also explored the fundamental role of embryonic factors in mediating tumorigenesis and reprogramming of cancer cells towards a stem-like phenotype, in diverse tumor types. Li *et al.* demonstrated that c-Met enhanced the glioblastoma CSC subset via a mechanism requiring Nanog (Li *et al.*, 2011). Other groups showed that a proper interplay between Nanog, Hedgehog signaling and p53 is also crucial for tumor growth and stem cell behavior in glioblastoma (Zbinden *et al.*, 2010). Also in hepatocellular carcinoma, Nanog was demonstrated to regulate stem cell renewal through up-regulation of the IGF1R-signaling pathway (Shan *et al.*, 2012). In this tumor, Oct4 has a critical role in stemness maintenance mediated by the sex-determining region Y (SRY) protein (Murakami *et al.*, 2015).

Expression of *OCT4* or transmembrane delivery of Oct4 protein induced the dedifferentiation of melanoma cells and acquisition of typical CSC-like features. Interestingly, forced Oct4 expression was also correlated with autoregulation of Oct4 expression and increased expression of other embryonic transcription factors such as Nanog and Klf4 (Kumar *et al.*, 2012).

An elegant study from Stolzenburg and colleagues demonstrated that selectively targeting Sox2 with a zinc finger-based artificial transcription factor suppressed mRNA expression in breast cancer cells, and resulted in reduced cell proliferation *in vitro* and *in vivo*. This study showed that also embryonic transcription factors are "druggable" molecules and important therapeutic targets in cancer (Stolzenburg *et al.*, 2012). Sox2, similar to other ESC transcription factors, plays important roles in stem cell functions in different tumor types such as skin squamous-cell carcinoma (Boumahdi *et al.*, 2014) and ovarian cancer (Li *et al.*, 2015), contributing also to chemoresistance.

Recently, several studies have demonstrated that Klf4 expression is cancer type-dependent and its expression during tumorigenesis may be depending on the tumor stage and play either a tumor suppressive or oncogenic role. For instance, the tumor suppressive role of Klf4, which is frequently down-regulated in colorectal cancer, was highlighted in *in vivo* models of colonic tumorigenesis. This study showed that Klf4 is involved in acinar-to-ductal cell reprogramming and has a critical role in suppressing the development of colonic neoplasia (Ghaleb *et al.*, 2016). Also Wei *et al.* showed that Klf4 is up-regulated in and required for acinar-to-ductal metaplasia during early pancreatic carcinogenesis and its ablation reduces the formation of pancreatic intra-epithelial neoplasia induced by mutant Kras (Wei *et al.*, 2016). However, for instance in esophageal squamous cell carcinoma, Klf4 expression is decreased and its deletion induced squamous cell dysplasia in mouse models. Also, recent studies indicate that Klf4 is decreased in high-grade dysplasia and early stage esophageal squamous cell tumors, but its expression increased with advanced cancer stage, and Klf4 expression in these tumors was inversely correlated with survival (Yang and Katz, 2016). Altogether these studies highlight the fact that using the expression of these transcription factors as diagnostic or therapeutic targets should be done taking in consideration the context and tumor type.

1.2.5.1 Self-renewal - Notch signaling pathway

Notch signaling is an important developmental-related signaling pathway controlling stem cell self-renewal. Notch controls the cell fate of ESCs during embryonic development and also plays a leading role in the differentiation of somatic stem cells into more specialized cell types. Notch signaling pathway is complex, plays different roles in diverse functional activities, and different cells and tumor types may express diverse Notch receptors and ligands. Post-translational modifications of pathway molecules and also crosstalk with other signaling pathways might also modify and

determine the net effect of Notch signaling (Andersson and Lendahl, 2014). Interactions between Notch transmembrane ligands (e.g. DLL1/3/4, Jagged1/2) from one cell with transmembrane receptors (Notch1-4) in another cell triggers a proteolytic cascade that culminates in the release of an intracellular fragment that can interact with CSL transcription factors that regulate the expression of target genes, such as p21, cyclin D1, cMyc and those belonging to the HES and HEY family (Gu *et al.*, 2012) (**Figure 1.8**).

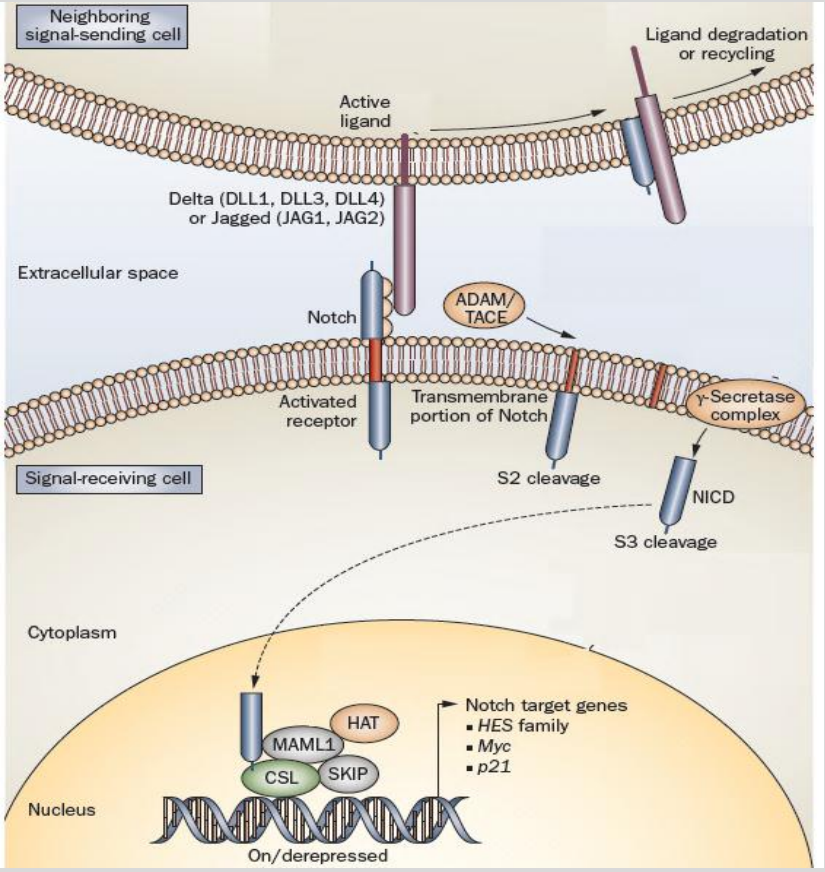


Figure 1.8. Notch signaling pathway. Cell-to-cell contact enables the activation of Notch signaling. Delta or Jagged ligands interact with Notch receptors and lead to a signaling cascade that culminates in the transcriptional activation of Notch-associated target genes. Adapted from (Takebe *et al.*, 2011).

In this signaling cascade, the enzyme γ-secretase plays a central role in the cleavage of the active Notch intracellular domain (NICD), and several compounds aiming to specifically target γ-secretase were already developed and are in clinical investigation (Takebe *et al.*, 2015). Several evidence associates alterations in the Notch signaling pathway to cancer development. Depending on the tissue type and cellular conditions, Notch signaling may serve both as oncogene if involved in stem cell self-renewal, or as

a tumor suppressor if involved in the regulation of cellular terminal differentiation (Previs *et al.*, 2015).

Notch pathway regulates cell-fate determination and maintains tissue homeostasis, playing a role also in CSCs from various malignancies (Takebe *et al.*, 2011). Examples include embryonal tumors, such as medulloblastoma, glioblastomas, pancreatic cancer and osteosarcoma. In fact, in these cancers Notch ligands and receptors have been found up-regulated and Notch inhibition associated with the suppression of typical Notch target genes such as Hes1, reduction of sphere formation and also of cells displaying CSC markers' expression, and reduction of tumorigenic potential of stem-like cells (Fan *et al.*, 2006; Fan *et al.*, 2010; Abel *et al.*, 2014). Importantly, the Notch pathway has also been shown to be a possible driver of osteogenic sarcoma (Tao *et al.*, 2014) and its inhibition suppresses osteosarcoma growth (Tanaka *et al.*, 2009).

- **Notch signaling and resistance to therapies**

Several studies suggest that Notch pathway is related to chemo- and radioresistance, and the pharmacological inhibition of this pathway might be an interesting manner to counteract the capacity of CSCs from various malignancies to survive conventional therapy (Pannuti *et al.*, 2010).

Wang and colleagues showed that inhibition of the Notch pathway by a γ-secretase inhibitor or Notch shRNA renders glioblastoma CSCs more sensitive to radiation therapy, by specifically impairing the clonogenic survival of CSCs, but not of the non-stem glioma cells, and by reducing the activity of the survival-related molecules such as AKT and Mcl-1. Of relevance, these authors also showed that Notch knockdown impaired xenograft tumor formation. This study suggested that Notch pathway may serve as another potential therapeutic target for reducing glioblastoma radioresistance (Wang *et al.*, 2010b). McAuliffe *et al.* conducted studies in ovarian CSCs and demonstrated that activation of the Notch signaling pathway, particularly via Notch3, are critical for the stemness regulation and cisplatin resistance. Moreover, Notch inhibition increased cells' sensitivity to cisplatin; also, the combinatorial treatment (cisplatin and γ-secretase inhibitor) targeted both the ovarian CSCs and the bulk of tumor cells, demonstrating a synergistic cytotoxic effect in Notch-dependent tumor cells (McAuliffe *et al.*, 2012). Another group has shown that treatment of breast cancer cells with the small herbal molecule psoralidin suppressed the proliferation of both parental ALDH-negative cells and ALDH-positive CSCs isolated from the MDA-MB-231 cell line. The cytotoxic effects of psoralidin were mediated by down-regulation of Notch1

signaling and also of epithelial-mesenchymal markers such as β-catenin and vimentin (Suman *et al.*, 2013).

Many agents targeting the Notch pathway have entered clinical trials [as reviewed recently in (Takebe *et al.*, 2015)] and hold potential for eradicating CSCs resistance, especially in combination with chemotherapy or other targeted agents.

- **Notch signaling in osteosarcoma**

As reported for other solid tumors, the prominent role of Notch signaling in controlling osteosarcoma CSCs has also been subjected of research by several independent groups. In general, activation of Notch pathway correlates with altered cell proliferation, chemoresistance and metastatic abilities of osteosarcoma cells (Hughes, 2010).

Tanaka *et al.* showed that osteosarcoma patient specimens overexpress Notch receptors and ligands, such as *NOTCH2*, *JAGGED1*, *HEY1*, and *HEY2*. Moreover, Notch inhibition associated with reduced cell proliferation *in vitro* and tumor progression *in vivo*, accompanied by cell cycle arrest and down-regulation of cell cycle promotors such as cyclin D1 and up-regulation of cell cycle suppressors, such as p21 (Tanaka *et al.*, 2009). Using a murine *in vitro* model of osteosarcoma, Mu and co-workers showed that Notch genes (*NOTCH1, 2, 4*) and also *HES1* and *STAT2* target genes were up-regulated in the highly metastatic K7M2 cell line, compared to the less metastatic K12 cells. Also, Notch inhibition reduced ALDH activity in K7M2 cells (Mu *et al.*, 2013). Tao and co-workers developed a mouse model of osteosarcoma based on the conditional expression of the intracellular domain of Notch1 (NICD). These authors showed the fundamental role of Notch activation in osteosarcoma since expression of NICD in immature osteoblasts was enough to favor the formation of tumors consistent with the pathophysiology of osteosarcoma with complete penetrance. Moreover, Notch expression combined with loss of *TP53* synergistically accelerated osteosarcoma development, further demonstrating the importance of Notch activation paralleled by mutations in *TP53* as drivers of osteosarcomagenesis (Tao *et al.*, 2014).

Activation of Notch pathway has also been reported after exposure of osteosarcoma cells to chemotherapeutics such as doxorubicin and cisplatin. It has been shown that doxorubicin at non-toxic doses inhibits proliferation of osteosarcoma cells through up-regulation of Notch target genes such as *NOTCH1*, *HEY1*, *HES1*, and *HES5* (Ji *et al.*, 2015), while sub-lethal doses of cisplatin select for cisplatin-resistant cells exhibiting a mesenchymal profile and stem cell-related genes, such as Stro-1 and CD117, a

phenotype that was down-regulated by γ-secretase inhibition. Moreover, Notch inhibition prevented tumor recurrence in resistant xenograft tumors (Yu *et al.*, 2016).

Overall, these studies indicate that targeted inactivation of the self-renewal Notch pathway may be useful for the elimination of CSCs and the overcoming of drug resistance in osteosarcoma.

1.2.5.2 Self-renewal - Hedgehog signaling

The Hedgehog signaling pathway is also recognized as a key regulator of embryonic development, controlling cellular differentiation, proliferation and self-renewal. Depending on the context, Hedgehog signals may act as morphogens, inducing different cell fates, or act as mitogens, regulating cell proliferation and organ development. In adult somatic cells, Hedgehog remains active and involved in the regulation of tissue homeostasis and maintenance of stem cell renewal (Gupta *et al.*, 2010).

Hedgehog signaling in human cells (**Figure 1.9**) initiates when Hedgehog ligands desert, indian or sonic hedgehog (DHH, IHH, SHH respectively) bind to transmembrane receptors such as Patched 1 (PTCH1). This binding results in the activation of smoothened (SMO), which is normally repressed by PTCH (*off state*). SMO activation enables the transduction of Hedgehog signals to the cytoplasm (Taipale *et al.*, 2002), through the activation of transcriptional activity of GLI transcription factors, namely the activators GLI1 and GLI2 (*on state*). GLI1 expression is highly dependent on active Hedgehog, and is therefore used as readout of Hedgehog pathway activation. In the absence of ligands, GLI transcription factors are proteolytically processed to generate the transcriptional repressor GLI3. In human cells, Hedgehog target genes include molecules involved in the signaling pathway itself, such as GLI1 and PTCH1, but also other cell-specific genes involved in cell proliferation, apoptosis and vascularization, such as cyclin D, Myc, Bmi1, Bcl-2, VEGF and Snail, depending on the contexts and cell types (Scales and de Sauvage, 2009).

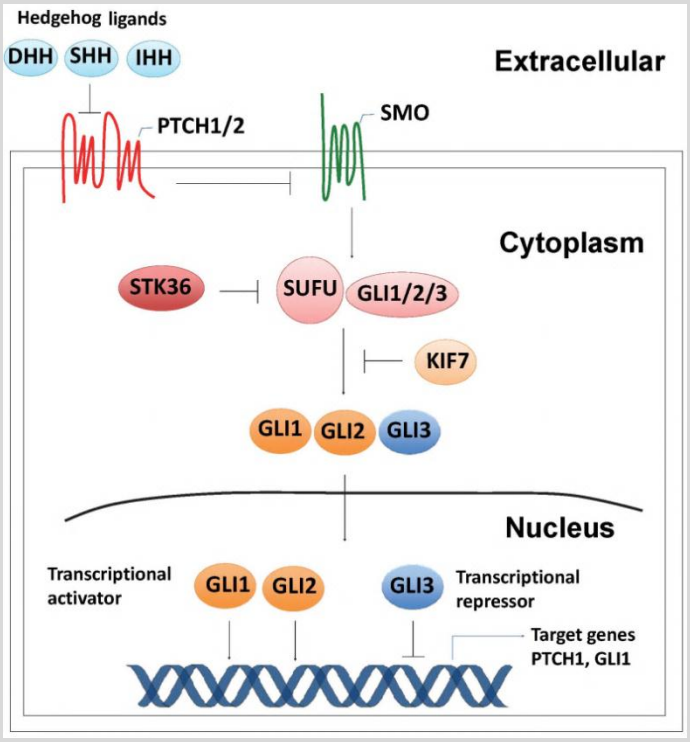


Figure 1.9. Hedgehog signaling pathway. Diagram depicting the ligands, receptors and transcriptional regulators of Hedgehog signaling pathway. See text for further details. Adapted from (Lim *et al.*, 2013).

- **Hedgehog signaling and resistance to therapies**

Similar to Notch signaling, also the Hedgehog pathway has been shown to be constitutively active in several cancer types and implicated in drug resistance, which seems to be mediated by paracrine signaling, interactions between the tumor cells and the surrounding stroma and also pathway activation in CSCs (Gupta *et al.*, 2010).

A decade ago, Sims-Mourtada and colleagues explored the connection between Hedgehog and ABC transporters. Hedgehog inhibition increased the sensitivity of esophageal adenocarcinoma cells to docetaxel, methotrexate and etoposide. Moreover, Hedgehog was shown to regulate P-glycoprotein and BCRP expression (Sims-Mourtada *et al.*, 2007). Inhibition of Hedgehog signaling with IPI-926, a compound that depletes tumor-associated stromal tissue, was also shown to enhance the delivery of gemcitabine in a mouse model of pancreatic cancer (Olive *et al.*, 2009). Also in myeloid leukemia, activation of Hedgehog was correlated with enhanced chemoresistance, and pathway inhibition interfered with P-glycoprotein-mediated drug resistance (Queiroz *et al.*, 2010).

More recently, several other groups further explored the connection between Hedgehog activation and resistance to chemotherapy. For instance, SMO antagonists

sensitized resistant ovarian cancer cells and ALDH-positive cells to paclitaxel and combination treatments reduced tumor burden *in vivo* (Steg *et al.*, 2012b). In gastric cancer, CD44-positive/Musashi-1-positive cells, which account for enhanced resistance to doxorubicin due to high drug efflux activity, overexpressed SHH and GLI1, but were sensitive to pharmacological and genetic GLI inhibition associated with *ABCG2* down-regulation (Xu *et al.*, 2015a). Other groups showed that Hedgehog pathway activity may transcriptionally control the expression of Twist1 and Snail, independently of ABC transporters; this study also found that *TWIST1* is a direct target of the transcription factor GLI (Kong *et al.*, 2015). Recently, in advanced non-small-cell lung carcinoma, GLI2 expression was found to be more often positive in patients refractory to chemotherapy than patients with controlled disease, which further reinforces that Hedgehog pathway activation is associated with resistance to chemotherapy (Giroux Leprieur *et al.*, 2016).

- **Hedgehog signaling in osteosarcoma**

Activation of the Hedgehog pathway is an important event in many types of human cancer and osteosarcoma is not exception, as evidenced by several studies.

Hirotsu and colleagues firstly demonstrated that SMO was a therapeutic target in osteosarcoma, as SMO genetic depletion or pharmacological inhibition prevented cell proliferation *in vitro* and *in vivo*. This study found that several cell lines and also biopsy specimens overexpressed signaling molecules such as SMO, PTCH1 and GLI, implying an activation of the pathway in osteosarcoma (Hirotsu et al., 2010).

Studies from the Setoguchi lab widely explored the role of GLI2 in human osteosarcoma. In 2011, this group reported that GLI2 was overexpressed in biopsy specimens and *in vitro* GLI2 knockdown prevented cell proliferation, by inducing cell cycle arrest and down-regulation of cell cycle promoters, such as cyclin D1 and phosphorylated Rb proteins (Nagao *et al.*, 2011). Moreover, GLI2 also seems to be involved in metastatic dissemination since genetic knockdown or pharmacological inhibition of GLI2 inhibited cell migration and invasion and decreased lung metastasis (Nagao-Kitamoto *et al.*, 2015a). Subsequent studies showed that osteosarcoma cell invasion mediated by GLI2 was regulated by ribosomal protein S3. Knockdown of GLI2 reduced the expression of this protein and cell migration, while forced expression of ribosomal protein S3 rescued that phenotype. This study indicated this signaling axis as a new marker of invasive osteosarcoma, since expression of ribosomal protein S3 was higher in osteosarcomas with lung metastases compared with specimens that were not disseminated (Nagao-Kitamoto *et al.*, 2015b). More recently, another report from this

group showed that Hedgehog inhibitors such as arsenic trioxide and vismodegib synergistically reduced cell proliferation when used in combination with standard chemotherapeutics, and treatment combination was also effective in a mouse xenograft model, establishing Hedgehog pathway inhibitors as an attractive therapeutic method in osteosarcoma (Saitoh *et al.*, 2016). Other reports further confirmed that Hedgehog signaling is an important therapeutic target in osteosarcoma and overexpression of pathway members such as GLI2 correlates with poor clinical outcomes (Yang *et al.*, 2013).

1.2.5.3 Self-renewal - Canonical Wnt or Wnt/β-catenin signaling pathway

The regulation of stem cell properties in cancer cells is regulated by several signaling pathways, among which the Wnt/β-catenin is probably the most relevant and studied.

Wnt/β-catenin signaling has a stage- and cell type-dependent function in bone development and also in cancer formation. The sequestration of Wnt ligands by e.g. Wnt inhibitory factor 1 (WIF1) or secreted frizzled-related proteins (SFRPs), or the interaction of lipoprotein-related protein 5 and 6 (LRP5/6) co-receptors with extracellular antagonists such as dickkopf-1 (DKK-1) and sclerostin (SOST) directly inhibit Wnt signaling (**Figure 1.10, left panel**). Moreover, the central signaling mediator β-catenin is normally phosphorylated, ubiquitinated and conducted to proteasomal degradation at the cytoplasmic level (*off state*), a process that occurs under control of the β-catenin destruction complex, a multi-protein molecular structure consisting of adenomatous polyposis coli (APC), casein kinase 1α, Axin 1/2, glycogen synthase kinase 3β (GSK-3β) and tankyrase 1/2.

Wnt/β-catenin signaling initiates when activating ligands, such as Wnt3A (Willert *et al.*, 2003), bind to members of the Frizzled receptor family and lipoprotein-related protein 5 and 6 (LRP5/6) co-receptors (Cong *et al.*, 2004) – activation of the *on state* (**Figure 1.10, right panel**). The interaction of a Wnt ligand with these membrane receptors triggers a signal cascade that reduces the degradation of β-catenin. During this *on state* β-catenin protein is not conducted to ubiquitin-proteasome-mediated degradation and its levels stabilize in the cytoplasm. The rise in protein levels and subsequent successive phosphorylation steps, culminate in the translocation of β-catenin to the nucleus. Once at the nuclear level, β-catenin partners with T-cell factor/lymphoid enhancer factor (TCF/LEF) family of transcription factors (Fodde and Brabletz, 2007), thereby promoting transcriptional activation of several specific target

genes, such as cyclin D1 and c-Myc, which regulate cell cycle, proliferation and differentiation (Tetsu and McCormick, 1999; He *et al.*, 1998), and also Axin2 and DKK-1, negative modulators of the signaling pathway (Jho *et al.*, 2002; Niida *et al.*, 2004).

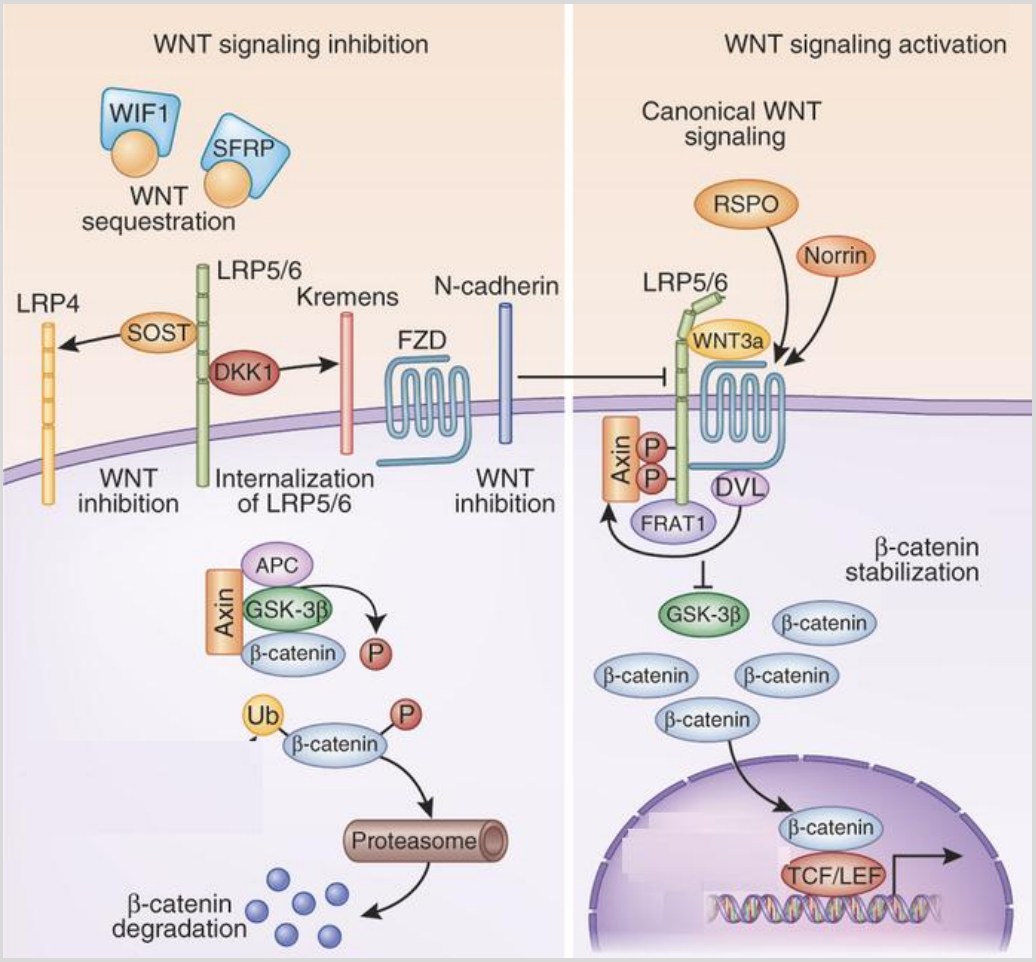


Figure 1.10. Wnt/β-catenin signaling pathway. Overview of the Wnt/β-catenin pathway, also known as canonical Wnt pathway. Adapted from (Baron and Kneissel, 2013).

- **Targeting Wnt/β-catenin pathway in cancer**

Wnt/β-catenin pathway can be abnormally activated in cancer by several manners, due to the large number of signaling molecules involved in the pathway, as previously described. Moreover, Wnt is implicated in several tumors of both epithelial and mesenchymal origin, with special emphasis for leukemia, colon, breast and prostate cancers. There is therefore a great interest and potential for the development of Wnt antagonists. Drugs targeting the Wnt pathway are currently on the market or under development, and are categorized in non-steroidal anti-inflammatory drugs (NSAIDs), derivatives of vitamin D, antibody-based treatments, and small molecule inhibitors (Yang *et al.*, 2016).

Small-molecule antagonists of the Wnt pathway ultimately leading to β-catenin degradation appear to be a promising strategy to specifically target tumor cells (Bao *et al.*, 2012; Lau *et al.*, 2013; Tian *et al.*, 2013; Wessel Stratford *et al.*, 2014). Tankyrases are members of the PARP-family of poly-ADP-ribosylation proteins, and promote the ubiquitination and degradation of Axin proteins. Axin is the concentration limiting factor in regulating the efficiency of the degradation complex exerting a key regulatory node in the Wnt/β-catenin signaling cascade. Tankyrase inhibitors, such as IWR-1, stabilize Axin by inhibiting the poly-ADP-ribosylating enzymes preventing PARsylation and ubiquitylation, thereby enhancing the stability of the degradation complex with consequent attenuation of Wnt/β-catenin signaling (Huang *et al.*, 2009). Pre-clinical studies, using tankyrase inhibitors, have shown that Axin stabilization via inhibition of tankyrase exerts significant anti-tumoral effects both *in vitro* and in xenografted animals with colon (Lau *et al.*, 2013) and lung cancer (Busch *et al.*, 2013).

Other compounds such as curcumin, piperine (polyphenols), sulforaphane (natural compound extracted from broccoli) and salinomycin (an antibacterial potassium ionophore) have been shown to inhibit, for instance, sphere forming efficiency and ALDH activity of breast cancer cells (Kakarala *et al.*, 2010), and to decrease tumor growth after serial transplants in mice (Gupta *et al.*, 2009; Li *et al.*, 2010). Salinomycin and sulforaphane also seem to be active against osteosarcoma cells, via inhibition of Wnt/β-catenin signaling, decrease in sphere forming efficiency, sensitization to doxorubicin and induction of cell cycle arrest (Tang *et al.*, 2011b; Kim *et al.*, 2011). A comprehensible description of clinical trials with drugs targeting the Wnt pathway was recently published (Zhan *et al.*, 2016).

- **Wnt/β-catenin signaling in osteosarcoma**

Aberrant activation of Wnt/β-catenin has been linked to the development of numerous carcinomas, and linked to the self-renewal of CSCs in a variety of solid tumors (de Sousa *et al.*, 2011; Mao *et al.*, 2014). However, reports regarding the activation status of Wnt/β-catenin in osteosarcoma are contradictory and no definitive causal relationship has been established so far.

▪ **Wnt *activation* in osteosarcoma.** Some authors reported an abnormal activation of Wnt/β-catenin mediated by an autocrine mechanism in osteosarcoma samples based on the detection of Wnt ligands, LRP5/6 co-receptors or cytoplasmic β-catenin staining. Elevated β-catenin levels have been associated with a metastatic phenotype in osteosarcoma (Kansara and Thomas, 2007). For example, osteosarcoma tumors

overexpressing LRP5, a Wnt co-receptor, are associated with a poorer prognosis and decreased patient survival (Hoang *et al.*, 2004). Another study also suggested that abnormal activation of Wnt/β-catenin signaling is involved in the formation of lung metastasis (Guo *et al.*, 2008). Furthermore, elevated levels of β-catenin are correlated with osteoprogenitor proliferation and the development of osteosarcoma lung metastasis (Iwaya *et al.*, 2003). Also Vijayakumar *et al.* reported that Wnt signaling was active in 50% of human sarcomas and cell lines examined (Vijayakumar *et al.*, 2011). Rubin *et al.* showed that re-expression of Wnt inhibitory factor 1 (WIF-1), a secreted Wnt-antagonist, inhibited Wnt signaling and reduced tumor growth and metastasis in osteosarcoma mouse models (Rubin *et al.*, 2010). Therefore, deregulation of the Wnt signaling pathway may lead to osteosarcoma by hampering terminal osteogenic differentiation and promoting cell proliferation. These results show a possible therapeutic benefit of Wnt-pathway disruption in the treatment of metastatic osteosarcoma. The Wnt-pathway is a putative therapeutic target since it appears that aberrant activation of the pathway leads to the transcription of oncogenes and cell cycle promoters and therefore to cell proliferation and enhanced survival.

▪ **Wnt *inactivation* in osteosarcoma.** Contradictory to other reports, previous data from the LUMC Bone Tumor Group, showed a down-regulation of Wnt/β-catenin in osteosarcoma biopsy samples and osteoblastomas, compared to normal osteoblasts, by evaluating nuclear β-catenin rather than cytoplasmic β-catenin. Nuclear β-catenin was absent in 90% of the biopsies and cell lines tested and the remaining cases showed weak nuclear staining. This report suggested that the loss of Wnt/β-catenin pathway activity contributes to osteosarcoma development and that only nuclear staining can determine the degree of Wnt activity, since it is within the nucleus that transcription occurs for target gene expression, which was furthermore confirmed by a Wnt induced reporter assay and inhibition of proliferation upon reactivation of Wnt/β-catenin signaling (Cai *et al.*, 2010). Also data from genome wide expression analysis of osteosarcomas pointed to Wnt/β-catenin down-regulation in this bone tumor (Cleton-Jansen *et al.*, 2009). Inactivation of Wnt signaling was also previously observed in another study, further suggesting a tumor suppressor role for Wnt/β-catenin signaling (Matushansky *et al.*, 2007). Inactivation of the Wnt antagonist, Wif-1, has been associated with radiation-induced osteosarcoma in mice, further implying deregulation of Wnt signaling in osteosarcomas (Kansara *et al.*, 2009).

Some reconciling explanations for the contradictory findings reported may be the heterogeneous nature of osteosarcoma cells, the diverse methods used to detect Wnt/β-catenin activity (e.g. β-catenin staining, reporter activity) and the differentiation stages of the cells examined, with special emphasis for the fact that most reports do not discuss the implications of tumor heterogeneity in Wnt signaling activation or inactivation.

Several studies already reported results targeting the Wnt pathway in osteosarcoma and other tumors (de Sousa *et al.*, 2011; Gurney *et al.*, 2012; Barham *et al.*, 2013; Le *et al.*, 2014; Takebe *et al.*, 2015). When targeting the Wnt-pathway, activating mutations in downstream molecules, for example β-catenin, can be of negative influence as it may bypass Wnt inhibition and preserve the invasive phenotype of the cells (Kansara and Thomas, 2007). A pre-clinical study by Leow *et al.* has shown that inhibition of the Wnt/β-catenin pathway with curcumin and PKIF118-310 diminished nuclear β-catenin levels, resulting in a decreased intrinsic and activated β-catenin/TCF transcriptional activities and therefore expression of β-catenin target genes. This led to an inhibition of migratory potential through down-regulation of MMP-9, and a decrease in expression of cyclin-D, c-myc and survivin. This resulted in anti-proliferative effect and an increase in cell death and decreased cell migration and invasion (Leow *et al.*, 2010). It also appears that blocking Wnt/LRP-5 signaling can reduce tumor invasiveness by reversing the EMT (Guo *et al.*, 2007).

None of the aforementioned studies addressed the role of the Wnt/β-catenin pathway in CSC subpopulations in osteosarcoma. All of them addressed exclusively cell lines and human tumor samples and considered the bulk tumor cells, which therefore do not allow inferences about the regulatory role of the Wnt/β-catenin signaling in CSCs, as they are postulated to represent a small fraction of the whole cell population. The critical role of Wnt signaling in modulating the delicate balance between self-renewal and differentiation of MSCs (Ling *et al.*, 2009), as well as in regulating the stemness networks in other adult stem cells, points out for a deregulation of this developmental pathway also in osteosarcoma CSCs.

1.3 Drug Resistance in Cancer Stem Cells and Osteosarcoma

Several lines of evidence acquired from human cancer tissues and pre-clinical studies indicate that different mechanisms play a determinant role in cancer resistance to chemotherapeutics. The knowledge of the different signaling pathways (**Figure 1.11**) that determine chemoresistance is vital for the development of novel molecular targets that enhance chemosensitivity and may allow the selection of appropriate therapeutic strategies for human cancers. In this section, we analyze some important mechanisms and signaling pathways that are interrelated with cancer drug resistance, particularly in CSCs, by giving appropriate examples.

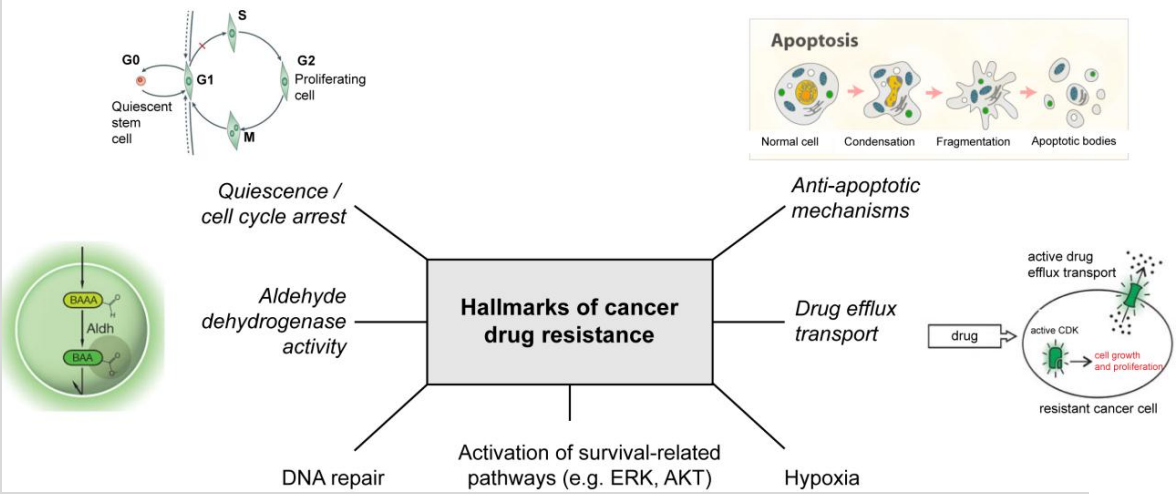


Figure 1.11. Mechanisms contributing to cancer drug resistance. Diagram depicting some of the main mechanisms that may contribute to drug resistance in cancer. Mechanisms and signaling pathways indicated in *italic* are explored in the results presented throughout this thesis. Composed from images adapted from (Cheung and Rando, 2013; Garaycoechea *et al.*, 2012), http://portal.faf.cuni.cz/Groups/Experimental-Pharmacology-and-Drug-Interactions/Research-Projects/Drug-resistance-in-cancer/, http://www.abnova.com/support/resources.asp?switchfunctionid=%7BCBB86AB6-2EA6-422F-BBBD-1CB8B9DCE6FA%7D (assessed September, 5 2016).

1.3.1 Quiescence and cell cycle arrest

Dormancy or quiescence in tumors may be derived from a defective angiogenic process, in which tumor mass expansion is limited due to inability to recruit new and functional blood vessels, and therefore the tumor mass is poorly vascularized. This process is closely associated with cancer cell cycle arrest in which blockade of tumor mass expansion results from quiescence of tumor cells. Microenvironmental stimuli or intracellular hits leading to increased cell proliferation may result in escape from dormancy and expansion of the tumor mass, leading to the emergence of clinically relevant disease (Almog, 2010). Quiescence is molecularly regulated by the signaling pathways that govern cell cycle progression, namely the tumor suppressors p53 and RB proteins, cyclin-dependent protein kinase inhibitors, such as p21, p27, and p57, and also Notch-related pathways and some micro-RNAs (Cheung and Rando, 2013).

- **Quiescence and cell cycle restriction in cancer stem cells and osteosarcoma**

Quiescence is a common characteristic of drug-resistant cells and has been linked with stem cell traits in several tumors. Similar to normal stem cells, CSCs are quiescent, slow-cycling cells and therefore circumvent the production of high levels of intracellular reactive oxygen species (ROS), which accounts for their self-renewal capacity and resistance to chemotherapeutic drugs and also to ionizing radiation (Moore and Lyle, 2011). For instance, quiescent glioblastoma CSCs have been revealed as selectively resistant to temozolomide treatment and being able to regenerate tumor growth after chemotherapy (Chen *et al.*, 2012). Another study has shown that rapidly-proliferating glioma cells keep their slow-cycling counterparts in a reversible state of quiescence, which is related to high chemoresistance (Campos *et al.*, 2014). Dey-Guha *et al.* reported that fast-cycling breast cancer cells could produce slowly proliferating progeny by asymmetric cell division. These "G0-like" cancer cells behaved like quiescent stem cells and were able to stably survive *in vivo* in a "out of cycle" state for a long period of time (Dey-Guha *et al.,* 2011). Leukemia CSCs are among the types of stem-like populations most well-explored in terms of quiescence or dormancy (Essers and Trumpp, 2010). In fact, leukemic CSCs have been shown to exist in a quiescent state in the marrow niche and are resistant to tyrosine kinase inhibitors. This study also demonstrated that inhibiting Bcl-2 using sabutoclax enhanced the sensitivity of those cells to tyrosine kinase inhibition at doses that spare normal progenitors (Goff *et al.*,

2013). The Fbxw7–c-Myc axis has also been proved to be crucial for the maintenance of quiescence and stemness in leukemia CSCs. Moreover, Fbxw7 ablation circumvented CSCs' quiescence and sensitized the cells to imatinib treatment (Takeishi *et al.*, 2013). Also, restriction of cell cycle progression by p21 expression seems crucial for maintaining self-renewal and preventing excessive DNA-damage in leukemia CSCs (Viale *et al.*, 2009). In other tumor types, e.g. medulloblastoma, it was reported that Sox2-positive cells, quiescent and resistant to chemotherapy, were responsible for the propagation of sonic hedgehog medulloblastomas, being also enriched following anti-mitotic chemotherapy and SMO inhibition (Vanner *et al.*, 2014).

Quiescent stem cells among tumors seem to depend on apoptosis-related proteins for their survival. In fact, slow-proliferating lung CSCs retaining the PKH26 dye were shown to be significantly more sensitive to the BH3-mimetic ABT-737 than rapidly proliferating PKH26-low cells, especially due to Bcl-xL inhibition. This study also showed that *in vivo* ABT-737 therapy promoted a cell-cycle arrest of lung CSC-derived tumors implying the long-term benefits of ABT-737, as it preferentially eliminated the quiescent cells in non-small cell lung cancer (Zeuner *et al.*, 2014). Lagadinou *et al.* also reported that Bcl-2 inhibition with ABT-263 severely impaired oxidative phosphorylation and selectively eradicated G0 quiescent leukemia stem cells, which also possessed low ROS levels, indicating that the association between lower ROS and quiescence is crucial for CSCs survival (Lagadinou *et al.*, 2013).

Quiescent cell populations have also been found in osteosarcoma and shown to be involved in chemotherapy resistance. Shimizu and colleagues demonstrated that IGF2 or insulin exposure create an autophagic state of cellular dormancy in highly tumorigenic osteosarcoma cells cultured under serum-free conditions, which then conferred resistance to drugs like doxorubicin, cisplatin and methotrexate (Shimizu *et al.*, 2014). This dormant cell state correlated with enhanced cell survival and autophagy, and the presence of extracellular glutamine, which when inhibited *in vivo*, conferred sensitivity to chemotherapeutics in osteosarcoma xenografts in mice. Recently, also Jiang and co-workers reported that microRNA-329 inhibits osteosarcoma cell proliferation and induced G0/G1 cell cycle arrest as well as inhibition of tumorigenicity *in vivo*, effects that seem to be mediated by the DNA repair protein Rad10 (Jiang *et al.*, 2016).

1.3.2 Activity of aldehyde dehydrogenases (ALDH)

In the context of drug resistance, a key characteristic feature of normal and malignant stem cells is the overexpression of enzymes belonging to the ALDH superfamily, particularly ALDH1A1, ALDH2 and ALDH3A1 (Douville *et al.*, 2008). These enzymes mediate the conversion of xenobiotic and intracellular aldehydes, such as drugs, ethanol and vitamins, into their less harmful corresponding carboxylic acids, thereby acting as important mediators of defense against cytotoxic compounds that can induce DNA damage, inactivation of enzymes and even cell death (Xu *et al.*, 2015b). ALDHs are responsible for the metabolic regulation of retinoic acid and ROS, and this seems to underlie the functional roles of ALDHs in CSCs.

The regulation of retinoic acid metabolism by ALDH has been reported in breast CSCs, with its modulation being sufficient to induce breast CSC differentiation (Ginestier *et al.*, 2007). Also in osteosarcoma, it has recently been shown that all-trans retinoic acid inhibits osteosarcoma growth by promoting osteogenic differentiation, mediated in part by activation of Smad signaling pathway (Yang *et al.*, 2016). Moreover, Mu and colleagues reported that retinal treatment preferentially affected ALDH-high osteosarcoma cells by decreasing their proliferation, invasion capacity, and resistance to oxidative stress. These effects were more pronounced in highly metastatic osteosarcoma cells, accompanied by altered expression of metastasis-related genes and down-regulation of Notch signaling markers (Mu *et al.*, 2015).

The role of ALDH in ROS metabolism also seems to be involved in CSC resistance to cytotoxic drugs. In fact, CSCs with relatively low ROS levels are more resistant to chemotherapeutic drugs in several tumor types (Diehn *et al.*, 2009b; Achuthan *et al.*, 2011; Kobayashi and Suda, 2012; Raha *et al.*, 2014).

- **ALDH in cancer stem cells' chemoresistance and cancer prognosis**

As mentioned in previous sections, ALDHs are actively involved in the chemoresistance of CSCs and their expression generally correlates with a poor prognosis.

A plethora of studies have investigated the role of ALDH expression and activity in diverse types of tumors. In 2012, Croker and Allan explored the response to standard therapy and to inhibitors of ALDH using the breast cancer cell lines MDA-MB-231 and MDA-MB-468. They found that ALDH-positive/CD44-positive cell populations were significantly more resistant to doxorubicin, paclitaxel and ionizing radiation than the

negative counterparts and expressed higher levels of P-glycoprotein, a candidate contributor for the resistance to therapy. Moreover, pre-treatment of those cells with the ALDH inhibitor ATRA sensitized them to chemo- and radiotherapy and reduced their capacity for colony formation (Croker and Allan, 2012). Kim and co-workers showed that pancreatic cancer also has a CSC population enriched for ALDH activity. Cells displaying this phenotype were resistant to gemcitabine but sensitive to disulfiram, an ALDH inhibitor. In *in vivo* experiments, these two drugs showed additive effects, reversing the therapy resistance phenotype. This study had further clinical significance, since surgical specimens receiving preoperative chemo-radiation therapy displayed higher positivity for ALDH compared with therapy-naïve specimens (Kim *et al.*, 2013). Several other authors also explored the involvement of ALDH in resistance to e.g. 5-fluorouracil in gastric cancer (Nishikawa *et al.*, 2013) and to tyrosine kinase inhibitors, such as gefitinib, in lung cancer cells (Huang *et al.*, 2013) revealing that this class of detoxifying enzymes consist in potential treatment targets.

Other studies have conveyed important clinical significance for ALDH expression. Ajani and co-workers found a significant association between extremely resistant esophageal/gastroesophageal junction carcinoma specimens established from patients who had chemo-radiation therapy and surgery, and high ALDH-1 labeling indices, demonstrating that ALDH-1 expression may be predictive of response to therapy. Complementary *in vitro* data also showed that higher ALDH-1 expression correlated with resistant/aggressive phenotype of several esophageal carcinoma cell lines (Ajani *et al.*, 2014).

Expression of different ALDHs has been reported as an important indicator of poor prognosis and patient survival. Several studies performed in patient samples and xenografts, using flow cytometry, immunohistochemistry and whole genome microarray expression revealed that expression of ALDHs is involved in disease progression and also metastatic dissemination. In fact, in breast cancer, perhaps the tumor type in which ALDH activity has been most well studied, studies from Ginestier and colleagues were the first to identify CSC populations based on ALDH activity. This study found a positive correlation between ALDH activity and the cell fraction most tumorigenic and endowed of self-renewal capacity in a series of breast carcinomas, and expression of ALDH1 was associated with a poor prognosis (Ginestier *et al.*, 2007). Three years later, a report again from Wicha group described that cellular invasion *in vitro* and metastasis formation in a xenograft model of inflammatory breast cancer, were mediated by a cell subset displaying high ALDH activity. Moreover, expression of ALDH1 was proposed as

an independent predictive factor for early metastasis formation and poor survival (Charafe-Jauffret *et al.*, 2010). Other subsequent studies have shown that ALDH activity in breast CSCs is mainly due to isoform ALDH1A3, whose expression in patient samples correlates with tumor grade, metastasis and cancer stage (Marcato *et al.*, 2011). Nevertheless, expression of ALDH1A1 isoform seems to be the most significant factor for determining ALDH activation in breast cancer and association with stage and response to chemotherapies (Khoury *et al.*, 2012; Sjöström *et al.*, 2015; Liu *et al.*, 2015).

- **ALDH and osteosarcoma**

ALDH activity has also been investigated in osteosarcoma and interconnected with drug resistance. Honoki and coworkers in 2010 were the first to report that Aldefluor™-positive cells were present in the human sarcoma cell lines, MG-63 and HT1080. On the other hand, sphere-forming MG-63 cells were resistant to doxorubicin and cisplatin and also had increased *ALDH1* expression, which may contribute to that phenotype (Honoki *et al.*, 2010).

Recent investigations indicate that activity of ALDH1 may be modulated by DKK-1, a Wnt antagonist, and ALDH1 can be involved in osteosarcoma cancer cell survival and resistance to chemotherapy (Krause *et al.*, 2014). In this study, transcriptional activation of ALDH1 was dependent on the activation of the non-canonical Jun-mediated Wnt pathway, suggesting that signaling pathways other than those controlling self-renewal (e.g. Wnt/β-catenin signaling) can also participate in the modulation of ALDH activity. Another study associated the resistance of Saos-2 and U2OS osteosarcoma cells to doxorubicin with activation of ALDH1-positive/CD133-positive cells. This resistance phenotype was inhibited by forced expression of miR-143, which suggests that it may play a role in tumor suppression in osteosarcoma by counteracting stemness properties such as ALDH expression (Zhou *et al.*, 2015).

Other authors have also identified chemoresistant chondrosarcoma cells that are sensitive to ALDH inhibitors such as disulfiram (Greco *et al.*, 2014). These authors also found a significant correlation of ALDH activity and existence of distant metastases in ten cases of human bone sarcomas (including osteosarcomas, chondrosarcomas and Ewing sarcomas), suggesting that ALDH has an important role in the metastatic potential of bone tumor cells. Awad and colleagues, in 2010 also identified high expression of ALDH activity in Ewing sarcoma cell lines, and isolated ALDH-positive cells that were also positive for the Oct4 pluripotency marker and resistant to doxorubicin, but sensitive to the ABC transporters' inhibitor verapamil (Awad *et al.*, 2010). These

studies reveal the complexity of ALDH activity in bone sarcomas and reinforce the need for circumventing these detoxification systems in order to promote the sensitivity of bone tumor cells to conventional chemotherapeutics.

1.3.3 Drug efflux transporters

Cancer cells can become resistant to cytotoxic drugs through efflux of the drug from the cell. Resistance to multiple drugs can arise due to the overexpression and function of so-called drug-efflux transporters belonging to the adenosine triphosphate (ATP)–binding cassette (ABC) superfamily. ABC transporters are complex molecular pumps, which mediate the translocation of a wide array of substrates across cellular membranes against a concentration gradient through ATP hydrolysis. Multidrug resistance (MDR) may develop due to the overexpression of ABC transporters and cause relapses to therapy, which together with metastatic dissemination is a major contributor to death by cancer (Moitra *et al.*, 2011).

The ABC family of drug efflux transporters comprises about 48 different proteins, which are primarily located on the plasma membrane and contribute to reduce the intracellular concentration of diverse drugs. Among these transporters, P-glycoprotein (P-gp/*ABCB1* or *MDR1*) and breast cancer resistance protein (BCRP/*ABCG2*) are some of the most well studied and are membrane transporters abundantly expressed on tissues as diverse as the intestine mucosal membrane, kidney proximal tubule epithelia, liver, placenta, and luminal blood–brain barrier. The primary functions of these transporters involve the protection against xenobiotics and cellular toxicants, as they export a variety of drugs from different drug classes, including doxorubicin, cisplatin and methotrexate, among many others (Chen *et al.*, 2016). Although the specificity of P-glycoprotein substrates is different from BCRP, they substantially overlap (Colabufo *et al.*, 2010).

High expression of ABC transporters has been detected in a variety of CSCs in diverse tumor types, preferentially in immature cancer cells, and correlated with resistance to a wide array of chemotherapeutic compounds as reviewed before (Dean, 2009; Higgins, 2007; Chen *et al.*, 2016).

- **ABC transporters and osteosarcoma**

High expression of P-glycoprotein was detected in high-grade osteosarcoma clinical samples and associated with a decreased probability of remaining event-free after diagnosis (Baldini *et al.*, 1995). This study also showed that increased levels of P-glycoprotein were associated with a significantly increased risk of adverse events in

patients treated with surgery and chemotherapy. Previous studies from our group have shown that chemosensitivity of osteosarcoma cells to doxorubicin was strongly dependent on *ABCB1* expression (Gomes *et al.*, 2006). Moreover, multidrug resistance mediated by P-glycoprotein was monitored non-invasively by *in vivo* bioluminescence imaging in orthotopically xenografted osteosarcoma mouse models, derived from osteosarcoma cell lines expressing different levels of P-glycoprotein (MNNG-HOS – high, and 143B – low expression) (Gomes *et al.*, 2007). Others have shown that inhibition or overexpression of the transcription factor Trps1, which regulates chondrocyte proliferation and differentiation, led to *ABCB1* repression or up-regulation in osteosarcoma cells. Moreover, expression of Trps1 directly correlated with *ABCB1*/P-glycoprotein in clinical samples (Jia *et al.*, 2014). Also studies testing other less conventional compounds, such as diallyl trisulfide via NF-κB suppression (Wang *et al.*, 2014c) and icariin by down-regulation of the PI3K/AKT pathway (Wang *et al.*, 2015b), showed decreases in P-glycoprotein expression, thereby reducing the multidrug resistance and inducing apoptosis of osteosarcoma cells.

Our previous recent studies also showed that inhibition of drug efflux transporters with verapamil enhanced the cellular uptake of doxorubicin by osteosarcoma MNNG-HOS spheres that overexpress P-glycoprotein and BCRP (Martins-Neves *et al.*, 2012), being encompassed by an up-regulation the pro-apoptotic protein Bak and suppression of anti-apoptotic Bcl-2, favoring the commitment of osteosarcoma CSCs towards apoptosis (Gonçalves *et al.*, 2015).

A recent ongoing clinical trial in non-metastatic high-grade osteosarcoma patients is re-evaluating the effect of mifamurtide combined with chemotherapy in relation to P-glycoprotein expression and the overall survival will serve as the primary outcome (NCT014559484, ongoing recruitment, 2011-2020).

1.3.4 Other chemoresistance mechanisms

Detailed aspects concerning apoptosis, ERK and AKT pathways, DNA repair mechanisms and hypoxia are not within the scope of this thesis. However, given their important contribution to resistance to chemotherapy, in this section we give a few examples of the role of these signaling pathways in CSCs and osteosarcoma.

- **Apoptosis**

Programmed cell death or apoptosis occurs in diverse cellular phases such as normal development, organogenesis, ageing or inflammatory response, and serves as a natural

barrier to cancer. Apoptotic mechanisms involve a complex signaling cascade and are composed of both upstream regulators and downstream effector components (Plati *et al.*, 2011). Currently, two distinct, although tightly interconnected, signaling pathways control apoptotic cell death. In the intrinsic or mitochondrial pathway, the counterbalance between pro- (e.g. Bcl-2, Bcl-xL, Mcl-1) and anti-apoptotic (e.g. Bax, Bak) members of the Bcl-2 family of proteins determines the trigger to mitochondrial apoptosis and dictates whether a cell will be committed towards cell death or not, depending on their expression levels and binding activities (Verena, 2012; Baig *et al.*, 2016). Another distinct way of controlling apoptosis occurs due to the activation of cell-surface death receptors (e.g. DR3, DR5, TRAIL, TNF receptors), which are responsive to diverse death ligands (e.g. TRAIL, TNF, FAS) expressed by immunocompetent cells (Ashkenazi, 2015; Baig *et al.*, 2016). In case that the net chief signal is pro-apoptotic, then the apoptotic program, either derived from mitochondrial changes or from activated cell-surface death receptors, culminates in the activation of normally latent proteases (effector caspases-3, -6 and -7) that will be responsible for the execution phase of apoptosis, in which the cell is progressively disassembled and its contents, so-called apoptotic bodies, engulfed by neighboring and phagocytic cells (Adams and Cory, 2007; Elmore, 2007).

Despite that most chemotherapeutic drugs exert their biological effects through induction of apoptotic cell death, tumor cells use a variety of strategies to circumvent apoptosis, being therefore a major impediment to effective cancer treatment. Moreover, apoptosis evasion is an even more evident hallmark of CSCs (Fulda and Pervaiz, 2010). For instance, overexpression of Bcl-2 and Bcl-xL was found in leukemia stem cells (Konopleva *et al.*, 2002), and the BH3-mimetic ABT-737 effectively eliminated those cells (Baev *et al.*, 2014). Also in colorectal cancer the induction of the initiator caspase-9 was demonstrated to target preferentially the colorectal CSCs (Kemper *et al.*, 2012).

Osteosarcoma cells are no exception in what concerns evasion from apoptosis. Expression of key anti-apoptotic proteins such as Bcl-2 (Ferrari *et al.*, 2004), Bcl-xL (Wang *et al.*, 2010b) and survivin (Trieb *et al.*, 2003), has been detected in cell lines and patient samples and correlated with enhanced cell survival proliferation *in vitro* and also associated with poor prognosis and metastatic dissemination. Conversely, activation of pro-apoptotic proteins even if mediated by other survival-related molecules (Eliseev *et al.*, 2008; Wang *et al.*, 2010a) promotes apoptosis in osteosarcoma cells.

- **Survival-related pathways – ERK and AKT**

The EGFR-Ras-Raf-MEK-ERK signaling network and the PI3K/PTEN/AKT signaling pathway control cell growth, regulate cell survival, cell differentiation and apoptosis, thus being considered important targets for cancer therapy (McCubrey *et al.*, 2006; Roberts and Der, 2007).

Deregulation and oncogenic activation of survival-related pathways is of utmost importance for CSCs proliferation and persistence among tumors. Therefore, it is not surprising that ERK and AKT pathways are up-regulated in CSCs and involved in tumorigenic features such as apoptosis resistance, *in vivo* tumorigenicity and EMT. Indeed, ERK activity has been observed in breast (Luo *et al.*, 2015) and prostate cancer (Kyjacova *et al.*, 2015a) and embryonal rhabdomyosarcoma stem cell subsets (Ciccarelli *et al.*, 2016). Also AKT activation has been connected with CSC features in prostate (Chang *et al.*, 2013) and skin cancer (Segrelles *et al.*, 2014) and also glioblastoma multiforme (Daniele *et al.*, 2015).

Activation of the ERK pathway was reported in osteosarcoma (Pignochino *et al.*, 2009). Moreover, recent genetic screens also identified AKT as an osteosarcoma-promoter pathway (Moriarity *et al.*, 2015). Some studies indicate these two survival-related signaling pathways have therapeutic potential in osteosarcoma treatment (Kuijjer *et al.*, 2014; Baranski *et al.*, 2015). Molecularly, the ERK pathway also seems to be involved in EMT and metastasis formation (Hou *et al.*, 2014) and activated by the down-regulation of p16 protein (Silva and Aboussekhra, 2016). Nevertheless, the role of these pathways in osteosarcoma CSCs is still not widely explored and deserves further investigation.

- **DNA repair mechanisms**

DNA-damaging agents, such as most conventional chemotherapeutics used in osteosarcoma treatment, elicit diverse types of lesions in the DNA molecules (e.g. single- and double-strand breaks). Cancer cells recognize those lesions and bypass the cytotoxic stress induced by anticancer agents, by activating various DNA repair pathways, such as nucleotide excision repair, base excision repair, homologous recombination repair and nonhomologous end joining (Maugeri-Saccà *et al.*, 2012). Several studies unraveled the molecular basis of these DNA repair pathways and provided rationale for the development of DNA repair inhibitors, which have been demonstrating therapeutic benefits and synergizing with those DNA-damaging drugs (Damia and D'Incalci, 2007; Zhu *et al.*, 2009). The PARP inhibitor olaparib, for treating

BRCA1 or BRCA2 mutated tumors, represents the first drug based on this principle (O'Connor, 2015). Some studies suggest that a prompt activation of DNA damage response and enhanced DNA repair capacity are key contributors to increased CSCs' resistance to therapy, due to their extraordinary ability to repair the genetic code compared with their offspring (Wang, 2015), as exemplified in glioma (Bao *et al.*, 2006), breast (Diehn *et al.*, 2009b), endometrial (Kato *et al.*, 2011), non-small cell lung cancer (Lundholm *et al.*, 2013) and prostate CSCs (Yan and Tang, 2014).

Suppression of Rad51, the key protein of DNA homologous recombination repair, correlated with osteosarcoma HOS cell cycle arrest and limited *in vivo* tumor growth, while also sensitizing cells to ionizing radiation and chemotherapy (Du *et al.*, 2011). Also the nucleotide excision repair variants XPD rs13181 and rs1799793 are related to higher event-free survival in osteosarcoma patients receiving neoadjuvant chemotherapy, and their expression provided a therapeutic advantage from cisplatin chemotherapy, probably by reducing DNA repair activity (Biason *et al.*, 2012). Recent studies suggest that homologous recombination deficiency associates with MG-63 and ZK-58 cells sensitivity to the PARP inhibitor talazoparib alone or in combination with chemotherapeutics (Engert *et al.*, 2016). Despite that more studies are required to a better understanding of DNA repair pathways in osteosarcoma, promising pre-clinical results testing DNA repair inhibitors in this and other tumors seems to hold potential for targeting osteosarcoma bulk cells and CSCs. For instance, two recent related studies reported that a set of primary osteosarcomas possessed mutation signatures reminiscent of BRCA deficiency (Kovac *et al.*, 2015) and osteosarcoma cells with this *BRCAness* phenotype were sensitive to PARP inhibition (Engert *et al.*, 2016).

- **Hypoxia**

Poorly organized networks of vascularization among solid tumors may determine the existence of hypoxic zones. The low oxygen tension present in these areas, despite generating toxic ROS, provides selective pressure for tumor growth and survival advantage for aggressive cells (Philip *et al.*, 2013). Moreover, hypoxia is considered an independent prognostic indicator of poor outcome and risk for metastasis development in osteosarcoma and other tumors (Höckel and Vaupel, 2001; Zhao *et al.*, 2015).

The central mediator of hypoxia, HIF-1, activates the transcription of genes involved in key aspects of cancer biology, representing an important therapeutic target (Semenza, 2003). Also, some studies suggest that moderate hypoxia might be beneficial for CSC's survival (Heddleston *et al.*, 2010), via the modulation of key survival-related

pathways controlling cell proliferation and self-renewal namely Wnt/β-catenin signaling (Santoyo-Ramos *et al.*, 2014), side-population and Oct4 expression (Das *et al.*, 2008), CD133, nestin and Sox2 (McCord *et al.*, 2009) and also Aldefluor™ activity (Soehngen *et al.*, 2014).

Expression of key hypoxia-related markers has been observed in osteosarcoma and related to drug resistance (El Naggar *et al.*, 2012) and e.g. down-regulation of Wnt/β-catenin (Scholten *et al.*, 2014). Osteosarcoma as a solid tumor is highly susceptible to hypoxia activated pro-drugs such as TH-302, which has been shown to cooperate with doxorubicin against osteosarcoma-induced bone destruction and limiting pulmonary metastases formation (Liapis *et al.*, 2015). The leading role of hypoxia in metastatic osteosarcoma dissemination seems to be mediated by the HIF-1α-CXCR4 pathway axis, which plays a crucial role during osteosarcoma cell migration (Guo *et al.*, 2014; Guan *et al.*, 2015). Overexpression of the long non-coding RNA HIF-2α-promoter upstream transcript inhibited osteosarcoma CSCs proliferation and migration, sphere-forming ability of MG-63 cells and decreased CD133-positive cells (Wang *et al.*, 2015a). Further studies are required concerning the role of hypoxia in osteosarcoma CSCs' biological features and may provide novel therapeutic targets for osteosarcoma, as observed for other solid tumors.

1.4 Chemotherapy-Induced Enrichment of Stem-like Cells in Human Cancer

The existence of cancer stem cells (CSCs) within solid tumors is nowadays a well-accepted concept and these cells are well-known for their resistance to conventional chemotherapies and ionizing radiation. Most studies exploring the leading role of CSCs in cancer resistance employ the approach of first, isolating CSCs with some established *in vitro* methodology, or test *in vivo* for their capacity to repopulate a complete tumor, and second, testing whether the isolated cells are more resistant to some specific drug or compound, in comparison to some normal control cells or corresponding more differentiated tumor cells. Conversely, it is conceivable that chemotherapeutics, by exerting the stressing pressure on tumor cells, might select a population of stem-like cells, which are spared from its cytotoxic effects, due to their intrinsic resistance to the chemotherapy; moreover, this selective pressure may also induce stem-like cell properties in regular cancer cells and lead to the outgrowth of previously differentiated and therapy-sensitive cancer cell clones.

Chemotherapeutic drugs, in addition to shrink the tumor mass, may also exert a selective pressure on tumor cells leading to the outgrowth of the fittest surviving clones. Recently, the ability of tumor cells to adapt to specific microenvironmental changes by changing their phenotype, a phenomenon called as phenotypic switching (Kemper *et al.*, 2014), is believed to constitute a defense mechanism that allows tumor cells to evade surveillance of the immune system, survive unfavorable conditions, or escape radio- or chemotherapy. Moreover, several lines of evidence support the concept that treatment of cancer cells with common conventional therapeutics may be a novel source of cancer cell plasticity and stemness within tumors (Pisco and Huang, 2015). Several studies suggest that both ionizing radiation (Ghisolfi *et al.*, 2012) and chemotherapy (Hu *et al.*, 2012; Abubaker *et al.*, 2013) can promote or enhance stem cell-related phenotypes in tumor cells, which were previously differentiated cancer cells (Auffinger *et al.*, 2014), or negative for specifics CSC biomarkers or for the activity of relevant signaling pathways controlling stem cells' renewal and survival (Lagadec *et al.*, 2013).

Some studies have already uncovered some of the mechanisms by which classic chemotherapies can promote phenotypic shifts in non-CSCs populations to a CSC-like state in cancer cell lines and bulk tumors, and correlated with clinical chemoresistance

(Steg *et al.*, 2012a) and poor prognosis (Lee *et al.*, 2011). However, the mechanisms leading to the enrichment of stem-like cells in osteosarcoma are still a matter of examination. In this part of **Chapter 1**, we focus on the revision of several studies that have already described stemness-induction by chemotherapy and other compounds, and also by ionizing radiation.

1.4.1 Stemness-induction or CSC enrichment with doxorubicin-based treatments

Doxorubicin is among the group of widely used conventional drugs in the treatment of sarcomas and other solid tumors. This DNA-intercalating agent has been shown to be a potent apoptotic inducer in cancer treatment but resistance almost invariably occurs. This may be attributable to active drug extrusion by ATP-binding cassette (ABC) transporters, also enhanced detoxification mediated by ALDHs, but the emergence of resistant cell clones may also occur due to the selection of stem-like cells, which are spared from the treatment due to their intrinsic resistant profile. Moreover, there are several studies indicating that doxorubicin is among the drugs with high propensity to induce stemness signatures in previously differentiated cancer cells of various human tumors.

Doxorubicin was shown to select for drug-surviving cells in non-small cell lung cancer cell lines, which expressed several stem-related markers, namely CD133, Oct4, ALDH activity and canonical Wnt activity, accompanied by functional characteristics such as sphere-formation and enhanced clonogenicity and tumorigenicity (Levina *et al.*, 2008). Another interesting study in small cell lung cancer has made use of cell lines established longitudinally from the same tumor sample of a patient receiving a combination of drugs (doxorubicin, cyclophosphamide and etoposide) and also irradiation (Berendsen *et al.*, 1988). In a review report, Hamilton and Olszewski described some results obtained from whole genome sequencing of these cell lines, which allowed the analysis of gene expression changes occurring during tumor relapse and treatment failure. They found that chemoresistant cell lines (GLC16/19) had increased expression of classic stem cell markers, such as CD133, ALDH1A1, and members of the Wnt and Notch pathways, compared to the chemotherapy-naïve GLC14 cell line (Hamilton and Olszewski, 2013).

Doxorubicin is also able to induce stemness characteristics in breast cancer cells, such as expression of *ABCB1* and CD44 markers and functional behavior, e.g.

mammosphere formation and increased invasiveness, accompanied by augmented tumorigenicity (Calcagno *et al.*, 2010). Importantly, also in breast cancer tissue patient samples, the proportion of CD44/ALDH1-positive cells was significantly increased after primary doxorubicin-inclusive systemic therapy and correlated with tumor aggressiveness and progression (Lee *et al.*, 2011). Moreover, others have shown that chemotherapy treatment of human breast cancers significantly increased the percentage of CD44-positive/CD24-negative cells, and increased the mammosphere formation efficiency of cells from biopsy samples taken before and after treatment (Li *et al.*, 2008).

Zheng and colleagues explored the long-term effects of doxorubicin in neuroblastoma cells and found that drug treatment selected for a cell sub-population able to form tumorspheres, with increased clonogenic and invasive abilities, which also expressed augmented levels of the pluripotency marker *SOX2* (Zheng *et al.*, 2013).

Recently, also doxorubicin at non-toxic doses was shown to activate *in vitro* the Notch pathway in osteosarcoma cells (Mei *et al.*, 2015), although further inhibition-approach studies should be performed to confirm the Notch pathway as another relevant candidate underlying the chemotherapy-induced phenotype in osteosarcoma cells. Moreover, also cisplatin increased the expression of Nanog, Bmi-1 and Oct4 ESC markers and seems to modulate the angiogenic-related autocrine signaling in several sarcoma cell lines, leading to the emergence of a tumorigenic side-population fraction within the previously much less tumorigenic HOS cells (Tsuchida *et al.*, 2008).

1.4.2 Stemness-induction or enrichment of CSC with platinum-based drugs

The induction of cancer stemness with platinum-based drugs has also been reported in several different types of solid tumors, in particular cisplatin, which is used in a wide variety of solid malignancies. Cisplatin-mediated DNA damage generally leads to a consistently rate of favorable initial responses, but treatment often results in the development of resistance, due to reduced drug uptake, increased drug inactivation, and increased DNA adduct repair, which dictate the overall extent of cisplatin resistance.

Using a cohort of matched primary/recurrent ovarian tumors, Steg and co-workers have shown that cisplatin-resistant ovarian tumor cells expressed a stemness signature with increase in the ALDH1A1, CD44 and CD133 markers, and also mediators of the Notch, Hedgehog and Wnt pathways, especially at the completion of primary therapy.

Moreover, knockdown of stem cell genes such as CD105, GLI1 and specially GLI2 augmented cancer cells sensitivity to cisplatin *in vitro* (Steg *et al.*, 2012a).

Yang and colleagues uncovered a mechanism by which the enrichment and survival of colorectal CSCs may occur. It was reported that autophagy, which may mediate both cell survival and cell death depending on the conditions, was induced after oxaliplatin treatment and protected the stemness and chemoresistance of colorectal cancer cell lines. Oxaliplatin treatment alone induced the expression of typical stem cells markers such as *OCT4*, *SOX2*, *NANOG* and *LIN28* in CD44-positive cells (Yang *et al.*, 2015a).

The effects of low dose cisplatin treatment in liver cancer cells were studied by Zhang *et al.* using the HepG2 cell line. They found that the percentage of ALDH-positive and CD133-positive cells was greatly increased in cells treated with 1-4 μg/mL cisplatin, but not as much with 5 μg/mL cisplatin, which indicates that low doses of chemotherapeutics may efficiently enrich for CSCs *in vitro* (Zhang *et al.*, 2014).

In head and neck squamous cell carcinoma, cisplatin has also been shown to enhance the stem cell fraction. Studies from Nör and co-workers unveiled that cisplatin treatment increased the fraction of ALDH/CD44-positive CSCs in the UM-SCC-22B cell line. Moreover, cisplatin promoted sphere formation and self-renewal and induced Bmi-1 and Oct4 expression, indicative of stemness induction. These authors also observed that interleukin-6 contributed to the cisplatin-induced sphere formation of cells isolated from primary cancer cells collected after surgery (Nör *et al.*, 2014), which makes evidence for the participation of microenvironmental niche factors in therapy-induced phenotypic alterations in cancer cells.

In lung cancer, cisplatin has also been shown to mediate the acquisition of an EMT-related phenotype and CSC properties via the AKT/β-catenin pathway (Wang *et al.*, 2014a), but also to select for CD133-positive stem-like cells, via activation of the Notch pathway (Liu *et al.*, 2013). Alternatively, cisplatin treatment appears able to regulate DNA damage repair in lung cancer and contribute to the development of a multidrug-resistance phenotype (Oliver *et al.*, 2010). Using another approach, Barr and colleagues demonstrated that cisplatin treatment of lung cancer cells *in vitro* resulted in the enrichment of the CD133-positive cell fraction, both after acute cytotoxic exposure and in cells with a stable cisplatin-resistant phenotype. These resistant cells displayed a stem cell-like gene signature with augmented ALDH activity and expression of pluripotency markers Nanog, Oct4 and Sox2, and also a profile consistent with the EMT, with c-Met and β-catenin being up-regulated as well (Barr *et al.*, 2013). Recently, also Moro and co-workers reported that the combinatorial treatment of non-small cell lung cancer cell

lines with cisplatin and the differentiating agent all-trans retinoic acid prevented the enrichment of CD133-positive CSC, which occurred after cisplatin treatment alone (Moro *et al.*, 2015).

Hu and colleagues showed that Sox2 and Oct3/4 expression increased upon carboplatin treatment, and knockdown of these genes sensitized hepatocellular carcinoma cells to treatment and reduced cells' capacity for sphere formation, a well-known functional readout of stem cell populations (Hu *et al.*, 2012).

In ovarian carcinoma cells, cisplatin promotes a more migratory and mesenchymal phenotype, accompanied by up-regulation of the typical CSC markers *OCT4* and *NANOG* (Latifi *et al.*, 2011). Later on, this group reported that cisplatin, paclitaxel and the combination of both drugs resulted in the enrichment of ovarian cancer cells with increased expression of *NANOG* and *OCT4*, although significant differences were not observed between single and combinatorial treatments (Abubaker *et al.*, 2013).

Sub-lethal doses of cisplatin have also been shown to preferentially select for chemo-resistant stem-like cells in osteosarcoma, through activation of Notch signaling. Cisplatin-resistant cells displayed more mesenchymal and stemness-related characteristics than cisplatin-sensitive cells, such as Stro-1/CD117-positivity. These authors also observed *in vivo* that Notch inhibition prevented tumor recurrence from cisplatin-resistant xenograft tumors (Yu *et al.*, 2016).

1.4.3 Stemness-induction or enrichment of CSC with other chemotherapeutic drugs

Research for new therapeutic compounds has been intense during the past decades. A whole class of new targeted therapeutics has been approved by the **F**ood and **D**rug **A**dministration (FDA - USA) and **E**uropean **M**edicine **A**gency (EMA – Europe) and included in the clinical management of several different malignancies. However, development of resistance has also been observed and, similar to what seems to occur with conventional therapeutics, these new drugs also seem to be capable of inducing stemness properties in cancer cells.

Auffinger *et al.* have shown that patient-derived and established glioma cells exposed to therapeutic doses of temozolamide, the most commonly used chemotherapeutic in gliomas, were able to interconvert between non-CSCs and CSCs, expressing pluripotency and stemness markers such as CD133, Sox2, Oct4, and Nestin. Importantly, when implanted into nude mice, the tumors resulting from the newly

temozolomide-induced CSC were easily implanted and displayed a more invasive phenotype (Auffinger *et al.*, 2014).

Shien and colleagues established gefitinib-resistant EGFR-mutant lung cancer cell lines, which exhibited EMT features and CSC properties, such as self-renewal capability, increase of side-population phenotype and overexpression of ALDH1A1. Moreover, ALDH1A1 was up-regulated in clinical samples with acquired resistance to gefitinib (Shien *et al.*, 2013). Also Kim *et al.* showed that ALDH-positive pancreatic cancer stem-like cells were resistant to gemcitabine and further enriched after treatment with this drug (Kim *et al.*, 2013).

Tamoxifen, a drug highly used for the treatment of estrogen receptor-positive breast cancer, has also been shown to induce stem cell characteristics in cancer cells. By performing whole transcriptome analysis of MDA-MB-231 breast cancer cells exposed to tamoxifen for a short period of time, Notas *et al.* identified a subset of estrogen receptor-dependent genes associated with developmental processes and pluripotency that were induced after treatment. This gene expression signature was also correlated with disease relapse in estrogen receptor-positive breast cancer samples after tamoxifen treatment, as observed for other tumor types. Moreover, in a sub-group of these samples, ALDH1A1 stemness marker was overexpressed after surgery and tamoxifen treatment (Notas *et al.*, 2015). Tamoxifen was actually recently shown to activate ALDH1A1, by specifically binding to the aldehyde site of ALDH1A1, but not of ALDH2 or ALDH3A1 (Belmont-Díaz *et al.*, 2015). These reports reinforce the stemness-related phenotypic signature that may occur after treatment exposure in clinical samples, as observed in *in vitro* models.

Dylla and colleagues demonstrated that treatment of colorectal CSC-derived xenografts with the alkylating agent cyclophosphamide resulted in the enrichment of stem-like cells displaying the ESA/CD44/CD166-positive signature, despite that the number of proliferating cells appeared unaltered. Also, residual tumorigenic cells surviving cyclophosphamide treatment displayed increased levels of ALDH1A1 and c-Myc, well-known stem cell markers (Dylla *et al.*, 2008).

Some investigators have explored the effect of targeted therapeutics and found that survival-related signaling pathways, such as MAPK and ERK, might be involved in the acquisition of invasive and metastatic phenotypes. Recent studies from Lu *et al.* demonstrated that MAPK-ERK inhibitors and copper chelators have the anti-therapeutic effect of inducing breast CSC enrichment. The loss of MAPK-ERK signaling caused transcriptional activation of the pluripotency factor Nanog, which was required for the

induction of the stemness phenotype and enhanced tumorigenesis (Lu *et al.*, 2015). MAPK pathway inhibition of melanoma cells displaying a proliferative phenotype resulted in the acquisition of characteristics consistent with an invasive phenotype, indicating that targeted therapy with MAPK inhibitors may promote phenotype switching, potentially resulting in metastasis (Zipser *et al.*, 2011). Other groups further explored phenotypic switching in melanoma after targeted treatment with BRAF inhibitors and found that inhibitor–resistant melanoma cells acquired an invasive and metastatic phenotype via the reactivation of the MAPK-ERK pathway. This phenotype was also observed in RAS-mutant melanoma cells and accompanied by a cell morphology adjustment from rounded to elongated cells (Sanchez-Laorden *et al.*, 2014).

1.4.4 Stemness-induction or enrichment of CSC with radiation therapy

Ionizing radiation, in parallel with chemotherapeutics, is an important first-line approach in the treatment of solid tumors. As reviewed in (Vlashi and Pajonk, 2015), recent reports indicate that non-stem cancer cells exhibiting a remarkable degree of plasticity, may re-acquire CSC traits, particularly in the context of radiation therapy.

Ghisolfi *et al.* were among the first to report that ionizing radiation can induce stem cell-like properties in heterogeneous cancer cells. These authors found that exposure of non-stem cancer cells to ionizing radiation enhanced sphere formation in hepatocellular carcinoma cell lines, in both the parental unselected cell populations and also on the non-side population fraction isolated from the bulk cell lines. These functional alterations were accompanied by increased expression of the pluripotency-related markers Sox2 and Oct3/4. Importantly, genetic depletion of Sox2 or Oct3/4 resulted in inhibition of the radiation–induced sphere formation, and sensitized the cells to radiation treatment (Ghisolfi *et al.*, 2012)

Studies from Wang and colleagues using mice bearing syngeneic mammary tumors revealed that stemness induced by irradiation correlated with increased spontaneous lung metastasis. However, this phenotype was blocked by disulfiram and copper treatment, which via NF-κB inhibition, inhibited primary tumor growth, associated lung metastasis formation and also the stem cell properties induced by irradiation alone (Wang *et al.*, 2014b). Others have explored the effects of radiation in the Notch signaling pathway also in breast cancer (Lagadec *et al.*, 2013). In this report, it was

found that radiation induced expression of both receptors and ligands such as Notch2, 3 and 4 and also DLL1 and 3, an effect that was especially pronounced in CSC-enriched mammospheres. However, treatment with a γ-secretase inhibitor prevented the radiation-induced phenotype, and compromised the pool of radiation-surviving CSCs.

Recent studies from Bae and co-workers revealed that there is a similar gene expression pattern between ESCs and ionizing radiation-treated colorectal carcinoma and breast cancer cells, indicated by hierarchical clustering extracted from whole genome transcriptome analysis. Specifically, they found that expression of several genes associated with stem cell regulatory and survival-related pathways (e.g. TGF-β, Wnt/Hedgehog/Notch, ERK or MAPK signaling) was up-regulated in irradiated cells. This elegant study adds further evidence that radiotherapy, similar to chemotherapeutics, may also induce stemness signatures in cancer cells (Bae *et al.*, 2015).

Ionizing radiation is among the first-choice treatment for prostate carcinoma. Recent studies from Kyjacova and co-workers showed that also in this solid tumor radioresistance can result in the dissemination of surviving stem cell-like cells. Using metastasis-derived cell lines and a clinically relevant protocol of radiation exposure, these authors showed that a sub-population of surviving cells, non-adherent and anoikis-resistant emerged after treatment and presented active Notch signaling together with expression of typical stem cell markers CD133, Oct4, Sox2 and Nanog. Notably, the survival of these non-adherent cells required the activation of the survival-related ERK signaling pathway, as pharmacological or genetic knockdown of ERK1/2 resulted in anoikis-mediated cell death (Kyjacova *et al.*, 2015).

Therapy-induced non-genetic cell plasticity is a central process in the development of drug resistance. In this introductory part of this thesis, we reviewed some of the publications describing the mechanisms by which chemotherapeutics and ionizing radiation may induce stem cell-like states in cancer cells, as illustrated in **Figure 1.12**.

Cancer cells have the ability to proceed to different phenotypes and switch between those phenotypes without acquiring genomic alterations, after drug exposure which together with cell cytotoxicity also significantly alters the microenvironmental niche. Altogether, the reports mentioned in this sub-section suggest that drug-triggered cancer cell stemness and plasticity constitutes an important contributor for drug resistance, and make evidence that targeting stem cell pathways before or in parallel with conventional treatments might be an effective approach to prevent therapy-induced tumor progression.

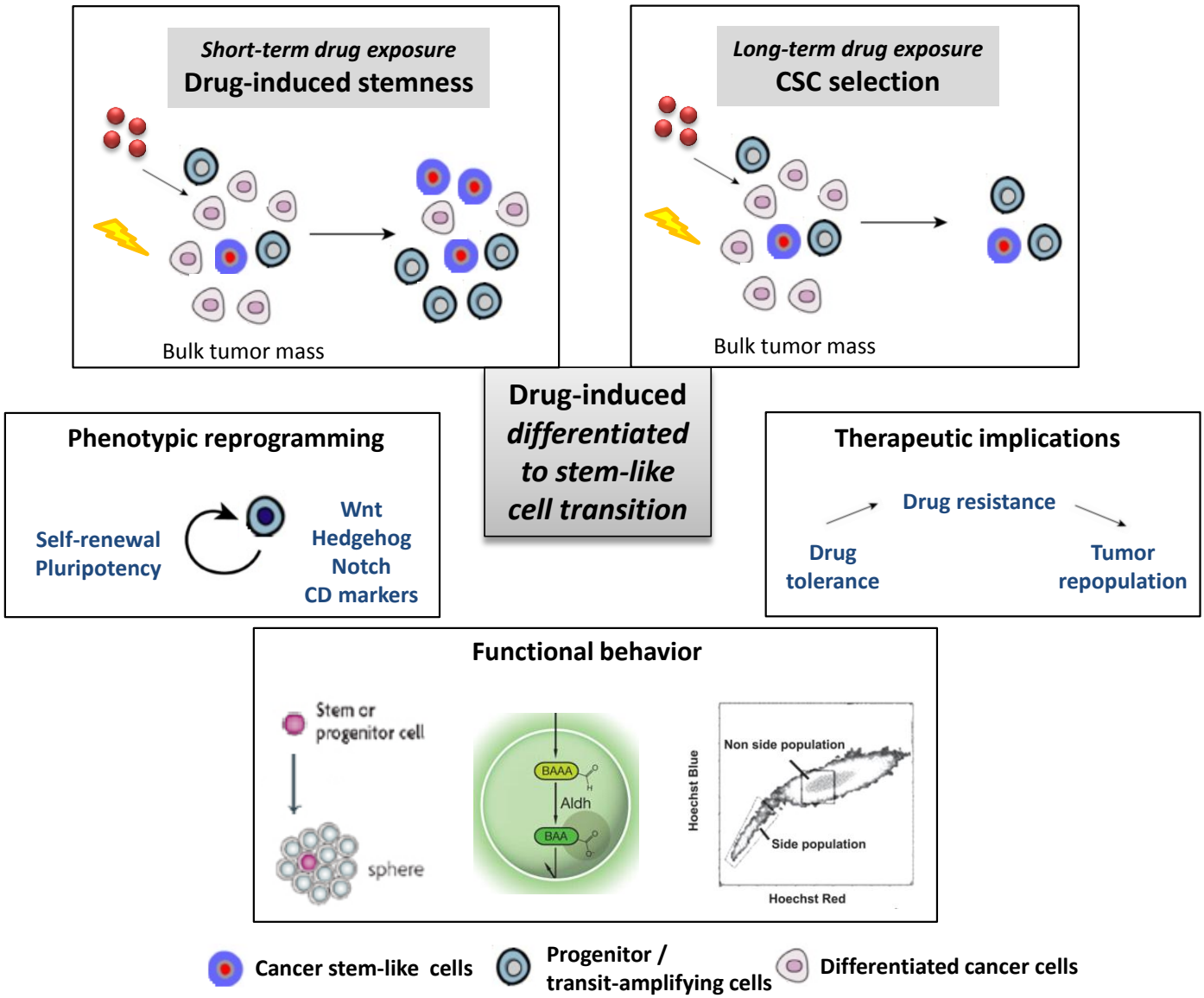


Figure 1.12. Induction of stemness by chemotherapeutic drugs or ionizing radiation in cancer cells. Exposure of cancer cells to conventional chemotherapeutic drugs may induce or select for stem-like cells, depending on the treatment schedule. Phenotypic reprogramming involving activation of pluripotency-related signaling pathways such as Wnt, Hedgehog and Notch, may culminate in the acquisition of a stem-like phenotype with an advantageous functional behavior that confers drug resistance and tumor repopulation ability. Pictures in the lowest panel adapted from (Visvader and Lindeman, 2008; Garaycoechea *et al.*, 2012; Kayama *et al.*, 2007).

1.5 Rationale and Specific Aims of this Thesis

Tumor heterogeneity is a well-recognized characteristic of osteosarcoma and constitutes one of the main causes of treatment failure. The existence of CSCs, already found in most, if not all, tumors types, seems to contribute extensively to the observed phenotypic and functional heterogeneity within the bulk tumor cell population, and associated to treatment resistance, tumor relapses and recurrences and disseminated metastatic disease, being therefore a leading therapeutic target. However, there are some difficulties in identifying a consistent phenotype for osteosarcoma CSCs. Moreover, identifying the mechanisms by which CSCs persist among these tumors and understanding how are they molecularly regulated is particularly important and offers the possibility of effectively targeting CSCs from a therapeutic perspective. Also, the exact mechanisms underlying the enrichment of stem-like cells induced by therapy and whether stemness-related markers are correlated with poor outcome in osteosarcoma are still open questions.

Specific aims

Based on these findings, the main goal of this thesis is to explore the role of CSCs in osteosarcoma by:

1. providing an in-depth functional and molecular characterization of CSCs in a panel of genetically profiled osteosarcoma cell lines derived from two distinct histological subtypes of high-grade osteosarcoma;
2. questioning the central role of the regulatory Wnt/β-catenin pathway in self-renewal, stemness maintenance and chemoresistance of osteosarcoma CSCs;
3. investigating the mechanisms underlying the chemotherapy-induced phenotypic cell state transition towards a stem-like phenotype, as a mechanism of adaptive resistance.

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2 Chapter

Osteosarcoma Stem Cells have Active Wnt/β-catenin and Overexpress SOX2 and KLF4

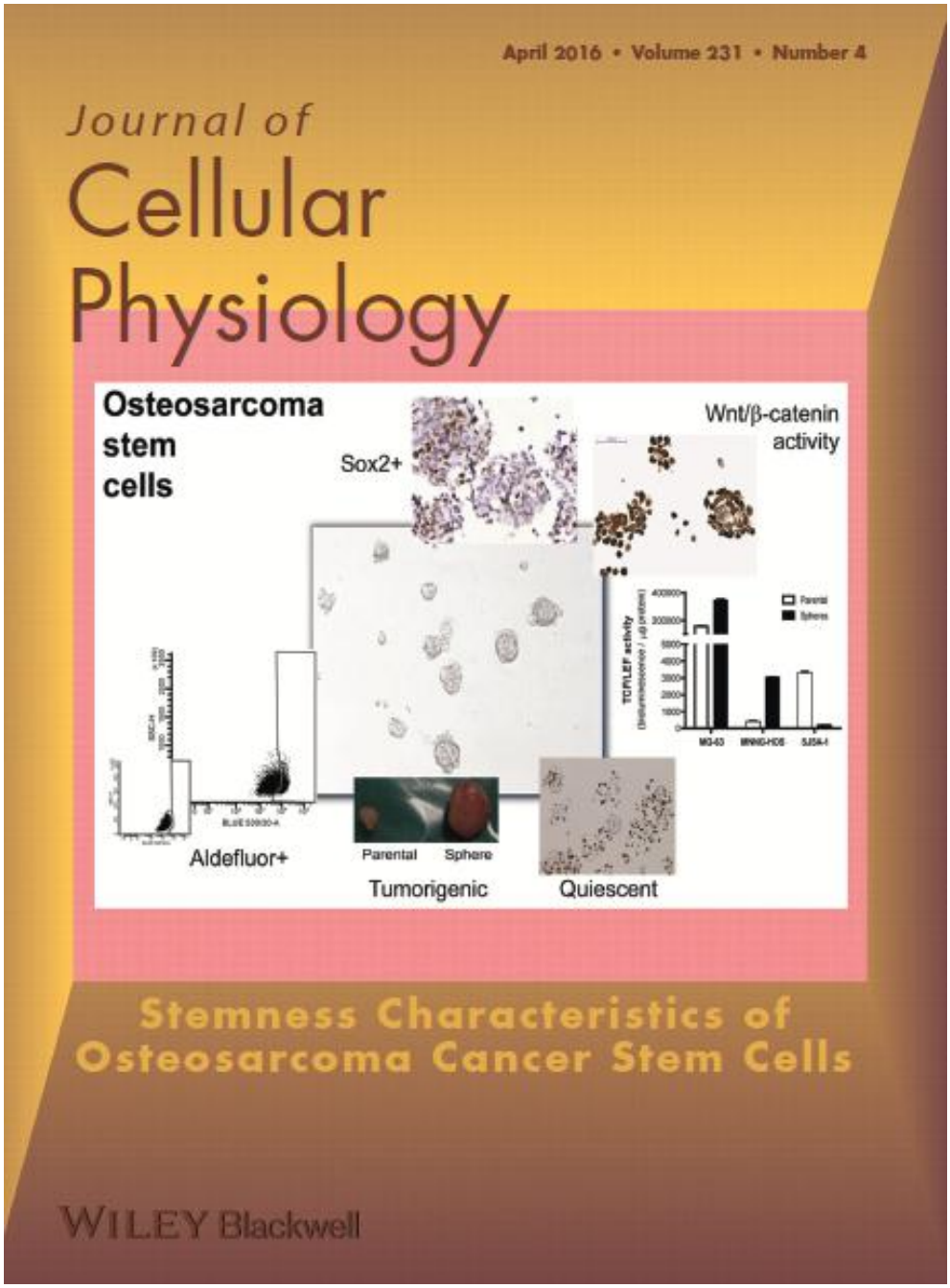
Sara R. Martins-Neves, Willem E. Corver, Daniela I. Paiva-Oliveira, Brendy E. W. M. van den Akker, Inge H. Briaire-de-Bruijn, Judith V. M. G. Bovée, Célia M. F. Gomes*, Anne-Marie Cleton-Jansen*

(* equal contribution)

Journal of Cellular Physiology, 231(4):876-886. DOI: 10.1002/jcp.25179

"Cancer affects all of us, whether you're a daughter, mother, sister, friend, coworker, doctor, or patient." –Jennifer Aniston

This chapter is published in its integral form in the volume 231, issue 4 of the *Journal of Cellular Physiology*. It was selected by the Editor, Dr. Gary Stein, to be featured on the cover of the journal, and the cover page is herein reproduced.



Osteosarcoma stem cells isolated using the sphere forming-assay are slow proliferating cells with a few number of Ki-67^{+} cells, display enhanced ALDH activity and tumorigenic potential and overexpress the pluripotency transcription factor Sox2. Importantly, biological evidence shows that the Wnt/β-catenin signaling is specifically activated in these cells, as indicated by nuclear β-catenin staining and increased TCF/LEF transcriptional activity.

2.1 ABSTRACT

Osteosarcoma is a bone tumor displaying significant cellular and histological heterogeneity, and a complex genetic phenotype. Although multiple studies strongly suggest the presence of cancer stem cells in osteosarcoma, a consensus on their characterization is still missing. We used a combination of functional assays (sphere-forming, Aldefluor and side-population) for identification of cancer stem cell populations in osteosarcoma cell lines. Expression of stemness-related transcription factors, quiescent nature, *in vivo* tumorigenicity and Wnt/β-catenin activation were evaluated. We show that different cancer stem cell populations may co-exist in osteosarcoma cell lines exhibiting distinct functional properties. Osteosarcoma spheres are slowly-proliferating populations, overexpress Sox2 and Klf4 stemness-related genes and have enhanced tumorigenic potential. Additionally, spheres show specific activation of Wnt/β-catenin signaling as evidenced by increased nuclear β-catenin, TCF/LEF activity and *AXIN2* expression, in a subset of the cell lines. Aldefluor-positive populations were detected in all osteosarcoma cell lines and overexpress *SOX2*, but not *KLF4*. The side-population phenotype is correlated with *ABCG2* drug-efflux transporter expression. Distinct functional methods seem to identify cancer stem cells with dissimilar characteristics. Intrinsic heterogeneity may exist within osteosarcoma cancer stem cells and can have implications on the design of targeted therapies aiming to eradicate these cells within tumors.

Keywords: osteosarcoma, cancer stem cells, Wnt/β-catenin, Sox2, Klf4, aldehyde dehydrogenase

2.2 INTRODUCTION

Osteosarcoma is a bone tumor, affecting children and adolescents. This specific incidence is partially explained by the rapid cell turnover occurring in the growth plates of long bones, where during skeletal growth, stem- and progenitor cells are actively dividing, generating bone matrix-producing cells (Tang *et al.*, 2008). Osteosarcomas produce osteoid, but are otherwise highly heterogeneous histologically. For instance, some osteosarcomas can be purely osteoblastic, while others demonstrate cartilaginous differentiation. Given its diverse histological classification, it is therefore hypothesized that osteosarcoma originates from a multipotent cell (Klein and Siegal, 2006; Mohseny *et al.*, 2009). Although surgery and neoadjuvant chemotherapy have remarkably improved the outcomes, the main clinical problem of this tumor is its unpredictable recurrence or metastasis, which is likely the result of a few resistant tumor cells.

The cancer stem cell (CSC) theory postulates that a small subset of tumor cells is responsible for tumor initiation and progression (Clarke *et al.,* 2006). These cells, also referred as tumor-initiating cells, can self-renew and generate all the cells comprising the tumors. Evidence suggests that CSCs are resistant to chemotherapy and may be the culprit cells causing relapses and metastatic dissemination, despite a good histological response to systemic therapy of the primary tumor (Oskarsson *et al.*, 2014).

The increasing complexity of CSCs was recently addressed, and it is therefore important to assign distinct biomarkers for identifying CSCs in solid tumors (Pattabiraman and Weinberg, 2014). Most studies attempted to identify CSCs based on the expression of specific surface antigens, but tumor heterogeneity renders it difficult to discover a universally accepted marker that can be used exclusively to identify CSCs. Currently, there are several surface markers (e.g. CD44, CD24, CD133) that were proven useful for identifying CSCs in different tumors, but discrepancies still exist (Al-Hajj *et al.*, 2003; Singh *et al.*, 2003; Schepers *et al.*, 2012). For example, CD133, a widely used CSC marker, has been questioned concerning its validity as a unique biomarker of tumor-initiating cells, and studies have demonstrated that CD133-negative cells are equally tumorigenic (Shmelkov *et al.*, 2008). Regarding osteosarcoma CSCs, a consensus is far from being achieved with several groups identifying unrelated biomarkers, which warrant a broader validation.

The difficulties in identifying a consistent and representative phenotype for osteosarcoma CSCs can reflect the lack of specific markers for osteosarcoma in general and the absence of exclusively expressed antigens on mesenchymal stem cells (MSCs),

possible osteosarcoma cells-of-origin (Mohseny *et al.*, 2009; Lv *et al.*, 2014; Mutsaers and Walkley, 2014,). These facts provide a rationale for the use of functional methodologies, rather than molecular markers that may not be specific on their own.

Tumor heterogeneity is recognized as an intrinsic feature of solid tumors (Pattabiraman and Weinberg, 2014). Such cellular heterogeneity provided one of the fundamental criteria for the original definition of a "cancer stem cell" – a cell capable of initiating the heterogeneous lineages found within the tumor mass (Clarke *et al.*, 2006). Theoretical observations suggest that cellular heterogeneity is not confined to the tumor as a whole, but exists within the stem-cell compartment itself, which is in contrast with the classical view of CSCs as being a functionally homogeneous population (Valent *et al.*, 2013). In line with this, some studies have investigated the role of distinct stem-cell populations in maintaining distinct phenotypic functions, namely tumor initiation, self-renewal and metastatic colonization, which can vary according to the tumor stage and histological subtype (Park *et al.*, 2010). However, information pertaining osteosarcoma CSC's heterogeneity remains scarce and deserves further investigation.

Here, we characterized osteosarcoma CSCs using different functional approaches for their identification and isolation. We show that osteosarcoma CSCs (as identified using the sphere assay) are enriched for a stem-like cell population, which concomitantly expresses Sox2 and Klf4 pluripotency-related transcription factors, and importantly has activation of the self-renewal related Wnt/β-catenin signaling. Moreover, we provide evidence that CSC populations have enhanced tumorigenic potential and display molecular and functional heterogeneity.

2.3 Materials and Methods

2.3.1 Ethics statement

All samples were handled according to the ethical guidelines as described in the Code for Proper Secondary Use of Human Tissue in The Netherlands of the Dutch Federation of Medical Scientific Societies. The animal studies were conducted according to the European Communities Council Directives and approved by the Institutional Ethics Committee of the Faculty of Medicine of University of Coimbra for animal care and use (Approval ID:38-CE-2011). Appropriate guidelines concerning animal welfare and care were incorporated in all animal experiments (Workman *et al.*, 2010).

2.3.2 Cell culture and spheres' isolation

Cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD) (HOS, MG-63, MNNG-HOS, SJSA-1 and U2OS), provided by the Leiden University Medical Center [L2531 (Lenos *et al.*, 2012), L3312 (Pahl *et al.*, 2012)] or from the EuroBoNeT cell line panel (MHM, OHS). HOS, MG-63, MHM, MNNG-HOS and SJSA-1 cells were derived from high-grade fibroblastic osteosarcoma, while L2531, L3312, OHS and U2OS were derived from high-grade osteoblastic osteosarcoma. All cell lines were cultured in RPMI-1640 medium (Invitrogen, Karlsruhe, Germany) supplemented with 10% v/v heat-inactivated fetal bovine serum (FBS; Invitrogen), 1% v/v penicillin/streptomycin (P/S; Invitrogen) and 1% v/v GlutaMAX™ (Invitrogen), under standard adherent conditions at 37°C in a humidified incubator with 5% CO_2/95% air. For all experiments, non-selected monolayer adherent cells, referred as parental cells, were harvested at 70-80% confluence. Mycoplasm contamination was screened twice a month as described (van Kuppeveld *et al.*, 1992). Cell line authentication was performed by short tandem repeat (STR) profiling using Cell ID™ System (Promega, Madison, WI, USA) and results compared to ATCC profiles or EuroBoNeT collection (Ottaviano *et al.*, 2010) (**Table 2.1**).

Table 2.1. DNA fingerprint analysis of polymorphic STR sequences performed on osteosarcoma cell lines used in this study.

Cell line	AMY	VWA	TH01	D21S11	D13S317	CSF1PO	D7S820	TPOX	D16S539	D5S818
HOS	XX	18-18	6-6	31.2-32.2	12-12	12-12	11-12	8-11	10-13	13-13
MHM	XX	18-18	6-9.3	30.2-31.2	12-12	11-12	11-12	9-11	10-13	12-13
MG-63	XY	14-18	6-9.3	31-31	13-13	12-13	11-12	11-11	11-12	8-11
MNNG	XX	18-18	9-9	31.2-31.2	12-12	12-12	11-12	8-11	10-13	13-13
SJSA-1	XY	19-19	6-6	29-30.2	12-12	11-11	10-10	8-9	9-10	11-11
OHS	XX	16-16	9-9	30-30	8-14	10-12	11-13	8-11	12-12	10-10
U2OS	XX	18-18	6-6	31.2-31.2	12-13	11-12	11-12	11-11	10-13	13-14

***Note**:* Results of STR profiling were compared to ATCC databases for each cell line or to previously published datasets with characterization of the osteosarcoma cell lines used [EuroBoNeT panel (Ottaviano *et al.*, 2010)].

Spheres, which are supposedly enriched for CSCs, were isolated from parental cell lines using the sphere-forming assay and sphere-formation media (Martins-Neves *et al.*, 2012). Single-cell suspensions of osteosarcoma cell lines were plated at a density of 2.5×10^4 cells/mL in sphere-formation media (SFM) using poly-HEMA-coated (Sigma-Aldrich®, St. Louis, MO, USA) 6-well plates (Greiner Bio-One). Fresh aliquots of human

epidermal growth factor (Peprotech EC, London, UK, 10 ng/ml) and recombinant human fibroblast growth factor-basic (Peprotech, 10 ng/ml) were added at least twice a week. After 7-10 days, spheres were collected by diluting SFM containing methylcellulose with hanks-balanced salted-solution, centrifuged and dissociated using undiluted accutase (Sigma) for 10min at 37°C. Spheres were immediately used for further experiments or transferred to adherent conditions. Sphere-forming efficiency was determined by dividing the total number of spheres formed by the total number of cells plated. Cells were periodically observed and photographed using an inverted microscope (Zeiss Axiovert 40C, Carl Zeiss Light Microscopy, Göttingen, Germany).

2.3.3 Single-cell clonogenic assay

Enzymatically-dissociated parental cells and corresponding spheres were plated in 96-well plates at a density of 1 cell/well and were maintained in regular culture conditions for 2 weeks. The number of colonies formed per plate was counted using an inverted microscope and expressed as a percentage.

2.3.4 TCF/LEF luciferase reporter assay

MNNG-HOS, MG-63 and SJSA-1 cells were transfected with pGL4.49 [luc2P/TCF-LEF RE/Hygro] Vector (Promega). Cells were plated in 6-well plates (30,000 cell/mL) and allowed to attach overnight. DNA complexes were prepared using the nonliposomal transfection reagent FuGENE® HD (Promega), at a ratio of 3μL reagent to 1.5μg DNA, in 100μL of media. After 15min stabilization, reagent:DNA mixture was gently added to cells cultured in fresh media. Plasmid was in contact with cells for 72h, after which fresh media containing hygromycin (250 μg/mL, Invitrogen) was added in order to select for stably transfected cells. Luciferase activity was measured using the IVIS® Lumina XR (Caliper Life Sciences Inc., PerkinElmer, Massachusetts, USA) in 96-well black plates after incubation with D-luciferin (30mg/mL, Caliper).

2.3.5 Tumorigenic potential

Tumorigenic potential of parental cells and spheres from MHM, MNNG-HOS and SJSA-1 cell lines was assayed in immunocompromised athymic Swiss nude mice (6-8 weeks, n=3 for each cell line) purchased from Charles River Laboratories. Mice were injected subcutaneously in opposite flanks with $1x10^5$ parental cells and corresponding sphere-forming cells re-suspended in hanks-balanced salted-solution. Tumor growth was monitored twice a week for up to 50 days using a caliper for tumor measurements

and tumor volume was calculated using the formula (LxW^2)x0.5. Animals were sacrificed by cervical dislocation when the tumors reached a maximum of 500mm3 and were harvested.

2.3.6 Immunohistochemistry

Formalin-fixed paraffin-embedded sections from monolayer cultures and spheres were subjected to antigen retrieval and stained overnight with antibodies (Ki-67, Sox2 and β-catenin, further details see **Table 2.2**), as previously described (Baranski *et al.*, 2015). Sections were then incubated with Immunologic PowerVision+ Poly-HRP-GAM/R/R IgG (Leica Biosystems, B.V, Rijswijk, Netherlands) for 30min and Dako liquid DAB+ Substrate Chromogen System (K3468) for 10min. Sections were counterstained with hematoxylin for 20sec at room temperature. Pictures were acquired with an automatic digital slide scanner (Panoramic MIDI, 3DHISTECH, Budapest, Hungary) and Panoramic Viewer used for graphics.

Table 2.2. Antibodies used in immunohistochemistry and respective staining conditions.

Antibody	Antigen retrieval	Dilution	Company
Ki-67 (clone MIB-1)	Citrate	1:800	Dako, Glostrup, Germany
Sox2 (clone D6D9)	Tris-EDTA	1:1,600	Cell Signaling Technology, MA, USA (#3579)
β-catenin	Citrate	1:2,000	BD Transduction Laboratories™, cat. 610153

2.3.7 Western blotting

Total protein extracts from parental cells and spheres were prepared with a standard cell lysis buffer, separated, electrotransferred and probed with antibodies, as previously described (Gonçalves *et al.*, 2015).

Blocked membranes were incubated overnight with primary antibodies, at 4°C. Antibodies used were Sox2 and Klf4 (Cell Signaling Technology MA, USA) at a dilution of 1:1,000 and 1:500, and β-catenin (BD Transduction Laboratories™) at a dilution of 1:500. Appropriate peroxidase–conjugated secondary antibodies were incubated at room temperature for 60min and proteins visualized by chemifluorescence (ECF™ Western Blotting Reagent Pack, GE Healthcare Life Sciences, Pittsburg, PA) using Typhoon™ FLA 9000 biomolecular imaging system (GE Healthcare). Quantification of protein bands was assessed by densitometry calculation using ImageJ software (National Institutes of Health, Bethesda, MD, USA) and normalized to β-actin (dilution

1:5,000; Sigma) used as protein loading control for total lysates, or normalized to lamin B1 (dilution 1:1,000; abcam®).

2.3.8 Analysis of cellular viability and proliferation

Spheres and parental cells were plated in 96-well plates (3×10^4 cells/mL) and maintained on their respective regular culture conditions. Cell proliferation was measured at different time-points for 0-72h using the WST-1 cell proliferation assay (Roche) and a light spectrometer operating in colorimetric mode (Victor^{3}V, 1420 multi-label microplate reader, Perkin Elmer, NK).

2.3.9 Analysis of mRNA expression

Total RNA was extracted from monolayer-growing cells or spheres, using TRIzol® (Invitrogen), according to the manufacturer's protocol and concentration was measured at an optical density of 260nm using NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies). Only samples with 260/280 ratios higher than 1.7 were used for subsequent applications.

First-strand complementary deoxyribonucleic acid (cDNA) was synthesized from 1µg of purified RNA using a mixture of AMV Reverse Transcriptase (AMV-RT) buffer (Roche Diagnostics, Mannhein, Germany), random primers (Invitrogen), dNTP Mix (Promega, Nederland), oligodT primers (Roche Diagnostics) and recombinant RNasin® (Promega). Twenty microliter per sample were incubated at 42°C for 60min (cDNA strand synthesis), and 65°C for 10min (AMV-RT inactivation), using the GeneAmp® PCR System 9700 thermal cycler (Applied Biosystems). cDNA samples were diluted 25x and tested in duplicates in real time quantitative reverse transcriptase – polymerase chain reaction (qRT-PCR) experiments. RNA from human embryonic stem cells (ESC) was a kind gift from Dr. Christine Mummery (Department of Anatomy and Embryology of Leiden University Medical Centre).

Primers for qRT-PCR reaction were designed to generate a PCR product of less than 200 bp, using the Primer-Blast tool (NCBI), and are listed in **Table 2.3**. A BLAST search was performed to exclude alternative primer annealing sites; all primers were firstly tested in appropriate control tissues and sequenced (Sanger Sequencing) to ensure detection specificity (Leiden Genome Technology Center, Leiden, The Netherlands). Primer sequences already validated and published are *AXIN2*, *DKK-1* (Cai *et al.*, 2010) and *ABCG2* (Gomes *et al.*, 2006). Levels of gene expression were tested by qRT-PCR and

normalized to the housekeeping genes *CAPNS1*, *SRPR* and *TBP*, which are stably expressed in osteosarcoma (Gomes *et al.*, 2006). Housekeeper genes were tested in a cDNA reference sample panel containing RNA from various normal and tumor human tissues. Quantitative RT-PCR was done in duplicates using iQ™ SYBR Green Supermix (Bio-Rad Laboratories, Inc.) and each sample contained 2.5 μL of 25x diluted cDNA. PCR thermal cycle conditions were as follows: an initial denaturation step at 95°C for 5min, followed by 39 cycles of 10s at 95°C, 10s at 60°C, and 10s at 72°C, and a final elongation cycle at 72°C for 10min; samples were run in CFX96™ Real-Time PCR Detection System (Bio-Rad Laboratories, Inc.) using a final volume of 12.5 μL per sample. Data analysis and normalization of gene expression were performed in Bio-Rad CFX Manager™ 3.0 software.

Table 2.3. Sequences of primers used in this study.

Gene	Forward (5'-3')	Reverse (5'-3')
Pluripotency-related genes		
KLF4	CCACACTTGTGATTACGCGG	TACGGTAGTGCCTGGTCAGT
NANOG	GATGCCTCACACGGAGACTG	GCAGAAGTGGGTTGTTTGCC
POU5F1 (OCT4)	TCTGCATCCCTTGGATGTGC	GTGTGGCCCCAAGGAATAGT
SOX2 (Park *et al.*, 2012)	CATGCACCGCTACGACG	CGGACTTGACCACCGAAC
Housekeeping genes		
CAPNS1	ATGGTTTTGGCATTGACACATG	GCTTGCCTGTGGTGTCGC
SRPR	CATTGCTTTTGCACGTAACCAA	ATTGTCTTGCATGCGGCC
TBP	CACGAACCACGGCACTGATT	TTTTCTTGCTGCCAGTCTGGAC

***Note**:* RNA from human embryonic stem cells was used as a positive control for pluripotency-related genes. *CAPNS1*: calpain, small subunit 1; *SRPR*: signal recognition particle receptor; *TBP*: TATA Box-binding protein.

2.3.10 Side-population analysis

Side-population experiments were conducted as previously described (Wu *et al.*, 2007).

Cells in logarithmic growth phase were harvested using trypsin/EDTA and resuspended in pre-warmed appropriate culture media (according to each cell type), at a concentration of 5×10^5 viable cells/mL. Single-cell suspensions were incubated with Hoechst-33342 dye (2.5μg/mL, Sigma) at 37°C for 90min in a water bath and in the dark, with occasional agitation to prevent cell aggregation. Negative control samples were treated with verapamil (50μM, VER, Sigma), a wide spectrum ATP-binding cassette (ABC) transporter inhibitor for 15min, before being incubated with Hoechst-33342.

Afterwards, cells were washed with ice-cold PBS, centrifuged at 4°C and resuspended in ice-cold PBS with propidium iodide (1μM), to identify and exclude dead cells. All samples were maintained at 4°C until flow-cytometry acquisition. Hoechst-33342 dye was excited at 355nm using a UV laser, and its dual wavelengths were detected using 450/50nm band-pass and 450LP filters (Hoechst-33342 Blue), and 655LP filter (Hoechst-33342 Red), for the discrimination of side-population cells. At least 10,000 events were acquired in the side-population region. Dead cells were excluded by gating propidium iodide-positive cells on forward vs. side scatter dotplots. Cell cycle profiles (Counts vs. Hoechst-33342 Red dotplot) were used to gate the G1 cell population at 150V voltage channel, in order to accurately identify side-population cells by matching test samples and negative controls (VER) in the same region of the dotplot. Data were acquired using BD™ LSR Ⅱ flow cytometer and BD FACSDiva™ software (Becton Dickinson Biosciences), and analyzed using WinList™ 3D 7.1 software (Verity Software House, Topsham, ME).

2.3.11 Aldefluor™ assay and cell sorting

Osteosarcoma parental cells and spheres were tested for activity of aldehyde dehydrogenase using the Aldefluor™ assay (STEMCELL Technologies, Durham, NC, USA), according to the manufacturer's instructions.

Harvested cells were suspended in 0.5mL of Aldefluor™ assay buffer, containing pre-activated fluorescent ALDH substrate (BODIPY™-aminoacetaldehyde, BAAA, 1.5μM/1×10^6 cells), and incubated for 40min at 37°C, in the dark. A negative control sample for each cell type was concurrently labeled with the ALDH-specific inhibitor diethylaminobenzaldehyde (DEAB 7.5μM/5×10^5 cells). Green fluorescence derived from the Aldefluor™ converted substrate BAAA was collected on the blue 530/30 band pass filter and at least 10,000 events were acquired. Non-viable cells were excluded based on propidium iodide positivity, added at a final concentration of 1μM (Sigma). All data were acquired and analyzed using the same instrumentation and software as for the side-population assay.

Aldefluor™-labelled cells were flow-sorted using a FACSAria I flow-sorter (BD Biosciences, Erembodegem, Belgium) with a nozzle of 40μm. ALDH-positive and ALDH-negative cells were directly sorted into 15mL Falcon tubes containing RNA*later*® solution (Ambion, Life Technologies, Bleiswijk, The Netherlands). After sorting, cells were centrifuged at 250*g* for 10min, before RNA was extracted using the ReliaPrep™ RNA

Cell Miniprep System (Promega Benelux BV, Leiden, The Netherlands), according to the manufacturer instructions. cDNA was prepared afterwards using the protocol described previously.

2.3.12 Statistical analysis

Statistical analyses were performed using SPSS Statistics version 20.0 (IBM Corporation, New York, USA). Significance was set at the level of $P<0.05$. Graphic illustrations were computed using GraphPad Prism version 5.04 (GraphPad Software, San Diego, CA).

2.4 RESULTS

2.4.1 Cell lines derived from fibroblastic osteosarcoma readily form spheres, whereas those derived from osteoblastic osteosarcoma do not

The sphere-forming assay has been used in the CSC field as a method to enrich for cells with a stem-like behavior, particularly when specific CSC biomarkers for a tumor are not defined, such as osteosarcoma (Mather, 2012). Therefore, we investigated the presence of CSCs in a panel of osteosarcoma cell lines using the sphere-forming assay.

Adherent parental cell lines (**Figure 2.1A,B**) were dissociated and plated at low-density in non-adherent serum-free conditions. Spheres formed after about 7-10 days of culture, depending on cell line specific growth rate. A clear distinction could be observed in the capacity of different cell lines to form spheres. Most cell lines derived from conventional high-grade osteosarcoma of fibroblastic histological subtype (from now onwards referred to as fibroblastic-like cell lines) formed tightly compact suspended-growing spherical colonies (**Figure 2.1A**), with a sphere-forming efficiency of 7.3±0.1% - HOS, 13.5±2.5% - MG-63, 8.9±0.7% - MHM, 11.1±0.1% - MNNG-HOS and 9.2±0.5% - SJSA-1. Although there were differences in morphology and size (cells with the highest sphere-forming efficiency formed larger spheres), all fibroblastic-like cells demonstrated the capacity to survive and proliferate in suspension for a long culture period. Unexpectedly, osteoblastic-like cell lines were not able to form spheres, growing instead in sparsely grouped cellular aggregates (**Figure 2.1B**) without the uniform and circular appearance of spheres. The cells contained in these clumps could not survive when transferred to serum-supplemented media in adherent conditions (not shown),

which shows they could not withstand the harsh conditions of the sphere-forming assay.

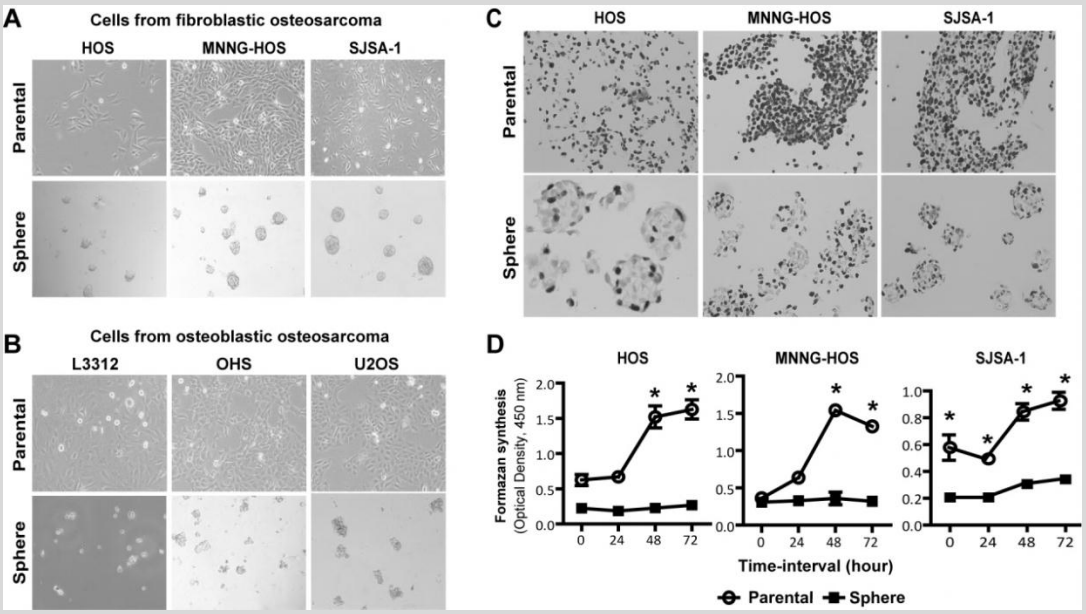


Figure 2.1. Fibroblastic osteosarcoma cell lines contain slow-proliferating sphere-forming cells. A. Morphological appearance of several cell lines derived from fibroblastic high-grade osteosarcoma, growing in adherent conditions (parental) and spheres growing in suspension. Magnification: 100×. **B.** Cells from osteoblastic high-grade osteosarcoma mainly form cellular clumps in the sphere assay. **C.** Parental cells are strongly positive for Ki-67 proliferation marker, whereas spheres show a more quiescent profile by having more Ki-67 negative cells. Magnification: 200× (except HOS spheres: 400×). **D.** Cell proliferation measured at different time-points. Points represent mean ± SEM of three independent experiments. *P<0.05, compared to spheres, independent samples t-test.

2.4.2 The majority of cells within spheres are is in a slow-proliferative state, but possesses enhanced single-cell clonogenic efficiency compared to parental cells

A slow-proliferative rate is a key feature of normal adult stem cells that has also been attributed to CSCs and may be partly responsible for resistance to conventional therapies (Moore and Lyle, 2011). We therefore reasoned that spheres contained cells in such a slow-proliferative state. Ki-67 staining revealed a different proliferation rate between adherent cells and spheres. Parental cells had a positive nuclear staining reaction, with the majority of cells being moderately to strongly stained (**Figure 2.1C**). In contrast, we consistently observed substantial heterogeneity among the Ki-67

staining profile of spheres (**Figure 2.1C**), with a major proportion of cells having an absent or weak nuclear staining reaction, and therefore being in a quiescent state. Scarce positively stained cells were randomly distributed throughout the sections. Noteworthy, larger spheres had a higher percentage of proliferating, Ki-67-positive cells (e.g. MNNG-HOS).

Measuring cell viability over time can be used as an indirect analysis of cell division and proliferation, with higher viability-related signals corresponding largely to higher cell density in *in vitro* cultures (Francoeur AM and Assalian A, 1996). For all the time-points analyzed, along the 72-hour period, spheres proliferated at consistently diminished rates in comparison to parental cells (**Figure 2.1D**), as assessed by WST-1 assay.

We also found that sphere-dissociated cells had enhanced clonogenic potential comparatively to parental cells, as demonstrated by the highest number of colonies formed at the single-cell level. Spheres formed on average 2-fold more colonies than parental cells (HOS 2.7-, MG-63 2.3-, MHM 2.7-, MNNG-HOS 1.7- and SJSA-1 2.9-fold), emphasizing a self-renewal advantage and increased malignant phenotype of osteosarcoma spheres (**Table 2.4**).

Table 2.4. Clonogenic efficiency of osteosarcoma parental cells and spheres.

Cell line	Parental	Spheres
HOS	16/96 (16.67%)	44/96 (45.83%)
MG-63	14/96 (14.58%)	32/96 (33.33%)
MHM	18/96 (18.75%)	48/96 (50%)
MNNG-HOS	28/96 (29.16%)	48/96 (50%)
SJSA-1	20/96 (20.83%)	58/96 (60.41%)

Note: Enzymatically-dissociated cells were plated at the single-cell levels in 96-well plates and grown at 37ºC for 2 weeks.

2.4.3 Spheres and osteosarcoma tissues overexpress the ESC-related genes SOX2 and KLF4

We explored whether cells capable of sphere-formation would express an ESC-specific gene signature, by analyzing four ESC-specific transcripts (*SOX2*, *KLF4*, *NANOG* and *OCT4*). Spheres showed molecular traits similar to undifferentiated stem cells, as analyzed by mRNA expression profiles. The most striking difference between spheres and parental cells was the significantly increased expression of *SOX2* and *KLF4* in spheres with fold-differences ranging from 2.75 to 18.50, and 2.01 to 10.32, respectively, depending on the cell line (**Figure 2.2A**). The other transcription factors *NANOG* and *OCT4* were more variable, being up-regulated in just one cell line (HOS: *NANOG*; MHM: *OCT4*), similarly expressed or even down-regulated in some spheres relatively to their corresponding parental cells.

We next assessed the expression of SOX2 and KLF4 at the protein level in a subset of cell lines, since these two transcription factors were more consistently overexpressed at the genomic level among our set of spheres. Immunohistochemistry staining of spheres showed a heterogeneous pattern of positivity for SOX2 with a significant proportion of cells having intense nuclear staining. Parental cells only showed few positive cells (black arrows) (**Figure 2.2B**). We could not analyze KLF4 expression by immunohistochemistry because the antibody showed aspecific staining in sections.

Western blot analysis showed a specific band of 35 and 65kDa respectively, for SOX2 and KLF4, and increased expression of these proteins was found in the spheres at least at 2-fold higher levels than in parental cells (except for HOS cell line), further reinforcing the presence of stem-like cells among osteosarcoma spheres (**Figure 2.2C**).

We tested whether osteosarcoma tissues had an ESC signature by measuring the mRNA expression level of master regulators of pluripotency in a panel of primary osteosarcoma tissues from clinical samples. Comparison of mRNA expression to parental cells and spheres showed that osteosarcoma primary tumors also express pluripotency components although at variable levels (**Figure 2.2D**, **Table 2.5**, **Table 2.6**), suggesting the presence of stemness-related genes in these tumors.

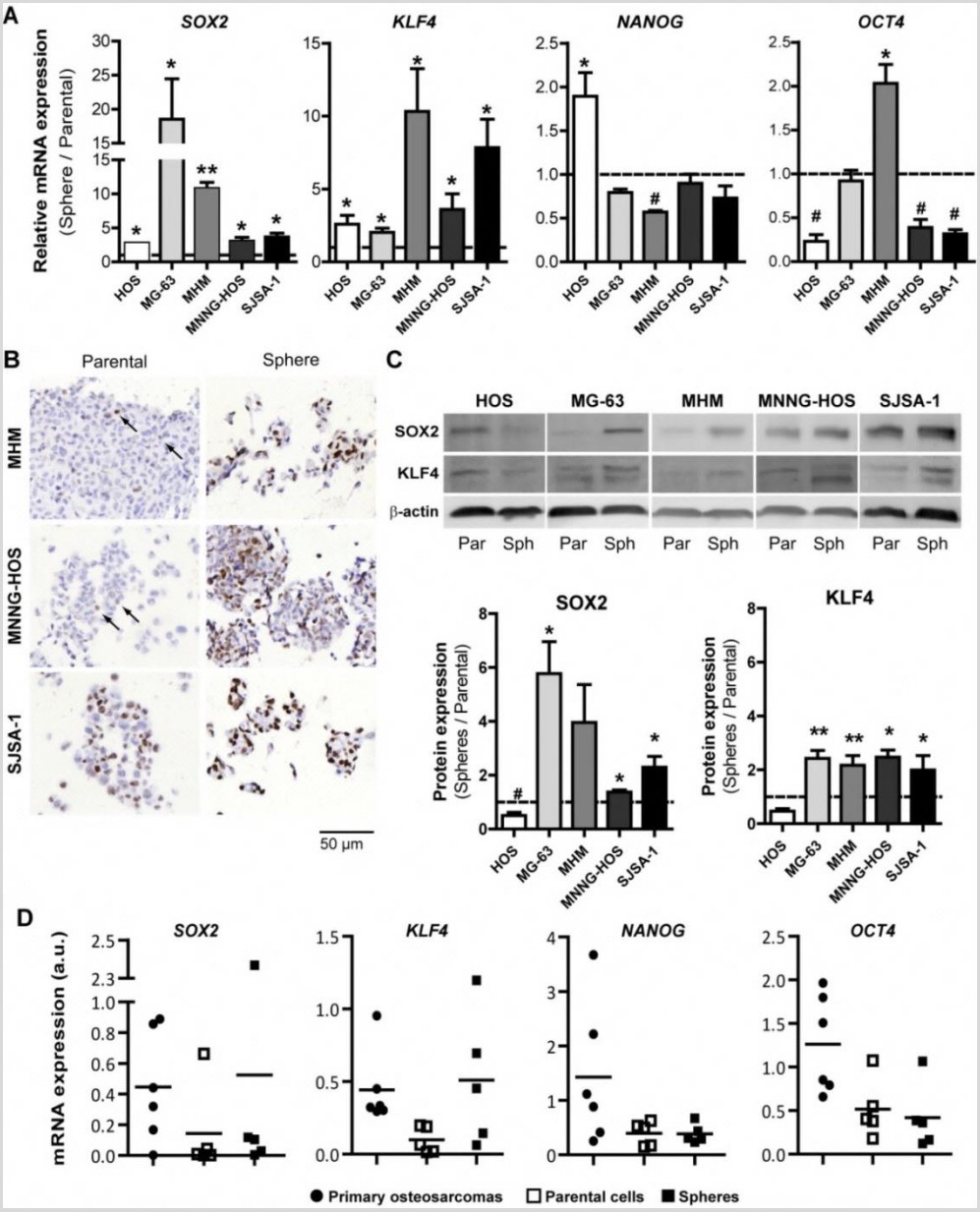


Figure 2.2. Spheres express genes involved in embryonic stem cell pluripotency. A. RT-PCR analysis of pluripotency-related gene transcripts, showing consistently higher expression of *SOX2* and *KLF4* in spheres. Values were calculated based on normalized Ct values; bars represent fold change expression ± SEM. **B.** Representative pictures of immunohistochemical staining showing more SOX2-positive protein expression in spheres than in parental osteosarcoma cell lines. **C.** Analysis of SOX2 and KLF4 protein expression by western blotting shows higher expression levels in spheres than in parental cells, for all the cell lines tested, except HOS. Bar graphs represent fold-difference of protein expression ± SEM, after normalization of band intensity to that of β-actin. *P<0.05, **P<0.01 significantly higher compared with parental cells; #P<0.05 significantly lower compared with parental cells (independent samples t-test). **D.** Osteosarcoma primary tissues display an mRNA expression signature associated with embryonic stem cells. Expression of

pluripotency-related genes was tested in six osteosarcoma primary tissues, and compared to that of fibroblastic-like cells. a.u., arbitrary units.

Table 2.5. Absolute mRNA expression levels of *SOX2* and *KLF4* transcripts in osteosarcoma primary tissues.

Osteosarcoma tissues	*SOX2*	*KLF4*
L2531	0.88880	0.44991
L2635	0.00205	0.33279
L2792	0.44195	0.32266
L2826	0.16748	0.29500
L2883	0.85550	0.95437
L2962	0.31882	0.30344

Note: Data shown was used to compute the scatter dot plots presented in **Figure 2.2D**.

Table 2.6. Absolute mRNA expression levels of *SOX2* and *KLF4* transcripts in osteosarcoma parental cells and corresponding spheres.

	SOX2		*KLF4*	
Cell line	**Parental**	**Spheres**	**Parental**	**Spheres**
HOS	0.00241	0.00476	0.02000	0.06200
MG-63	0.00200	0.02900	0.19000	0.45400
MHM	0.01000	0.10500	0.01600	0.14400
MNNG-HOS	0.04300	0.11800	0.19800	1.19700
SJSA-1	0.66300	2.36900	0.06700	0.69500

Note: Data shown was used to compute the scatter dot plots presented in **Figure 2.2D**.

Since osteoblastic-like cell lines did not form spheres, we explored the expression of pluripotency markers in a large cohort of samples using the R2 bioinformatic tool ('R2: Genomics Analysis and Visualization Platform (http://r2.amc.nl)'), which contains genome-wide gene expression data of high-grade osteosarcoma samples and cell lines (dataset Mixed Osteosarcoma - Kuijjer - 127 - vst - ilmnhwg6v2), to further evaluate if there is any relationship between the expression pattern of these markers and the histological subtype of osteosarcoma. We observed that the mRNA expression of all the embryonic stem cell markers does not particularly correlate with a specific osteosarcoma cell line subtype, with expression being diverse across the different cell lines (**Figure 2.3**).

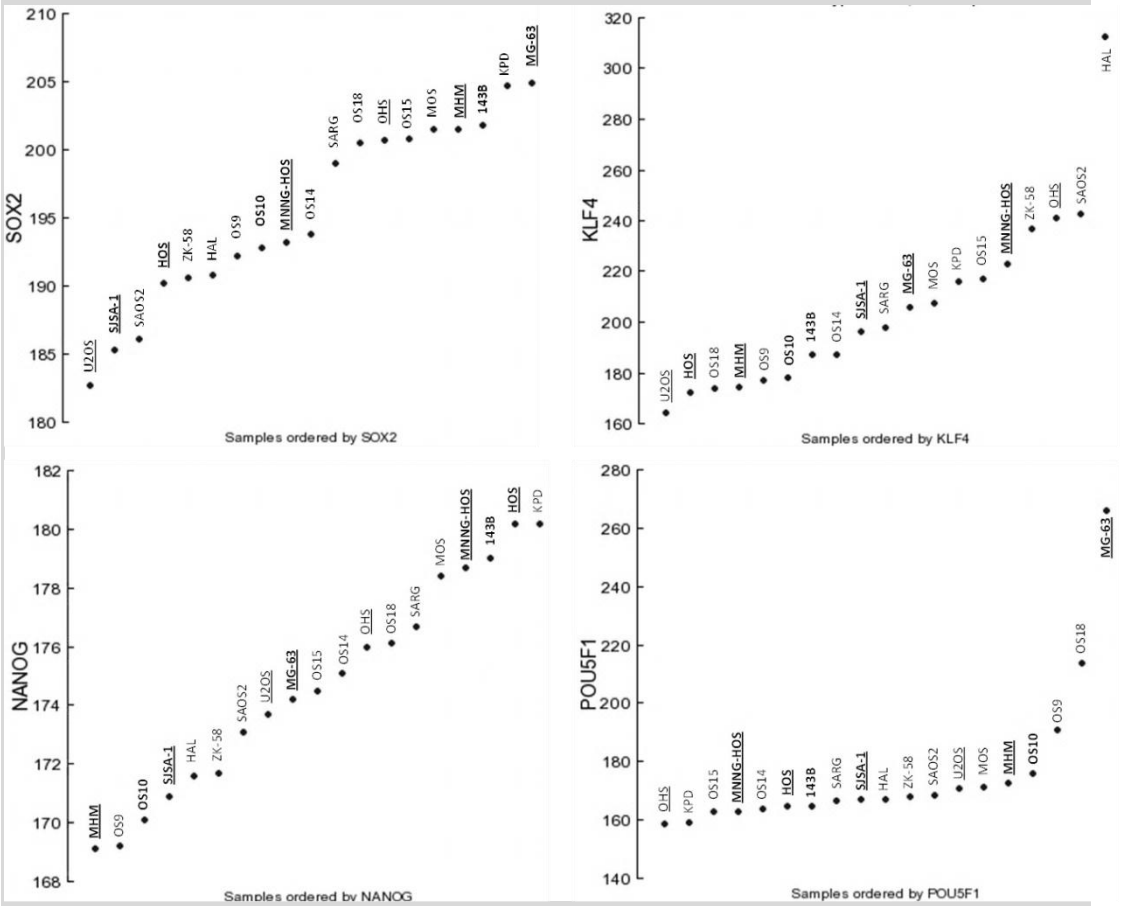


Figure 2.3. Expression of *SOX2*, *KLF4*, *NANOG* and *OCT4* (*POU5F1*) in the EuroBoNeT osteosarcoma cell line panel (Mohseny *et al.* 2011). Cell lines known to be derived from high-grade fibroblastic osteosarcoma are indicated in bold. Cell lines used in this study are also underlined. Data was extracted and analyzed using the R2 bioinformatic tool available at http://r2.amc.nl, using the dataset Mixed Osteosarcoma - Kuijjer - 127 - vst - ilmnhwg6v2. Known osteoblastic-like cell lines: KPD, MOS, OHS, OS9, OS14, OS15, OS18, U2OS and ZK-58; known fibroblastic-like cell lines: 143B, HOS, MG-63, MHM, MNNG-HOS, OSA and OS10.

Similar results were observed when comparing the high-grade fibroblastic and osteoblastic osteosarcoma tissue samples (**Figure 2.4**). Expression of the transcripts did not particularly vary across the different categories of sample classification, namely localization, sex, response to chemotherapy (based on Huvos grading system), site or metastatic dissemination (**Figure 2.5**).

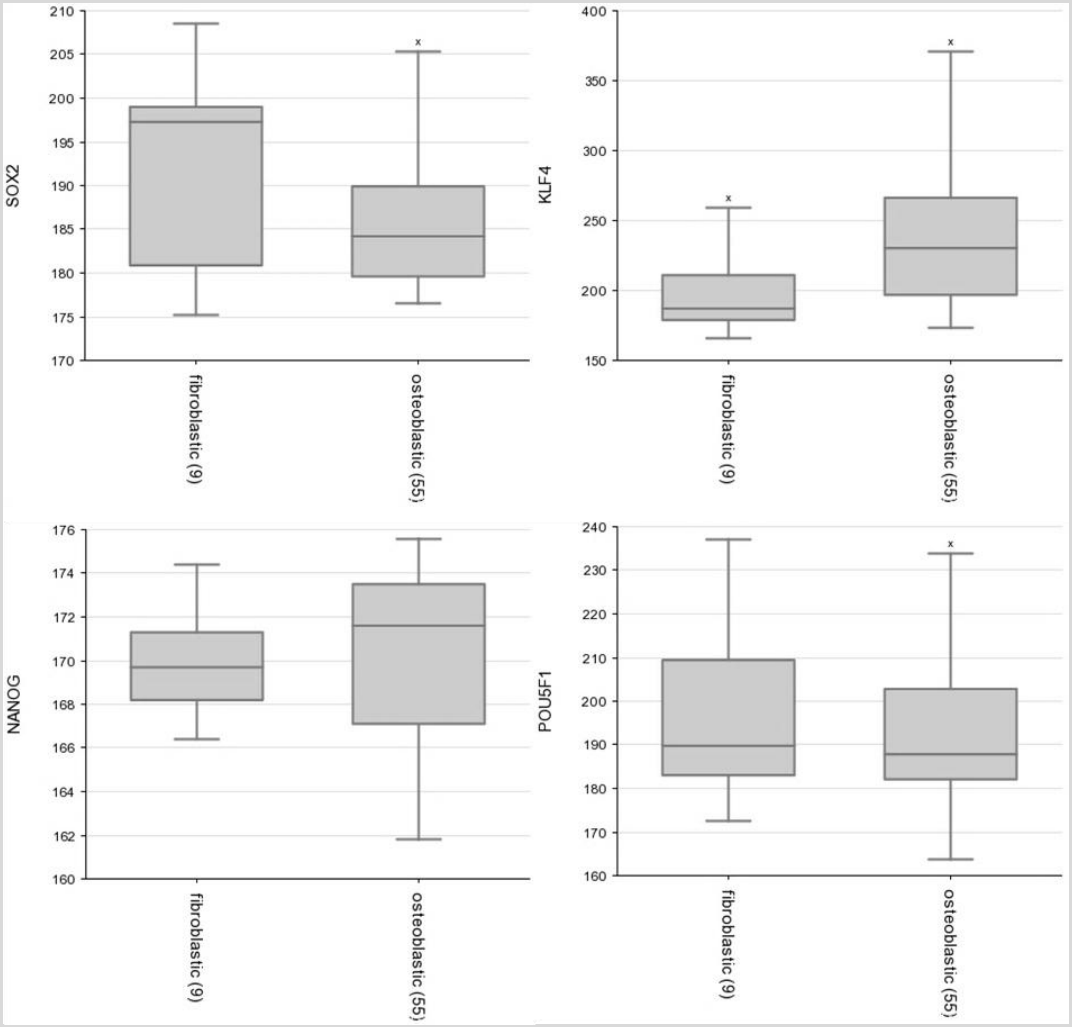


Figure 2.4. Expression of *SOX2, KLF4, NANOG* and *OCT4 (POU5F1)* in high-grade fibroblastic and osteoblastic osteosarcoma tissue samples. Data was extracted and analyzed using the R2 bioinformatic tool available at http://r2.amc.nl, using the dataset Mixed Osteosarcoma - Kuijjer - 127 - vst - ilmnhwg6v2.

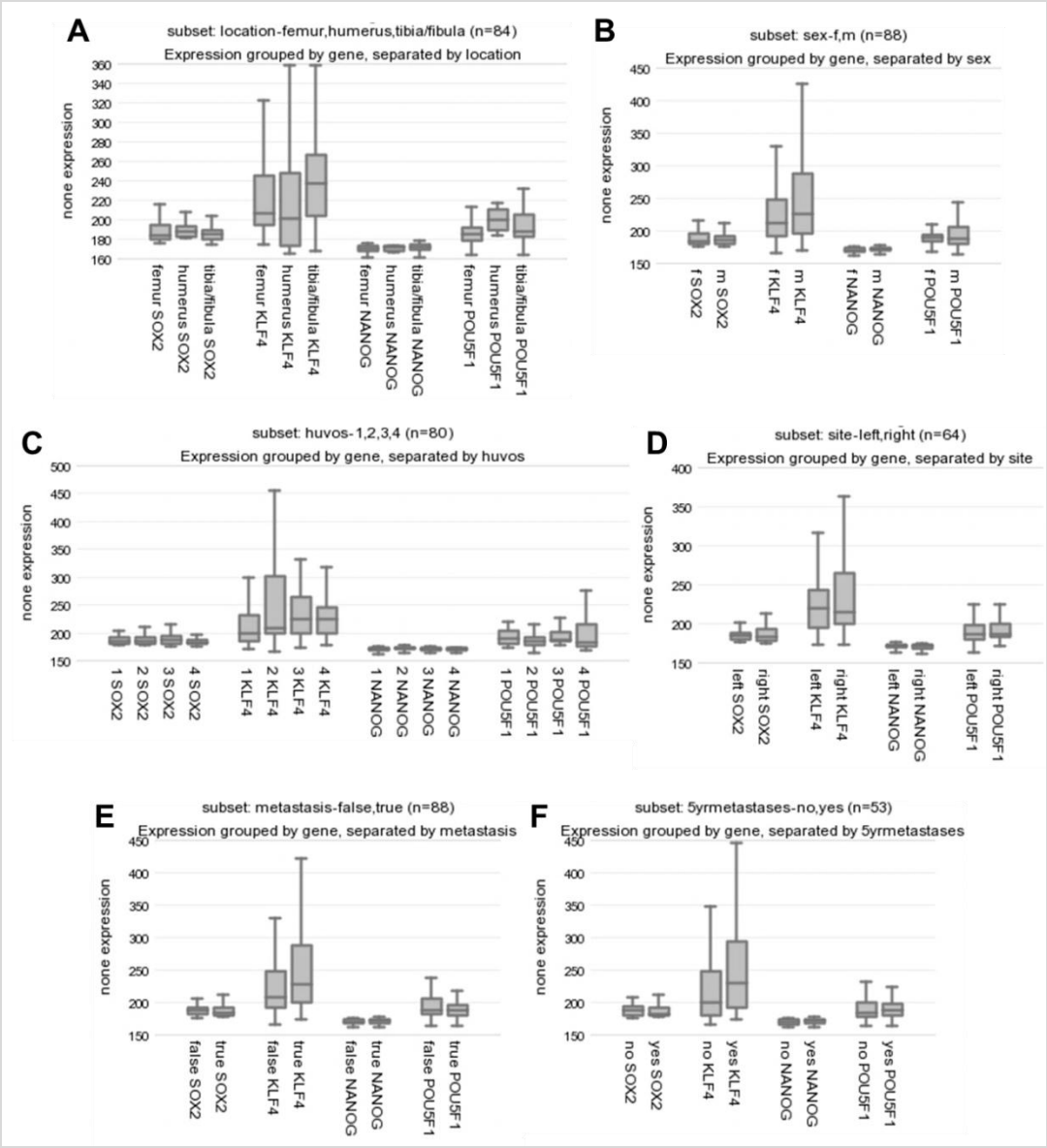


Figure 2.5. Expression of pluripotency markers in annotated high-grade osteosarcoma samples (dataset Mixed Osteosarcoma - Kuijjer - 127 - vst - ilmnhwg6v2). Expression of *SOX2*, *KLF4*, *NANOG* and *OCT4 (POU5F1)* does not significantly vary across the different categories of sample classification, namely localization (**A**), sex (**B**), Huvos grade (**C**), site (**D**) or metastatic dissemination (**E, F**).

An important concern about the use of specific SFM medium pertains to the "induction" of stemness features in differentiated cells, rather than the "selection" of cells already displaying those properties. We demonstrate that CSCs' isolation using this media formulation allows the selection of undifferentiated cells, since the mRNA expression of the pluripotency markers we studied was not induced by merely culturing parental cells in SFM in adherent conditions, as shown for HOS, MNNG-HOS and SJSA-1 cells (**Figure 2.6**).

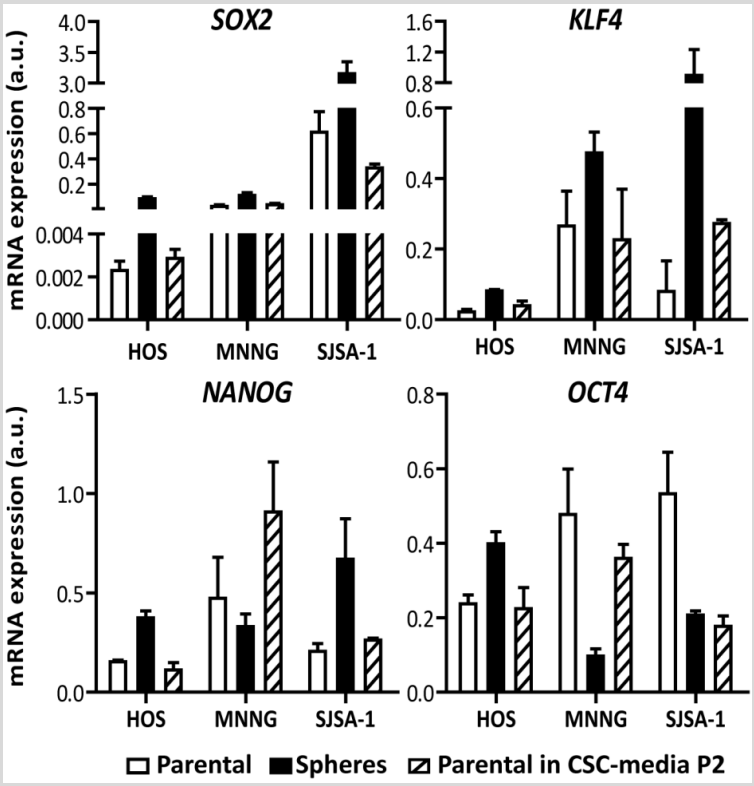


Figure 2.6. The combination of serum-free and suspended-growing conditions is required for the maintenance of a stem-like state. Three parental cell lines cultured in sphere-formation serum-free medium in adherent conditions for two passages (Parental in CSC-media P2) resemble the genotype of parental cells cultured in RPMI medium supplemented with serum, by showing mRNA expression comparable to these cells, and not to matched spheres. Data represents mean ± SEM of two independent experiments.

2.4.4 Wnt/β-catenin signaling is activated in osteosarcoma CSCs

Previous data indicated that inactivation of the developmental-related pathway Wnt/β-catenin is a key contributor for osteosarcomagenesis (Cleton-Jansen *et al.*, 2009; Cai *et al.*, 2010). Since this signaling pathway is also involved in stem cell maintenance and self-renewal (Reya *et al.*, 2003; Zhao *et al.*, 2007), we explored this pathway in osteosarcoma CSCs. Nuclear β-catenin accumulation is one of the hallmarks of activated Wnt/β-catenin signaling, and its expression was examined in parental cells and spheres by immunohistochemistry. Nuclear β-catenin was found in a significant fraction of cells in MG-63 and MNNG-HOS spheres (**Figure 2.7A**), while parental cells displayed only membranous expression of β-catenin. SJSA-1 (**Figure 2.7A**), HOS and MHM (not shown) spheres only showed minimal expression of nuclear β-catenin. These results were further confirmed by western blotting in nuclear lysates from MG-63, MNNG-HOS and SJSA-1

parental cells and spheres (**Figure 2.7B**). We found that MG-63, MNNG-HOS and SJSA-1 spheres had higher nuclear β-catenin levels than corresponding parental cells. Moreover, β-catenin-TCF/LEF-mediated transcriptional activity was increased in sphere-derived cells of two cell lines with higher nuclear β-catenin accumulation (MG-63 and MNNG-HOS) and lower in the SJSA-1 spheres relatively to corresponding parental cells (**Figure 2.7C**).

To further explore the Wnt/β-catenin activation in CSCs, we tested the mRNA expression of the specific Wnt/β-catenin target gene *AXIN2* (Jho *et al.*, 2002). MG-63, MNNG-HOS and SJSA-1 spheres showed higher expression of *AXIN2* than parental cells (**Figure 2.7D**), which is in line with active Wnt/β-catenin signaling in spheres, as tested by β-catenin nuclear expression. We also tested the expression of the secreted Wnt/β-catenin signaling antagonist *DKK-1* and found that parental cells had increased expression of this transcript (**Figure 2.7D**), in line with previous results showing its relatively high expression in a diverse panel of osteosarcoma cell lines (Cai *et al.*, 2010). Conversely, spheres had lower *DKK-1* expression, which can be a possible mechanism for the increased Wnt activity in this cell population.

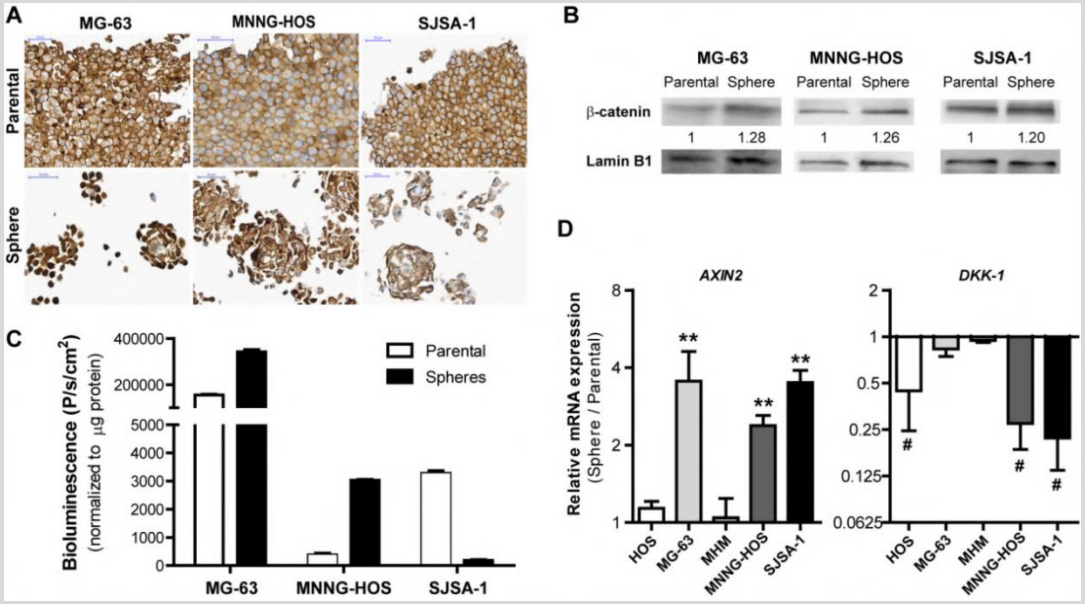


Figure 2.7. Wnt/β-catenin is active in spheres. A. Representative pictures show that parental cells have membranous and cytoplasmic, but not nuclear, β-catenin staining, while MG-63 and MNNG-HOS-derived spheres comprise a high fraction of cells displaying strong to moderate nuclear β-catenin. SJSA-1 spheres show minimal expression of nuclear β-catenin. Bar represents 50μm. **B.** Analysis of β-catenin expression in nuclear lysates from MG-63, MNNG-HOS and SJSA-1 parental cells and spheres. Nuclear β-catenin expression is higher in MG-63, MNNG-HOS and SJSA-1 spheres than in parental cells. Numbers below protein bands represent fold-expression

spheres/parental, after normalization of band intensity to that of lamin B1, used as nuclear loading control. **C.** Luciferase reporter assays using pGL4.49[luc2P/TCF-LEF RE/Hygro] reporter plasmid, in osteosarcoma parental cells and spheres. Bar graphs represent fold change bioluminescence (luciferase) activity ± SEM normalized to total protein content (in µg). **D.** Expression of *AXIN2* and *DKK-1*, genes involved in Wnt/β-catenin signaling. Bars represent fold change expression ± SEM in spheres *versus* parental cells. **P<0.01 and #P<0.05 significantly higher and lower, respectively, compared with parental cells (independent samples t-test).

2.4.5 Osteosarcoma spheres have enhanced tumorigenic potential

We next evaluated the ability of spheres and matched parental cells of three random cell lines to form tumors in immunocompromised mice, by subcutaneous injection of equal cell numbers ($1x10^5$) in opposite flanks (**Figure 2.8**). MHM spheres formed a tumor with a volume of 350 mm^3, after 50 days of cell inoculation, whereas parental cells could not form visible xenografts during this time-period. For MNNG-HOS and SJSA-1 cells, both spheres and parental cells formed tumors, although sphere-derived tumors appeared at an earlier time-point and reached higher tumor volumes. Tumors derived from MNNG-HOS cells were removed after 21 days of cell injection and reached volumes of 29 mm^3 (parental) and 190 mm^3 (spheres). SJSA-1-derived tumors were collected after 25 days of cell inoculation and reached volumes of 90 mm^3 (parental) and 210 mm^3 (spheres).

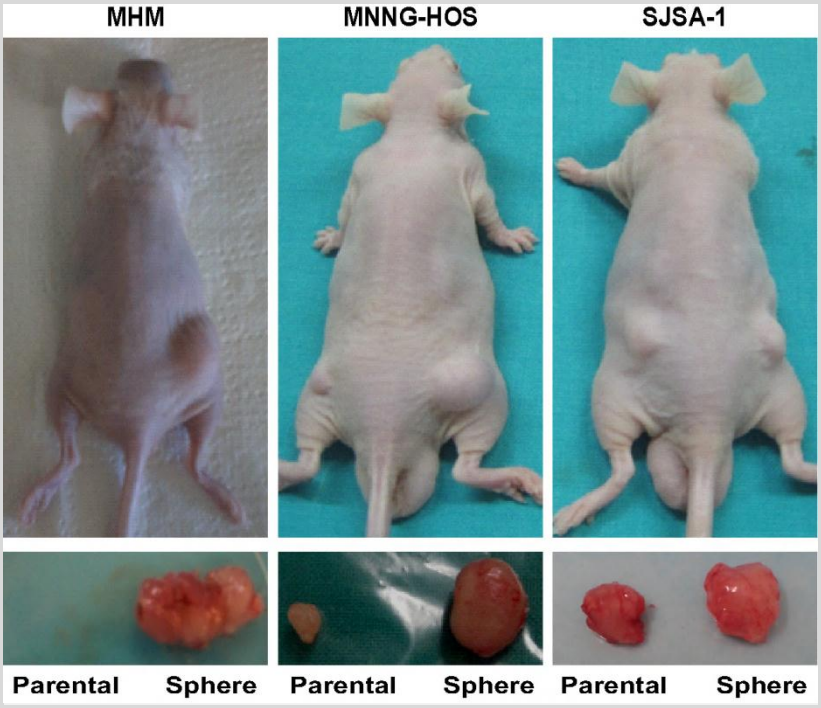


Figure 2.8. Osteosarcoma spheres have enhanced tumorigenic potential in immunocompromised mice. Representative images of Swiss nude mice inoculated with parental (left flank) and sphere-derived cells (right flank) from MHM, MNNG-HOS and SJSA-1 cell lines (upper panel). Images of tumors excised after 50 days (MHM), 21 days (MNNG-HOS) and 25 days (SJSA-1) post injection (lower panel).

2.4.6 Osteosarcoma cell lines contain side-population cells

An alternative method to identify cells with stem-like properties is based on the capacity of cells to actively extrude Hoechst-33342, the so-called side-population (Golebiewska *et al.*, 2011; Wu *et al.*, 2007). We found a distinct side-population located near the lower left quadrant of the FACS profile (**Figure 2.9A**) in all the cell lines examined. Osteoblastic-like cells showed side-population fractions varying from 0.8% to 1.68% (**Figure 2.9B**). The fibroblastic-like cells also contain a fraction of side-population that varied from 0.36% to 1.35%. The side-population phenotype is primarily defined by the capacity of cells to excrete cytotoxic compounds, via the drug efflux transporter *ABCG2*. Expression of this transcript was found at variable levels in all parental cells (**Figure 2.9C**), and largely correlated with the percentage of side-population subset found on osteosarcoma cells, as indicated by a positive and statistically significant ($p=0.02$) coefficient of correlation (Spearman $Rs=0.77$) (**Figure 2.9D**). We also investigated if sphere-forming cells were enriched for the side-population phenotype. Unexpectedly, side-population fractions on sphere cultures were lower than fractions of

corresponding parental cells (MG-63 and MNNG-HOS, **Figure 2.9E**, left panel), except SJSA-1 spheres, which showed an enrichment of the side-population, albeit not significant. Despite that spheres were not enriched in side-population they exhibited a higher *ABCG2* expression as compared to parental cells (**Figure 2.9E**, right panel).

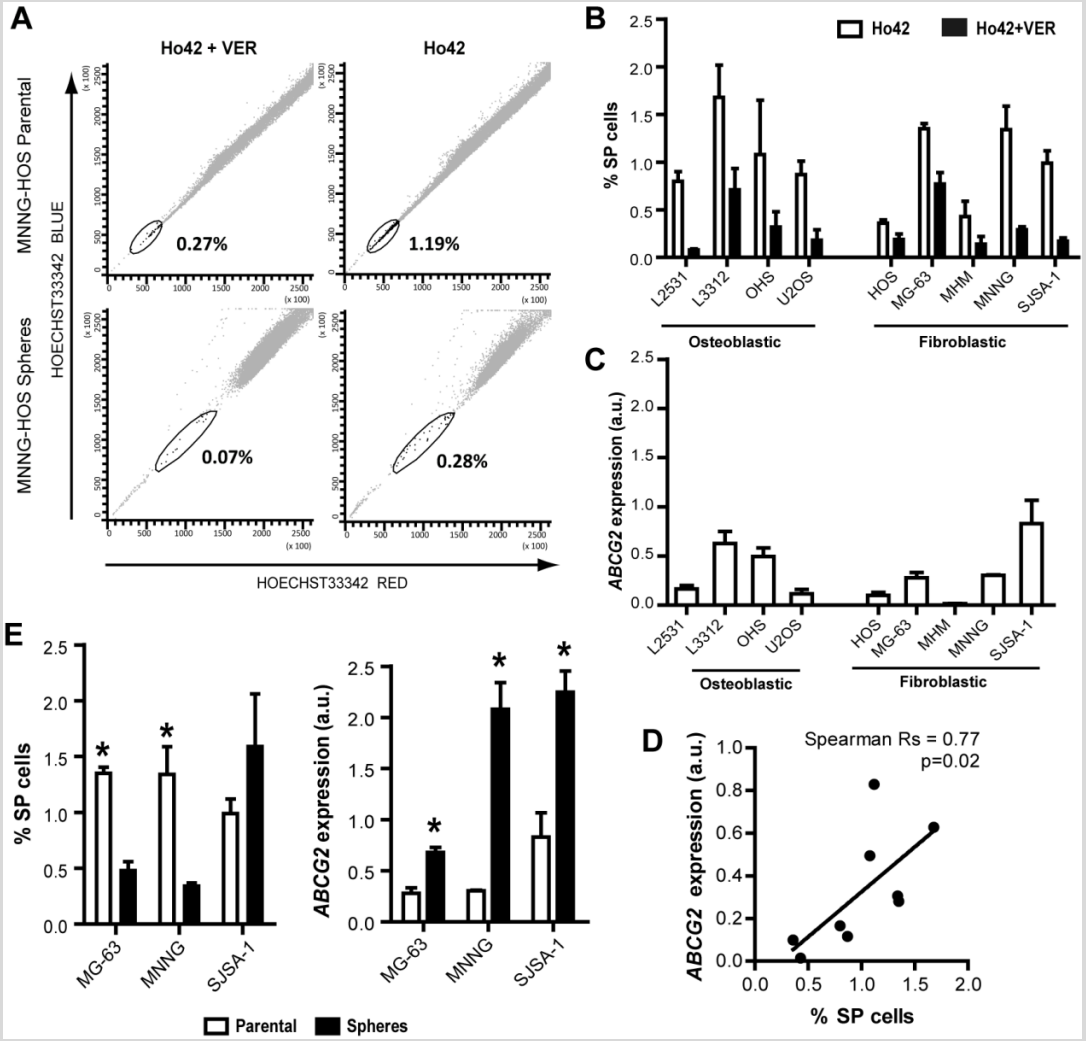


Figure 2.9. Osteosarcoma cell lines contain side-population cells. A. Representative dotplots of side-population detection, using flow cytometry. Side-population is shown as percentage of the total cell population. Verapamil is an inhibitor of ABC transporters, and used as a negative control. **B.** Variable percentages of side-population cells were found among osteosarcoma cell lines. **C.** RT-PCR analysis of *ABCG2* in parental cell lines. Values were calculated based on normalized Ct values. Data represents mean ± SEM from three independent experiments. **D.** Correlation of side-population percentages with *ABCG2* mRNA expression. Spearman test was used to estimate correlation between the two variables. **E.** Comparison of side-population fractions and *ABCG2* expression in the three cell lines that formed spheres with high efficiency (MG-63, MNNG-HOS and SJSA-1). *P<0.05, compared to corresponding spheres (left panel) or

parental cells (right panel) (independent-samples Mann-Whitney test). Ho42, Hoechst-33342; VER, verapamil; SP, side-population.

2.4.7 Osteosarcoma cells contain ALDH-positive cells, which also show an embryonic stem cell-related gene signature

Cells with stem-like properties possess enhanced mechanisms of self-defense, owing to the over-activation of detoxifying enzymes, such as aldehyde dehydrogenases (ALDH) (Ma and Allan, 2011). These enzymes also play a role in early differentiation and proliferation of stem cells through retinoic acid metabolism, being considered a CSC marker. We used the Aldefluor™ assay to identify CSCs in our panel of osteosarcoma cells, particularly in osteoblastic-like cell lines that did not form spheres. ALDH-positive cells were found within osteoblastic-like cell lines (**Figure 2.10A,B**) at variable ranges (0.30±0.12% in L2531 to 1.83±0.20% in L3312). Moreover, we identified ALDH-positive cells in fibroblastic-like cell lines that efficiently form spheres, although the levels of activity were different, with the HOS cell line showing approximately 4-fold higher activity than the average activity of MHM, MG-63, MNNG-HOS and SJSA-1. A further enrichment of this population was found within spheres from HOS, MNNG-HOS and SJSA-1 cells (**Figure 2.10B**).

To further evaluate whether gene expression pattern of ALDH-positive cells overlapped with that of spheres, we performed mRNA characterization in the ALDH-positive cell populations. We selected HOS parental cells, and MNNG-HOS and SJSA-1 spheres based on their more abundant Aldefluor™ activity. The other samples were not tested due to the reduced percentage of ALDH-positive cells (**Figure 2.10B**). *SOX2* was significantly overexpressed on ALDH-positive cells, while *KLF4* was significantly down-regulated compared to ALDH-negative cells (**Figure 2.10C**). Expression of *NANOG* and *OCT4* was more variable, although ALDH-positive cells sorted from spheres showed a trend to have higher expression of these transcription factors.

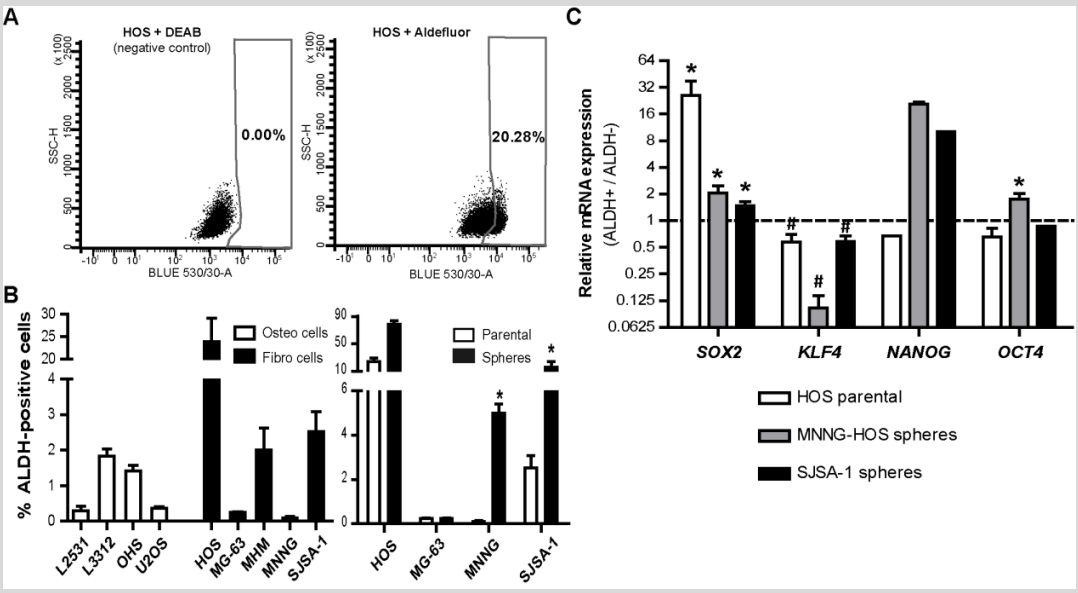


Figure 2.10. Flow-cytometric analysis of osteosarcoma cell lines based on ALDH activity. A. Representative dotplots showing detection of ALDH-positive events. **B.** Cell lines contain a variable fraction of ALDH-positive cells. Mean ± SEM of three independent experiments is shown. *P<0.05, significantly increased ALDH activity on spheres, compared to parental cells (Mann-Whitney test). **C.** Expression of pluripotency-related genes in ALDH-positive (ALDH+) as fold-difference *versus* ALDH-negative cells (ALDH-) sorted from HOS parental cells and from MNNG-HOS and SJSA-1 spheres. RT-PCR analysis was conducted on total RNA from indicated cells after fluorescence-activated cell sorting. Values are mean fold-difference ± SEM, after Ct values normalization. *P<0.05 and #P<0.05 represent, respectively, significantly higher and lower expression in ALDH-positive, compared to ALDH-negative cells (Mann-Whitney test). N.d., not detected.

2.5 Discussion

The isolation of CSCs from human solid tumors appears to be a controversial topic in the CSC biology, due to technical concerns and absence of specific biomarkers (Pastrana *et al.*, 2011; Golebiewska *et al.*, 2011). We used a panel of cell lines (Mohseny *et al.*, 2011; Kuijjer *et al.*, 2011) derived from two histological subtypes of high-grade osteosarcoma to identify CSC populations and conducted three distinct functional approaches for their isolation and enrichment, aiming to further characterize a stemness signature on this bone sarcoma. Despite many efforts towards the identification of specific surface antigens for putative osteosarcoma CSCs, no universal reliable marker combination has been reported so far. This can be attributed to the clonal variation within tumors, inexistence of accepted CSC markers due to mutations or epigenetic

silencing and the lack of knowledge regarding the role of such markers on maintaining CSCs on a stem-like state (Pattabiraman and Weinberg, 2014). Moreover, also MSCs lack specific markers.

We found that cell lines derived from distinct histological subtypes of high-grade osteosarcoma have differential capacity to survive within the sphere-forming assay. Only the fibroblastic cell lines generated floating spherical colonies, in opposite to osteoblastic-like cells that formed cellular aggregates upon few days on sphere media. A possible explanation for this observation is the higher degree of differentiation of osteoblastic tumors in comparison to fibroblastic tumors (Hauben *et al.*, 2002). Alternatively, this may be attributed to a more differentiated status of the precursor cells of osteoblastic tumors in comparison to the fibroblastic-derived progenitor cells (Mutsaers and Walkley, 2014). In this scenario, it appears that more primitive cells (fibroblastic-like) are more prone to survive the harsh conditions required for CSC-enrichment using this method.

Cell dormancy is an intrinsic feature of some somatic and cancer stem cells. It is generally accepted that cell quiescence protects against cytotoxic insults, and studies indicate that abrogation of quiescence can increase CSCs' sensitivity to chemotherapy (Saito *et al.*, 2010). We provide evidence that spheres are enriched for a slow-dividing subset of cells as indicated by the small fraction of Ki-67 positive cells and lower metabolic activity revealed by the WST-1 cell proliferation assay. These findings are in line with our previous report showing reduced uptake of the glucose analogue FDG by spheres (Martins-Neves *et al.*, 2012) as a consequence of the lower energy requirements. Also Palorini *et al.* observed a lower metabolic and mitochondrial activity in a 3AB-OS osteosarcoma stem-like cell population (Palorini *et al.*, 2014). These observations are in accordance with the recent findings that CSCs, like their normal counterparts, rely on glycolysis for energy supply. It is likely the low energetic metabolic requirements are implicated in pluripotency and stemness favoring self-renewal maintenance over differentiation capabilities (Moussaieff *et al.*, 2015). Others also showed that breast tumor-initiating cells are quiescent, exhibiting Ki-67-negativity and a G0/G1 cell-cycle state (Feng *et al.*, 2014). These results collectively correlate with the traditional CSC model, which postulates that CSCs are slow-cycling cells (Moore and Lyle, 2011).

To identify stemness-related markers on spheres we tested the expression of diverse pluripotency-related factors. Constitutive expression of *SOX2* maintains cellular self-

renewal, and coordinated with *OCT4* and *NANOG* supports ESC-pluripotency (Boyer *et al.*, 2005). Furthermore, ectopic *KLF4* expression reprogrammes pluripotency in somatic cells (Wei *et al.*, 2009). The characterization of sphere-forming cells showed a regular and marked expression of two self-renewal transcription factors (SOX2 and KLF4) in relation to their matched counterparts, at both gene and protein levels. We found that increased sphere-forming efficiency did not directly correlate with enhanced expression of self-renewal transcription factors. Indeed, HOS and MHM cells formed spheres less efficiently than e.g. MG-63 cells, but expressed more ESC-related genes than the latter. In addition to *SOX2* and *KLF4*, HOS and MHM overexpressed *NANOG* or *OCT4*. Interestingly, others also detected increased expression of pluripotency markers in smaller and younger spheres (Gibbs CP *et al.*, 2005). Only the HOS cell line showed a discrepancy between transcript and protein levels, a fact that can be caused by post-transcriptional and post-translational modifications. It can also be that other stemness-related proteins play a role on the maintenance of HOS spheres, such as *STAT3*, or even those belonging to ESC-related lineages, as previously proposed (Gibbs CP *et al.*, 2005).

Previous studies including our own observed an up-regulation of Oct4 and Nanog at both gene and protein levels in osteosarcoma CSCs and tissues (Gibbs CP *et al.*, 2005; Saini *et al.*, 2012); however, in the samples tested in this report we barely detected augmented expression of *OCT4* and *NANOG* mRNA in the spheres, in line with reports from other groups (Basu-Roy *et al.*, 2015; Wang *et al.*, 2009). This suggests that a variable repertoire of several ESC-like genes characterizes osteosarcoma stem-like cells.

OCT4 and *NANOG* were similarly expressed in our panel of parental cells and spheres suggesting that these factors are multifunctional and may also have an oncogenic role other than regulating self-renewal and differentiation. For instance, Chang *et al.* detected high expression levels of *OCT4* in bladder cancer specimens and found a correlation with tumor progression and metastasis through the activation of metalloproteinases, well-known regulators of cell motility (Chang *et al.*, 2008).

Noteworthy, we also found expression of pluripotency-related genes in primary osteosarcomas, although this expression does not particularly correlate with a specific histological subtype or other clinicopathological features such as location, sex, site or Huvos grading; nevertheless, the expression of these biomarkers suggests that they provide stemness signatures in these tissues, and also reinforce the notion that stem-like tumor cells may exist among osteoblastic-like samples and cell lines, despite that they cannot be enriched using the *in vitro* sphere assay.

Our data suggests the Wnt/β-catenin signaling is constitutively activated in osteosarcoma CSCs, but not in parental cells as we previously described. Nuclear expression of β-catenin, increased TCF/LEF transcriptional activity and increased *AXIN2* expression demonstrate the specific activation of Wnt/β-catenin signaling on MG-63 and MNNG-HOS spheres. Despite controversial results being reported (McQueen *et al.* 2011), our previous studies, conducted on osteosarcoma tissue sections and bulk cell lines, demonstrated that the Wnt/β-catenin signaling is generally inactivated in osteosarcoma (Cai *et al.*, 2010). However, here we report that the Wnt/β-catenin pathway is specifically activated in the subset of osteosarcoma CSCs and not in their parental cells, suggesting that it can play an important role on the self-renewal of stem-like cell populations, as previously reported (Mao *et al.*, 2014; Malanchi *et al.*, 2008). The discrepancy between nuclear β-catenin, TCF/LEF activity and *AXIN2* expression observed in SJSA-1 spheres maybe be explained by the fact that this cell line showed the highest constitutive expression of SOX2 (**Figure 2.2B**). In fact, SOX2 has previously been indicated as an antagonist of the Wnt/β-catenin pathway in osteosarcomas, with a possible mechanism being the direct binding of SOX2 to β-catenin (Basu-Roy *et al.*, 2012). This can account for the absence of nuclear β-catenin and subsequent activation of TCF/LEF transcriptional factors in SJSA-1 CSCs enriched with the sphere assay. Nevertheless, a residual activation of Wnt/β-catenin pathway might still exist that can contribute to high *AXIN2* transcript expression in these cells, or result from the activity of other pathways that can regulate *AXIN2* expression, such as the pro-tumorigenic pRb/E2F pathway (Hughes and Brady, 2005). The discrepancy between *AXIN2* and *DKK-1* expression (both negative regulators of the Wnt/β-catenin pathway) may be due to the fact that other distinct signaling pathways might also contribute to the regulation of their mRNA expression. In fact, *AXIN2* expression is also regulated by the pRb/E2F pathway, as previously mentioned, and *DKK-1* transcription is regulated by the JNK signaling cascade (Colla *et al.*, 2007) and by BMI1 (Cho *et al.*, 2013).

Enhanced tumorigenicity *in vivo* is described as a hallmark of CSCs. We demonstrated the tumorigenic ability of osteosarcoma spheres in immunocompromised mice. All animals injected with sphere-derived single cell suspensions developed large tumors with volumes 6-fold (MNNG-HOS) and 2-fold (SJSA-1) higher than the tumors induced by parental cells. Importantly, while MHM spheres also formed a large xenograft, parental MHM cells were not at all tumorigenic even after 50 days of cell injection, in accordance with previously published data (Mohseny *et al.*, 2011). These results suggest that spheres are enriched in a stem-like cell population having improved

capacity to generate tumors, while parental cells either fail to form tumors or require longer periods of time to form such tumors, probably representing the presence of a smaller subset of stem-like cells that are sufficient to induce tumor development. Our results agree with previous reports demonstrating the enhanced capacity of CSCs from mesenchymal neoplasms to form *in vivo* xenografts (Wu *et al.*, 2007). We did not test the tumorigenic potential of osteoblastic cells *in vivo*, since our aim was to evaluate this feature in enriched CSCs populations. However, previous reports described two of the osteoblastic cell lines we used in this study (OHS, U2OS) as tumorigenic (Mohseny *et al.*, 2011) when inoculated in immunocompromised mice at high cell density ($2x10^6$ cells), which reflects the existence of cells with tumor-initiating ability.

A small side-population fraction was detected in osteosarcoma cell lines derived from both fibroblastic-like and osteoblastic-like subtypes. We show that this can probably be attributed to enhanced activity of the *ABCG2* transporter in line with previous data (Martins-Neves *et al.*, 2012; Adhikari *et al.*, 2010). Interestingly, MG-63, MNNG-HOS and SJSA-1 cell lines, which had the highest sphere-forming capacity also had the highest side-population fraction, comparing to HOS and MHM fibroblastic-like cells. We evaluated if there was an enrichment of the side-population subset within the sphere cell population. Surprisingly, an even lower percentage of side-population cells was found in spheres compared to parental cells, despite that they showed higher *ABCG2* expression. This suggests that the side-population assay does not select for the same population obtained using the sphere assay. Moreover, other ABC transporters like *ABCB1* can also contribute to Hoechst dye exclusion (Golebiewska *et al.*, 2011). One limitation of this work was our inability to flow-sort sufficient side-population cells to further characterize these cells at least at the genomic level to confirm their stem-like nature.

Aldefluor™ activity has been used as a tumor stem cell marker (Wang *et al.*, 2011; van den Hoogen *et al.*, 2010). ALDH-positive cells represented a small subset, agreeing with the notion that CSCs constitute a minority of the tumor cells. Similar ranges of ALDH-positive populations were found on the cell lines, independently of their histological origin, with the exception of the HOS cell line (average of 25% ALDH-positive cells). We also provide evidence that Aldefluor™ activity is functionally involved in osteosarcoma stemness, as observed with the enrichment of ALDH-positive population in the spheres. ALDH-positive cells had higher *SOX2* expression than ALDH-negative cells, but lower *KLF4* expression. Differences between ALDH-positive and

ALDH-negative cells concerning *NANOG* and *OCT4* expression were more variable among the cell lines, as observed for spheres. Based on this, we suggest that distinct stem-like populations may rely on different stemness-related biomarkers. *SOX2* appears as the most prominent ESC-related gene playing an important biological role in osteosarcoma CSC populations isolated using the sphere and Aldefluor™ assays, as supported by previous reports (Basu-Roy *et al.*, 2012; Basu-Roy *et al.*, 2015).

Altogether, our data suggest that different CSC populations might co-exist within osteosarcoma cells, which seems dependent on the histological subtype. Importantly, osteosarcoma CSCs isolated with the sphere assay have constitutively activated Wnt/β-catenin signaling and enhanced tumorigenic potential. Distinct CSC subsets may assume dissimilar functions, according to their role in the maintenance of self-renewal (spheres) or chemo-resistance (ALDH activity and side-population). These findings may imply that therapies targeting CSCs should consider clonal heterogeneity, which can prevent their effective eradication.

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3 CHAPTER

SELECTIVE ERADICATION OF OSTEOSARCOMA STEM CELLS BY TARGETING WNT/β-CATENIN SIGNALING ACTIVITY

Sara R. Martins-Neves, Daniela I. Paiva-Oliveira, Carlos Fontes-Ribeiro, Judith V. M. G. Bovée, Anne-Marie Cleton-Jansen*, Célia M. F. Gomes*

(* equal contribution)

Submitted for publication

"Cancer can take away all of my physical abilities. It cannot touch my mind, it cannot touch my heart, and it cannot touch my soul." – Jim Valvano

3.1 ABSTRACT

Background: Wnt/β-catenin pathway regulates the self-renewal of cancer stem cells (CSCs) and is a key determinant of tumor progression and also resistance to chemotherapy. Previously, we reported that this pathway is constitutively activated in a subset of osteosarcoma CSCs and also that doxorubicin induced stemness properties in differentiated cells through activation of the Wnt/β-catenin pathway. Here, we investigated whether inhibition of this pathway might constitute a strategy to target CSCs and improve the efficacy of chemotherapy in osteosarcoma.

Methods: Pharmacological inhibition of Wnt signaling was performed with the tankyrase inhibitor IWR-1 in osteosarcoma sphere-forming stem-like cells. The effectiveness of IWR-1 on Wnt inhibition was measured by luciferase reporter assay, redistribution of β-catenin and analysis of Wnt target genes by quantitative RT-PCR and western blot. The inhibitory effects of IWR-1 were evaluated in vitro by measuring cell viability (WST-1), apoptosis (TUNEL) cell cycle progression and cytotoxicity to doxorubicin. The in vivo effects were evaluated in a xenografted mouse model using a pGL4 luciferase reporter vector by bioluminescence imaging and immunohistochemical analysis.

Results: IWR-1 was specifically cytotoxic for osteosarcoma CSCs. IWR-1 impaired CSC' self-renewal capacity by compromising landmark steps of the canonical Wnt signaling, namely translocation of β-catenin to the nucleus and subsequent TCF/LEF activation and expression of downstream targets. Moreover, Wnt inhibition hampered the activity and expression of important stemness-related genes i.e., *NANOG*, *POU5F1*, Sox2 and *ALDH* isoforms. *In vitro*, IWR-1 induced apoptosis of osteosarcoma CSCs and in combination with doxorubicin treatment elicited synergistic cytotoxicity, reversing CSCs intrinsic resistance to this chemotherapeutic drug. *In vivo*, IWR-1 alone and in synergy with doxorubicin significantly decreased tumor progression. This was associated with specific down-regulation of TCF/LEF transcriptional activity and of nuclear β-catenin, accompanied by decreased expression of the putative CSC marker Sox2.

Conclusions: Our results suggest that selective targeting of the Wnt/β-catenin pathway can eliminate CSCs. Combining conventional chemotherapy with Wnt/β-catenin inhibition in osteosarcoma treatment can simultaneously contribute to reduce chemotherapy doses and eradicate the aggressive CSC-enriched sub-populations in osteosarcoma.

Keywords: cancer stem cells, osteosarcoma, Wnt/β-catenin signaling, apoptosis, IWR-1, doxorubicin

3.2 INTRODUCTION

Osteosarcoma is the most common malignant primary bone tumor and has a peak incidence at puberty. In the seventies, the inception of multimodal chemotherapy combined with surgical resection significantly improved patient survival rates. However, 5-year overall survival of patients with localized and metastatic disease remained disappointingly leveled at 60-65% and 30%, respectively (Fletcher *et al.*, 2013). Despite intensification of drug dosages (Eselgrim *et al.*, 2006; Lewis *et al.*, 2007) and tentative addition of new therapeutic compounds (Meyers *et al.*, 2005; Hattinger *et al.*, 2010), the outcome of poor responders and overall survival did not significantly improve. Moreover, recurrence rates occurring after an initial favorable response to preoperative chemotherapy persistently fluctuated between 10-20% (Fletcher *et al.*, 2013).

The standard treatment for osteosarcoma patients proposed by the EURAMOS-1 protocol promotes significant rates of disease remission, but some patients still relapse and die, mostly because of lung metastases, even when presenting a favorable response to neoadjuvant chemotherapy. This scenario reflects the cellular heterogeneity observed in osteosarcoma tumor samples and the existence of a self-renewing sub-population that does not respond to chemotherapy, a cellular behavior that has been attributed to the so-called cancer stem cells (CSCs) (Adhikari *et al.*, 2010; Yan *et al.*, 2016). These cells are mainly characterized by expression of markers involved in pluripotency, such as Sox2 (Skoda *et al.*, 2016), and also activation of signaling pathways controlling stem cell self-renewal (Chen *et al.*, 2015), observations that are in agreement with our own previous studies (**Chapter 2**).

Wnt/β-catenin signaling plays pivotal roles in the context of embryonic development, stem cell pluripotency (Sato *et al.*, 2004), differentiation (Kielman *et al.*, 2002) and importantly, in cellular self-renewal (Reya *et al.*, 2003; Zhao *et al.*, 2007). Wnt/β-catenin signaling initiates when specific canonical Wnt ligands bind to cell membrane receptors. This signal triggers the inhibition of cytoplasmic GSK3 and causes translocation of β-catenin into the nucleus to induce the transcription of key target genes, such as *AXIN2*, *DKK-1*, *c-Myc* and *cyclin D1*, among others (Atlasi *et al.*, 2014). Given the pivotal role of the Wnt/β-catenin signaling in the regulation of cell stemness and also malignant behavior, this pathway has been linked to oncogenic events, participating in both tumor genesis (Cairo *et al.*, 2008) and proliferation (Vijayakumar *et al.*, 2011). Additionally,

evidence suggests an underlying causal role of Wnt/ β-catenin in the resistance to chemotherapy (Chau *et al.*, 2013; Flahaut *et al.*, 2009).

Hyperactivity of the Wnt/β-catenin signaling pathway is established as being causative of tumor development in some types of human malignancies, due to mutations hindering key molecular elements regulating the signaling cascade. This is best exemplified in colon cancer [APC mutations (Fodde *et al.*, 2001)] and in hepatoblastoma, bladder and prostate cancer [β-catenin mutations (Ahmad *et al.*, 2011; Jiang *et al.*, 2013; Cairo *et al.*, 2008)]. However, the activation/inactivation status of Wnt/β-catenin in osteosarcoma has been subject of debate and no definitive causal relationship has been established so far. Indeed, some reports provide evidence for an abnormal activation of Wnt/β-catenin pathway in osteosarcoma samples, based on the detection of Wnt ligands, LRP5/6 co-receptors or cytoplasmic β-catenin staining (Vijayakumar *et al.*, 2011). Contradictory to these findings, previous results from our group suggest that the Wnt/β-catenin signaling is down-regulated in osteosarcoma biopsy samples compared to normal osteoblasts, by evaluating nuclear β-catenin expression, the hallmark of canonical Wnt signaling activation (Cai *et al.*, 2010), and by the lack of reporter gene activation in multiple osteosarcoma cell lines (Guo *et al.*, 2008; Cai *et al.*, 2010). Also results from Matushansky *et al.* provide evidence for a down-regulation of Wnt/β-catenin signaling in several human sarcomas (Matushansky *et al.*, 2007). Subsequent studies from our group demonstrated that Wnt/β-catenin is specifically activated in the osteosarcoma stem cell subpopulation, but not in their differentiated counterparts (**Chapter 2**). Moreover, conventional chemotherapeutic drugs used in the treatment of osteosarcoma induced stemness properties in differentiated cells through activation of the Wnt/β-catenin pathway leading to expansion and survival of stem-like cells (**Chapter 4**). Importantly, data extracted from the public R2 database (Kuijjer *et al.*, 2013; Buddingh *et al.*, 2011) revealed that high expression levels of Wnt target genes (*CTNNB1* (β-catenin) *DKK-1* and *MYC*) correlated with poor overall survival and a poor therapeutic response in osteosarcoma patients. Based on these findings, we hypothesized that the Wnt/β-catenin pathway plays an essential regulatory role in self-renewal and survival of CSCs and that it might be a promising therapeutic target for a selective eradication of stem-like cells in osteosarcoma.

In the present study, we demonstrated that inhibition of Wnt/β-catenin using a tankyrase inhibitor (IWR-1) exerted a selective inhibitory effect in stemness properties, self-renewal and survival of osteosarcoma CSCs, and repressed tumor growth in a

xenograft mouse model. Moreover the suppression of Wnt/β-catenin activity in CSCs synergized and improved the efficacy of doxorubicin in tumor abrogation. Collectively, these data suggest that Wnt/β-catenin signaling is a potential therapeutic target for osteosarcoma and offer a pre-clinical proof-of-concept for the use of conventional chemotherapy combined with specific targeting of this signaling pathway in the clinical setting.

3.3 Material and Methods

3.3.1 Ethical statement

Human bone-marrow-derived mesenchymal stem cells (MSCs) were collected from healthy donors and handled according to the ethical guidelines of the national organization of scientific societies, as previously reported (Cleton-Jansen *et al.*, 2009). Animal studies were conducted at the University of Coimbra in an accredited facility, complying with the local and international guidelines on animal welfare and experimentation (Workman *et al.*, 2010). Research protocols were approved by the Institutional Ethics Committee of the Faculty of Medicine of University of Coimbra for animal care and use (Approval ID:38-CE-2011).

3.3.2 Cell culture and sphere formation assay

Osteosarcoma cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD) (MG-63, MNNG-HOS and SJSA-1) and cultured in RPMI-1640 medium (Invitrogen Life Technologies, Karlsruhe, Germany) supplemented with 10% v/v heat-inactivated fetal bovine serum (FBS; Invitrogen) and 1% v/v penicillin/streptomycin (Invitrogen). Cells were maintained under standard adherent conditions at 37°C in a humidified incubator with 5% CO_2 and 95% air. Mycoplasm contamination was screened using a genetic-based tool (van Kuppeveld *et al.*, 1992). Cell line authentication was performed by short tandem repeats (STR) DNA profiling using Cell ID™ System (Promega Corp., Madison, WI, USA) and compared with profiles of ATCC (**Table 3.1**).

Isolation of cancer stem-like cells from osteosarcoma cell lines MG-63, MNNG-HOS and SJSA-1 was performed using the sphere assay and reagents as previously described (Martins-Neves *et al.*, 2012). Self-renewal ability was evaluated by plating primary spheres in serum-free medium, after sphere dissociation with accutase. Efficiency of

sphere formation was estimated based on the total number of spheres formed divided by the total number of cells initially plated.

Table 3.1. DNA fingerprint analysis of polymorphic short tandem repeat (STR) sequences performed on osteosarcoma cell lines used in this study.

Cell line	AMY	VWA	TH01	D21S11	D13S317	CSF1PO	D7S820	TPOX	D16S539	D5S818
MG-63	XY	14-18	6-9.3	31-31	13-13	12-13	11-12	11-11	11-12	8-11
MNNG-HOS	XX	18-18	9-9	31.2-31.2	12-12	12-12	11-12	8-11	10-13	13-13
SJSA-1	XY	19-19	6-6	29-30.2	12-12	11-11	10-10	8-9	9-10	11-11

Note: Results of STR profiling were compared to ATCC databases for each cell line.

3.3.3 Cell treatment and viability assays

Small molecule compound IWR-1 (tankyrase inhibitor) and SB216763 (GSK3 inhibitor) were obtained from Sigma-Aldrich® (Zwijndrecht, Netherlands). Aliquots of 2mM in dimethylsulfoxide (DMSO) were stored at -20°C and working concentrations prepared freshly prior to use. Human Wnt3A peptide (ab23327, Abcam®, Cambridge, UK) was diluted in sterile saline solution (PBS) and used at a final concentration of 100ng/mL. Stock solutions of doxorubicin (hydroxydaunorubicin, LUMC Pharmacy) were stored at 4°C protected from light, and working dilutions were prepared in PBS, immediately before use.

Wnt/β-catenin inhibition was achieved using the small molecule IWR-1, which mediates disruption of Wnt activity by stabilizing the levels of Axin proteins (Chen *et al.*, 2009) via tankyrase inhibition (Huang *et al.*, 2009). Cells were dissociated, plated in 96-well plates (5,000 cells/well) and allowed to attach overnight. Parental cells and spheres were screened for their profile of sensitivity to IWR-1, by treatment with increasing concentrations of compound (2.5, 5, 7.5 and 10μM) for 48h. Afterwards, 10μL of WST-1 Cell Proliferation Reagent (Roche Diagnostics Netherlands B.V., 1:10 dilution) were added to each well, to estimate the number of metabolically viable cells remaining in culture after drug treatment. Quantification of the water-soluble formazan product, formed by WST-1 mitochondrial conversion in viable cells, was performed in a microplate reader operating in colorimetric mode (Perkin Elmer Victor 3 Model 1420-012 multi-label microplate reader). Cellular growth inhibition was calculated by dividing the absorbance of drug-treated cells by that of control untreated wells.

To examine whether IWR-1 could overcome the resistance profile of osteosarcoma spheres to doxorubicin, we pre-treated both parental cells and spheres with IWR-1 10μM or 0.5% DMSO, for 48h. Afterwards, cells were incubated with increasing concentrations of doxorubicin (0-100μM), in the presence of IWR-1 or DMSO for further 48h. Cell viability was estimated with WST-1 assay as previously described and data normalized to absorbance of untreated cells.

To test synergistic or additive effects of Wnt inhibition and doxorubicin cytotoxicity towards tumor cells, spheres were treated with increasing doses of IWR-1 (2.5-10μM) combined with increasing doses of doxorubicin (0.25-1μM), for 48h. Combination index (CI) values were estimated using the algorithms proposed by Chou and Talalay in the median-effect principle (Chou, 2010), implemented in the CompuSyn software (version 1.0 CompuSyn, Inc., Paramus, NJ). In this analysis, a CI < 1 indicates synergistic, CI = 1 additive and CI > 1 antagonistic interactions between doxorubicin and IWR-1.

3.3.4 Cell transfection

In order to check the effects of treatments in TCF/LEF transcriptional activation, MNNG-HOS, MG-63 and SJSA-1 cells were transfected with pGL4.49 luciferase reporter vector [Luc2P/TCF-LEF RE/Hygro] (Promega) using FuGENE® HD (Promega) followed by clonal selection as described in **Chapter 2, section 2.3.4**. This vector contains eight copies of a TCF-LEF response element that drives transcription of the firefly luciferase reporter gene Luc2P, and can be used as a reporter system for monitoring the activation of β-catenin triggered by stimuli treatment. Luciferase activity was measured with IVIS® Lumina XR (Caliper Life Sciences Inc., PerkinElmer, Massachusetts, USA) using D-luciferin (30 mg/mL, Caliper) as a substrate.

3.3.5 Analysis of mRNA expression

Osteosarcoma cells were plated in 6-well plates and treated with DMSO or IWR-1 (10 μM) for 96h. Cells were then dissociated, washed twice with HBSS and maintained at -20°C, until total RNA extraction using TRIzol® reagent (Invitrogen), according to the manufacturer's protocol. TRIzol® was also used to extract total RNA from homogenized tumor tissues excised from untreated and animals treated with doxorubicin, IWR-1 and the combination after 15 days. cDNA synthesis from purified RNA and qRT-PCR reactions were performed as described in **section 2.3.9, Chapter 2** using appropriate

housekeeping genes. Primers used on qRT-PCR reaction were previously validated and are listed in **Table 3.2**.

Table 3.2. Sequences of primers used in this study.

Gene	Forward (5'-3')	Reverse (5'-3')
LRP5 (Vijayakumar *et al.*, 2011)	ACAACGGCAGGACGTGTAAG	AGCACGATGTCGGTGAAG
LRP6 (Vijayakumar *et al.*, 2011)	AGGGTGGAATGAATGTGCTT	TGATGGCACTCTTTTGACTGA
AXIN2 (Cai *et al.*, 2010)	GAATGAAGAAGAGGAGTG	AAGACATAGCCAGAACC
DKK-1 (Cai *et al.*, 2010)	CCTTGGATGGGTATTCCAGA	CCTGAGGCACAGTCTGATGA
ALDH1A1 (Feldmann *et al.*, 2008)	CTGGTTATGGGCCTACAGCA	ATTGTCCAAGTCGGCATCAG
ALDH2 (van den Hoogen *et al.*, 2010)	CGAGGTCTTCTGCAACCAG	GCCTTGTCCACATCTTCCTT
ALDH7A1 (van den Hoogen *et al.*, 2010)	CAACGAGCCAATAGCAAGAG	GCATCGCCAATCTGTCTTAC
NANOG (Chapter 2)	GATGCCTCACACGGAGACTG	GCAGAAGTGGGTTGTTTGCC
OCT4 (Chapter 2)	TCTGCATCCCTTGGATGTGC	GTGTGGCCCCAAGGAATAGT
SOX2 (Park *et al.*, 2012)	CATGCACCGCTACGACG	CGGACTTGACCACCGAAC
RUNX2 (Chapter 2)	CAGAACCCACGGCCCTCCCT	CCCAGTGCCCCGTGTGGAAG
SPARC (Chapter 2)	CCAGCCCCCATTGGCGAGTT	TGCGCAGGGGGAATTCGGTC
GLI1 (Hameetman *et al.*, 2006)	TGCAGTAAAGCCTTCAGCAATG	TTTTCGCAGCGAGCTAGGAT
GLI2 (Hameetman *et al.*, 2006)	TTCTCCAACGCCTCGGAC	GTGGACCGTTTTCACATGCTT
PTCH1 (Hameetman *et al.*, 2006)	CCACGACAAAGCCGACTACAT	GCTGCAGATGGTCCTTACTTTTTC
SMO (Hameetman *et al.*, 2006)	AGCGCAGCTTCCGGG	CAGTTCCAAACATGGCAAACAG
CAPNS1	ATGGTTTTGGCATTGACACATG	GCTTGCCTGTGGTGTCGC
SRPR	CATTGCTTTTGCACGTAACCAA	ATTGTCTTGCATGCGGCC
TBP	CACGAACCACGGCACTGATT	TTTTCTTGCTGCCAGTCTGGAC

Note: *CAPNS1*: calpain, small subunit 1; *SRPR*: signal recognition particle receptor; *TBP*: TATA Box-binding protein.

3.3.6 Real-time measurement of cell proliferation

To analyze whether Wnt/β-inhibition affected cell proliferation and adhesion, we conducted a real-time measurement of cell index values based on cellular impedance, which were continuously monitored using the xCELLigence™ Real-Time Cell Analyzer (RTCA DP). Cell index was programmed to be measured every 15 min. Cells were firstly treated with DMSO or IWR-1 for 48h, enzymatically dissociated and then plated in electronic microtiter E-plate 16 (ACEA Biosciences, Inc., San Diego, CA, USA) at a total of 7,000 cells/well, for another 30h in the presence of compounds. Cells were maintained at 37°C during all the experiments. Data was acquired and analyzed using the RTCA software 1.2.1 (Roche Diagnostics).

3.3.7 Caspase 3/7 and TUNEL assays

Activation of executioner caspases 3/7 was estimated using the Caspase-Glo® 3/7 Assay System (Promega) according to the manufacturer instructions. Cells were plated in black 96-well plates (50,000 cell/mL) and treated with DMSO or 10μM IWR-1 for 96h. Bioluminescent signals from conversion of aminoluciferin by apoptotic cells were measured using a microplate reader (Synergy™ HT, Biotek Instruments, Winooski, VT) operating in luminescence mode. Raw data was collected as relative lights units.

For TUNEL assay, cells were treated with 10μM IWR-1 for 96h, collected as single-cell suspensions and fixed with paraformaldehyde 4%. After a washing step, cells were disposed in glass slides using the Tharmac Cellspin I Cytocentrifuge (Tharmac GmbH, Germany). Apoptotic cells were detected based on terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL staining) performed using the DeadEnd™ Fluorometric TUNEL System (Promega), following the manufacturer protocol. Images were acquired on a LSM 710 confocal microscope (Carl Zeiss MicroImaging, GmbH, Germany) using the Plan-Apochromat 20x/0.8 M27 objective and ZEN 2009 software. Three random fields were acquired per condition and cells manually counted using ImageJ software. Average percentage of TUNEL-positive cells was estimated by dividing the number of green stained cells (TUNEL-positive) by the total number of cells (estimated based on cell nuclei staining using 4',6-Diamidino-2-Phenylindole).

3.3.8 Cell cycle analysis

For cell cycle phase distribution, cells were stained with a propidium iodide solution (50 μg/mL) containing 10μg/mL RNase A, followed by flow cytometry analysis as previously described (Martins-Neves *et al.*, 2012).

3.3.9 Western blot analysis

Total cell extracts were prepared with a standard cell lysis buffer, separated and electro transferred, as previously described (Gonçalves *et al.*, 2015). Nuclear lysates were also prepared as described in **section 4.3.4, Chapter 4**. Blocked membranes were incubated with primary antibodies, overnight at 4°C according to the conditions specified in **Table 3.3**. Appropriate peroxidase–conjugated secondary antibodies were incubated at room temperature for 2h and proteins visualized by chemifluorescence (ECF™ Western Blotting Reagent Pack, GE Healthcare Life Sciences, Pittsburg, PA) using Typhoon™ FLA 9000 biomolecular imaging system (GE Healthcare). Quantification of protein bands was assessed by densitometry calculation using ImageJ software (National Institutes of Health, Bethesda, MD, USA) and normalized to β-actin or Lamin A, which were used as protein loading controls.

Table 3.3. Antibodies used in western blotting and respective protocol conditions.

Antibody	Incubation solution	Dilution	Band size (kDa)	Source	Company
Axin2	TBS-T 5% milk	1:500	95-98	Rabbit	Cell Signaling Technology, Danvers, MA, USA, #2151
Bak	TBS-T 1% milk	1:200	28	Rabbit	Santa Cruz Biotechnology, Inc. Heidelberg, Germany, Sc-832
Bcl-2	TBS-T 1% milk	1:200	26	Mouse	Santa Cruz Biotechnology, Inc. Heidelberg, Germany, Sc-7382
BCRP	TBS-T 1% milk	1:250	70	Mouse	clone BXP-21, Chemicon Int., Temecula, CA, USA
c-Myc	TBS-T 1% milk	1:1,000	57	Rabbit	Epitomics, Inc., Burlingame, California (clone N-term Y69) #1472-1
Cyclin D1	TBS-T 1% milk	1:1,000	36	Rabbit	Thermo Fisher Scientific, Fremont, CA, USA (clone SP4) #RM-9104
Lamin A	TBS-T 5% milk	1:1,000	70	Rabbit	Cell Signaling Technology, Danvers, #2032
Pgp	TBS-T 1% milk	1:100	170	Mouse	clone C219, Calbiochem®, Millipore, Germany
Sox2	TBS-T 5% BSA	1:1,000	35	Rabbit	Cell Signaling Technology, Danvers, (clone D6D9) #3579
β-actin	TBS-T 1% milk	1:5,000	43	Mouse	Sigma
β-catenin	TBS-T 1% milk	1:1,000	92	Mouse	BD Transduction Laboratories™, cat. 610153

Note: BSA, bovine serum albumin; TBS-T, Tris-buffered saline/Tween-20.

3.3.10 *In vivo* studies

Immunocompromised nude mice (n=12, 6-week old female Swiss nude) were obtained from Charles River Laboratories and housed in pathogen-free facility. pGL4-transfected MNNG-HOS cells ($2x10^6$ cells/100µL PBS) were injected subcutaneously in the left flank of the animals. Body weight and the tumor growth were monitored twice a week using caliper measurements of length (L) and width (W) and tumor volumes were calculated using the formula $(L \times W^2) \times 0.5$. Treatments started when tumor volumes reached on average 62.5 mm^3 (5x5mm). IWR-1 was formulated in DMSO and was administered intratumorally (5mg/kg) each 2 days, for 12 days. Doxorubicin was administered intraperitoneally (8mg/kg) at each 4 days, for the same time-period. Control animals were treated with vehicle following a similar administration schedule. At the end of the treatments, on day 15, mice were euthanized by cervical dislocation and tumors immediately collected for gene expression analysis and formalin fixation.

3.3.11 Immunohistochemical staining (IHC)

Mouse xenograft tumors derived from pGL4-MNNG-HOS cells were excised, formalin-fixed and embedded in paraffin. Antigen-retrieved tissue sections were stained overnight with anti-β-catenin and anti-Sox2 antibodies following procedures previously described in **section 2.3.6, Chapter 2**. Normal tonsil was used as a positive control for both antibodies.

3.3.12 Statistical analysis

Graphics were computed with GraphPad Prism version 5.04 (GraphPad Software, San Diego, CA). Statistical analyses were performed using SPSS Statistics version 20.0 (IBM Corporation, New York, USA). Significance was set at the level of $p<0.05$.

3.4 RESULTS

3.4.1 Growth inhibitory effect of IWR-1 in osteosarcoma stem-like cells displaying activated Wnt/β-catenin

We reported previously that Wnt/β-catenin signaling was specifically active in osteosarcoma stem-like cell populations (**Figure 3.1A**), as evidenced by increased nuclear β-catenin, TCF/LEF transcriptional activity, *AXIN2* expression and down-regulation of *DKK-1* Wnt antagonist (**Chapter 2**). In the present report, we extended the characterization of this signaling pathway to the mRNA expression of the Wnt signaling co-receptors *LRP5* and *LRP6* (MacDonald and He, 2012). We found a significantly increased expression of both receptors in spheres compared to parental cells (**Figure 3.1B**). Interestingly, the magnitude of fold-difference in the expression of *LRP5* was higher than that observed for *LRP6*, suggesting a more prominent role of LRP5 in stemness maintenance in osteosarcoma.

To investigate the biological effects of inhibiting Wnt/β-catenin signaling, we treated both parental cells and spheres (MG-63, MNNG-HOS and SJSA-1) with the tankyrase inhibitor IWR-1. Cells were exposed to increasing doses of IWR-1 (2.5-10μM) for 48 and 96h. IWR-1 was effective in reducing sphere viability in a concentration- and time-dependent manner. Reduction of cell viability was evident in spheres already at 48h and was more significantly pronounced at 96h for concentrations higher than 5μM, relatively to DMSO-treated cells. Indeed, at 96h, 10μM of IWR-1 elicited more than 70% reduction of cell viability in spheres, for the three cell lines analyzed (**Figure 3.1C**). In contrast, parental cells showed only minimal response to IWR-1. Also in MSCs, which were used as normal control cells and have been shown to display constitutively active Wnt/β-catenin contributing to their stemness maintenance (Etheridge *et al.*, 2004; Ling *et al.*, 2009), cell viability was not compromised after treatment with increasing dosages of IWR-1 for 96h (**Figure 3.1D**). These results indicate that Wnt/β-catenin is constitutively activated in and is essential for self-renewal of osteosarcoma stem-like cells, and can be inhibited using IWR-1 at concentrations that spare normal progenitor cells such as MSCs denoting active Wnt/β-catenin signaling. We also observed that Wnt/β-catenin activation using the GSK-3 inhibitor SB216763 resulted in increased cell viability overtime in spheres (**Figure 3.2**). These effects were not so pronounced in parental cells and in MSCs, further reinforcing the specific activity of Wnt/β-catenin in osteosarcoma stem-like cells.

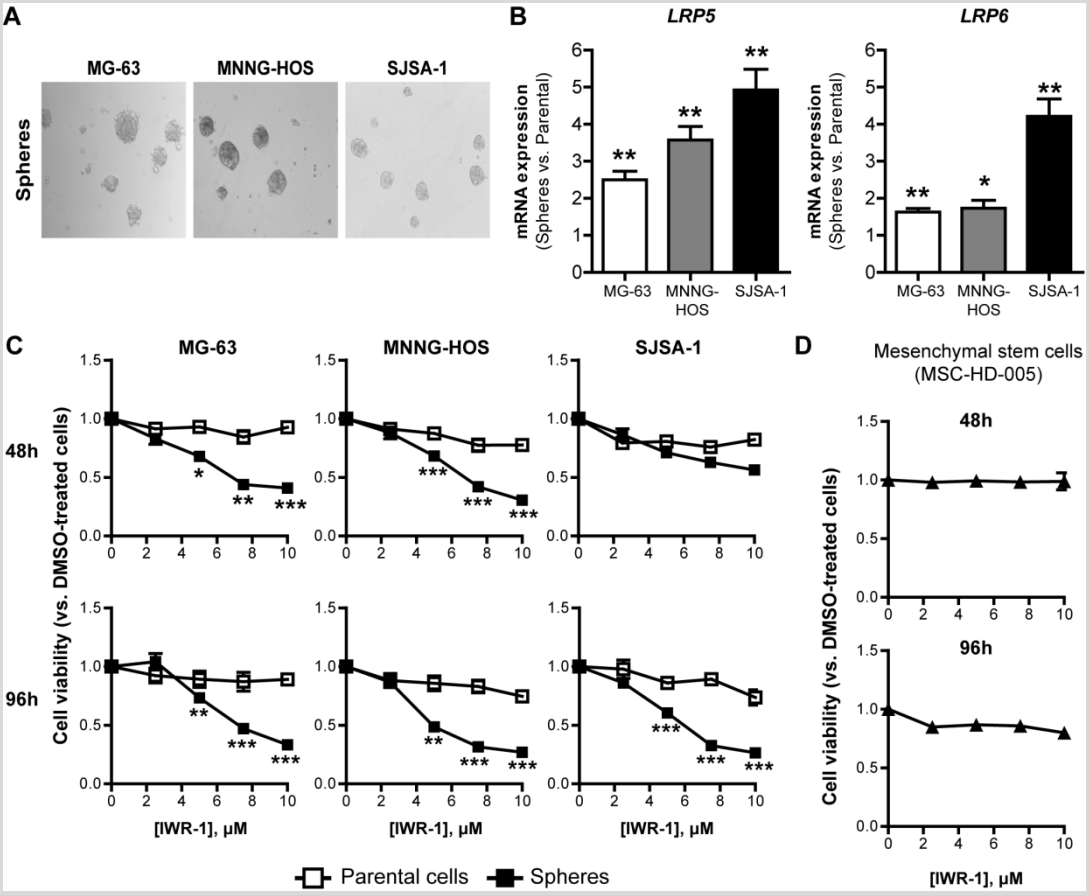

Figure 3.1. Wnt/β-catenin inhibition using IWR-1 has a cytotoxic effect in osteosarcoma spheres. A. Morphological appearance of MG-63, MNNG-HOS and SJSA-1 spheres. **B.** Expression of *LRP5* and *LRP6* Wnt receptors is increased in spheres, compared to parental cells. Gene expression levels were determined based on absolute Cq values, after normalization to three housekeeping genes. **C.** Effects of IWR-1 in the viability of spheres and parental cells, and in human mesenchymal stem cells (**D.**). Cell viability was measured using the WST-1 assay and data normalized to absorbance of DMSO-treated cells. Points represent mean ± SEM of three independent observations. *p≤0.05, **p≤0.01, ***p≤0.001 significantly different from parental or untreated cells (independent samples t-test).

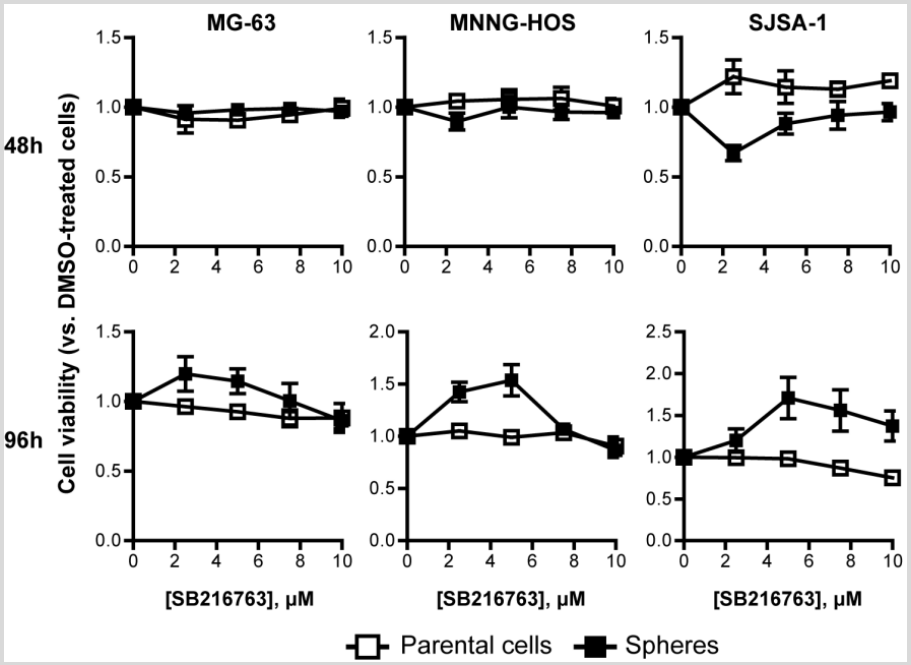

Figure 3.2. Canonical Wnt activation (through GSK3 inhibition) using SB216763 promotes survival and expansion of osteosarcoma spheres. Spheres' viability augments in the presence of SB216763, in comparison to untreated cells, at 96h of treatment. Activation of canonical Wnt during this time period did not significantly alter parental cell survival and proliferation. Cell viability was measured using the WST-1 assay and data normalized to absorbance of DMSO-treated cells.

β-catenin is also actively involved in mediating the interaction of adherens junctions, key molecular structures supporting cell adhesion (Gumbiner, 2005). To explore if IWR-1 would affect osteosarcoma cells at the level of adhesion and proliferation, we monitored both parental cells and dissociated spheres using the xCELLigence™ system, which estimates a cell index measurement based on the number and morphology of cells, and also strength of cellular adhesion. We observed that IWR-1 treatment was associated with decreased cell adhesion in spheres and compromised cell proliferation overtime. These effects were less pronounced in parental cells, in line with the fewer effects observed in cell viability, as compared to spheres (**Figure 3.3**). Based on these results, IWR-1 was used at a concentration of 10µM in subsequent studies.

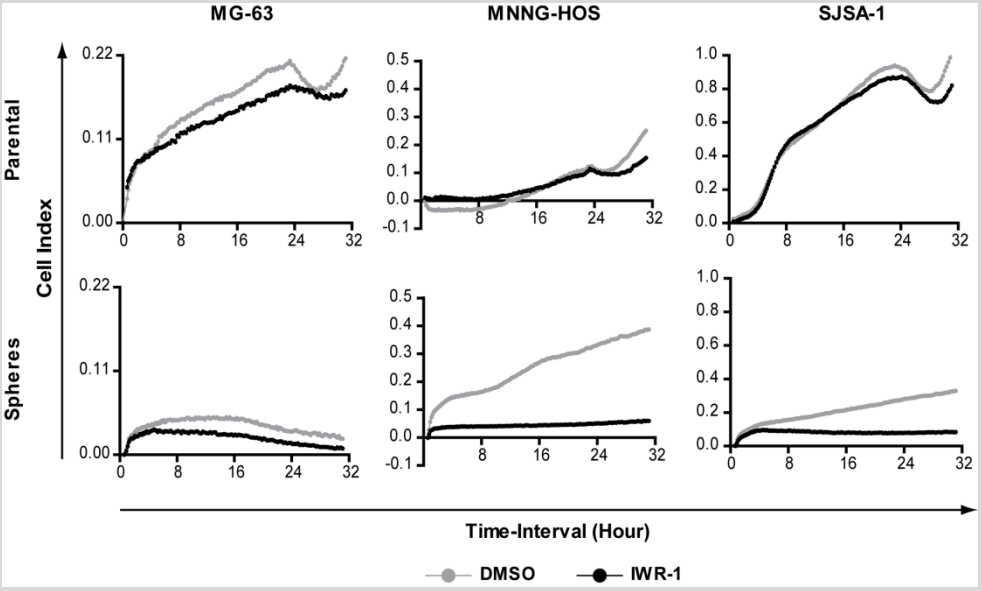

Figure 3.3. Real-time analysis of proliferation, viability and adhesion of osteosarcoma parental cells and spheres. Cells were treated with DMSO or IWR-1 for 48h, dissociated and then plated in E-plate 16 for another 30h in the presence of compounds. Data shows that IWR-1 abrogated sphere adhesion (during the first 8h) and proliferation (remaining time-period). This effect was less evident in parental cells.

3.4.2 Wnt/β-catenin inhibition is lethal to osteosarcoma CSCs via apoptotic-dependent mechanisms

Wnt/β-catenin inhibition using IWR-1 elicited specific harmful effects in viability of osteosarcoma sphere-forming cells (**Figure 3.1**). Apoptotic events depending on mitochondrial changes culminate in the activation of the downstream effector caspases 3 and 7 (Lakhani *et al.*, 2006). Accordingly, the activation of caspases 3/7 was significantly increased in spheres following IWR-1 treatment, reaching 2.15-, 1.27- and 3.23-fold differences relatively to DMSO-treated cells, in MG-63, MNNG-HOS and SJSA-1 spheres respectively. These effects were slightly negligible in parental cells (**Figure 3.4A**).

TUNEL staining was also used to identify DNA fragmentation, an important morphological change occurring during the late phases of apoptosis (**Figure 3.4B**). We detected a significantly increased number of TUNEL-positive cells upon IWR-1 treatment for 96h (**Figure 3.4C**), reaching 4.65-, 15.83- and 7.75-fold differences relatively to DMSO-treated cells, in MG-63, MNNG-HOS and SJSA-1 spheres respectively.

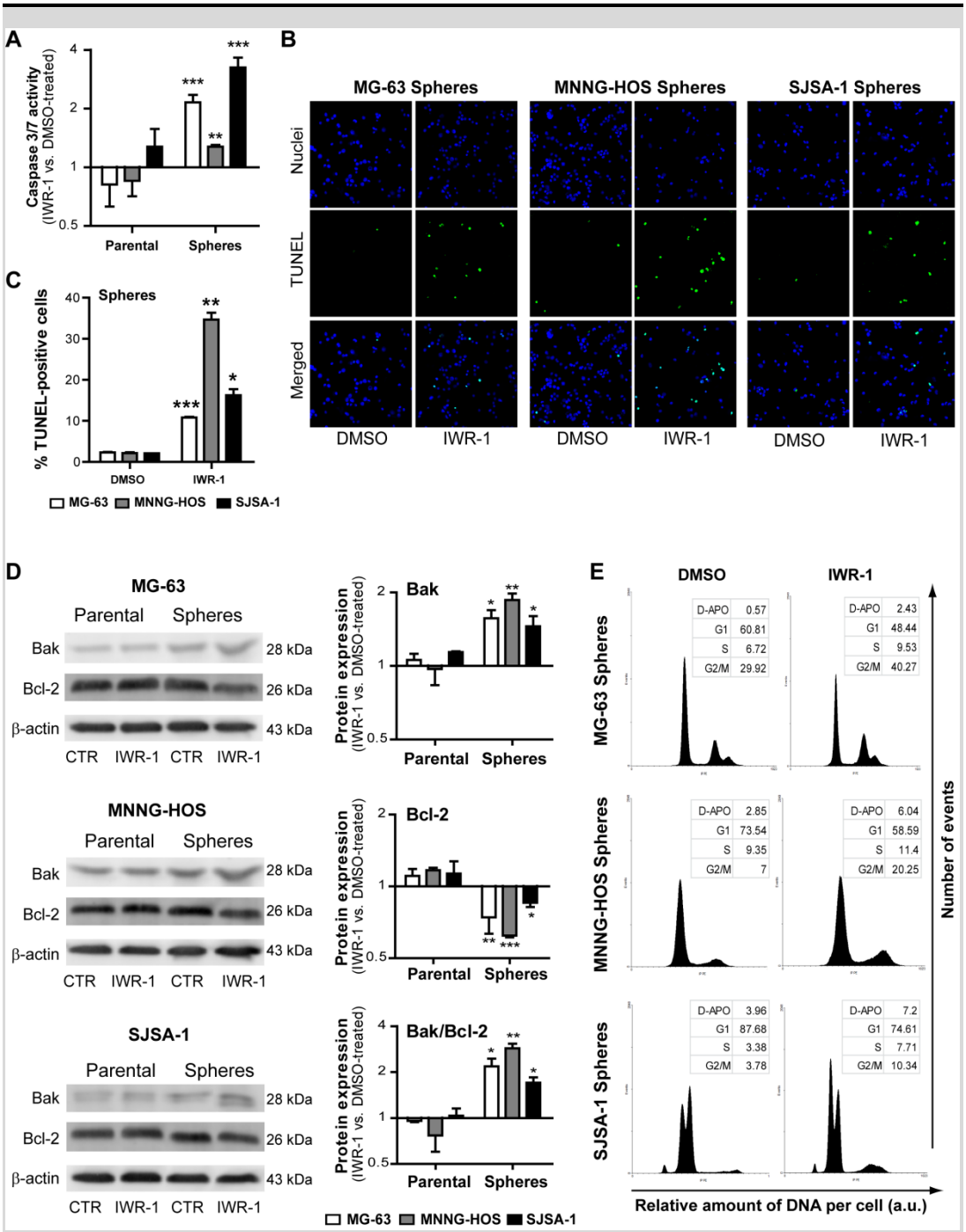

Figure 3.4. Wnt/β-catenin inhibition elicits apoptotic cell death in osteosarcoma spheres. **A.** Caspase 3/7 activity measured using the Caspase-Glo assay after IWR-1 treatment in spheres and parental cells, compared to DMSO-treated cells. Data represents relative light units (RLU). **B.** Representative cytofluorimetric images of TUNEL-stained spheres, showing high numbers of TUNEL-positive cells in IWR-1-treated cells. Cells were stained with TUNEL (green) and 4',6-diamidino-2-phenylindole (nuclei, blue) after 96h exposure to the compound. All images were taken at 20x magnification. **C.** Analysis of DNA fragmentation by TUNEL assay shows increased apoptotic cell death in spheres, upon IWR-1 treatment for 96h. Data represents mean ± SEM percentage of TUNEL-positive cells over total cell number (based on nuclei staining with 4',6-

diamidino-2-phenylindole). **D.** Western blot analysis of Bcl-2 and Bak in parental and sphere-forming cells upon treatment with 10 μM IWR-1 for 96h. The blots used in the figure are cropped. All the gels have been run under the same experimental conditions. **E.** Analysis of cell cycle alterations after IWR-1 treatment for 48h, using flow cytometry. A cell arrest effect in S-G2/M phases is observed in IWR-1-treated spheres. In **A.**, **C.** and **D.** *p≤0.05, **p≤0.01, ***p≤0.001 compared to DMSO-treated cells (at least N=3, independent samples t-test or Mann-Whitney test, after Shapiro-Wilk test for normality assessment). Abbreviation: *D-APO, debris-apoptotic cells*.

Additionally, Western blot analysis of key proteins involved in mitochondrial-dependent apoptosis showed a significant up-regulation of Bak and a down-regulation of Bcl-2 in spheres, after 10μM IWR-1 exposure for 96h (**Figure 3.4D**), leading to a Bak/Bcl-2 ratio >1 that prone cells to undergo apoptosis. Expression levels of these proteins did not change considerably in parental cells submitted to the same treatment schedule.

We also examined whether IWR-1 induced alterations in cell cycle in osteosarcoma CSCs. Treatment with 10μM IWR-1 for 48h induced a cell cycle arrest in the S phase in all sphere-derived cells, compared to DMSO-treated cells (MG-63: 9.53% *vs*. 6.72%; MNNG-HOS: 11.4% *vs.* 9.35%; SJSA-1: 7.71% *vs.* 3.38%) that was accompanied by a correspondent reduction of cells in the G1 phase (MG-63: 60.81% to 48.44%, MNNG-HOS: 73.54% to 58.59%; SJSA-1: 87.68% to 74.61%, **Figure 3.4E**). The percentage of cells in the G2/M phase was also increased in all IWR-1-treated spheres (MG-63, MNNG-HOS and SJSA-1 spheres - 40.27%, 20.25% and 10.34%, respectively) compared with the corresponding untreated cells (29.92%, 7% and 3.78%, respectively). The percentage of cells in the sub-G1 phase, which correspond to apoptotic cells, also increased in all IWR-1 treated cells. Altogether these data showed that IWR-1 inhibited the proliferation of CSCs by inducing cell cycle arrest and ultimately apoptosis.

3.4.3 IWR-1 down-regulates Wnt/β-catenin signaling activity in osteosarcoma CSCs

In order to test whether IWR-1 could selectively inhibit Wnt/β-catenin signaling, we analyzed the expression of β-catenin in nuclear, cytoplasmic and total protein extracts, in order to address at which cellular level IWR-1 elicited its specific effects. Treatment with IWR-1 induced a substantial decrease in nuclear β-catenin levels by 59%, 38% and 76% in MG-63, MNNG-HOS and SJSA-1 spheres, respectively. We also observed a reduction in nuclear β-catenin in MNNG-HOS and SJSA-1 parental cells, although less pronounced in comparison to corresponding spheres. The cytoplasmic levels increased in MNNG-HOS and SJSA-1 derived spheres and remained almost constant in parental cells (**Figure 3.5A**). Analysis of total β-catenin revealed an overall reduction in protein levels in IWR-1-treated spheres compared to DMSO-treated cells. In contrast, no significant variations of total β-catenin levels were observed in IWR-1-treated parental cells, compared to controls (**Figure 3.5A**). The calculation of the nuclear/cytoplasmic β-catenin ratios (**Figure 3.5A, lowest panel**) confirmed the preferential accumulation of β-catenin within the nuclear compartment of MG-63 and MNNG-HOS spheres, as indicated by the higher ratios against parental cells (MG-63: 6.10 *vs*. 1.27; MNNG-HOS: 10.70 *vs*. 3.46). Despite that this trend was not observed in SJSA-1 spheres (2.58 *vs*. 3.15), the treatment with IWR-1 led to a clear decrease in the nuclear/cytoplasmic β-catenin ratios in all sphere-derived cell populations, remaining nearly unaltered in parental cells. These results demonstrate the preferential effects of IWR-1 in the β-catenin redistribution in osteosarcoma spheres.

Additionally, analysis of mRNA expression data revealed a consistent down-regulation of the Wnt target gene *AXIN2*, by at least 50% and of the Wnt co-receptors *LRP5* and *LRP6* in all IWR-1-treated spheres compared to DMSO-treated cells. Regarding the Wnt-antagonist *DKK-1* a trend was observed towards a down-regulation in either IWR1-treated parental cells or spheres, with the exception of SJSA-1 spheres (**Figure 3.5B**).

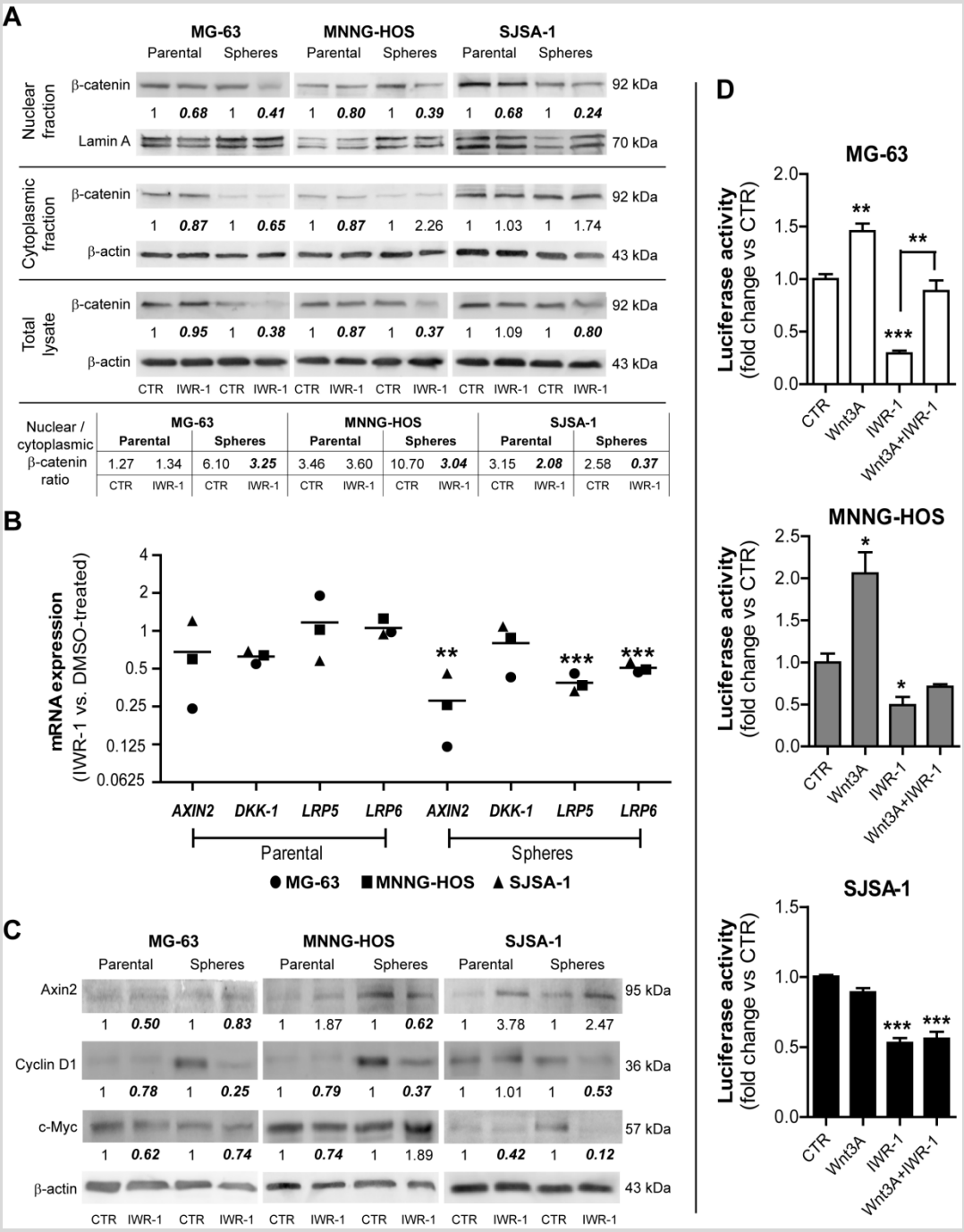

Figure 3.5. Axin stabilization (via tankyrase inhibition) with IWR-1 leads to decreased Wnt/β-catenin signaling in osteosarcoma cells. A. Western blot analysis of β-catenin protein redistribution in different cellular compartments, upon IWR-1 treatment for 48h. Numbers below protein bands represent relative protein expression in IWR-1 *versus* DMSO-treated cells after normalization of protein expression to Lamin A or β-actin (loading controls for the nuclear and cytoplasmic fractions, respectively). Lower panel represents the ratio of nuclear/cytoplasmic β-catenin after protein normalization to respective loading controls. **B.** Expression of the Wnt target genes *AXIN2* and *DKK-1*, and *LRP5*/*LRP6* receptors analyzed by quantitative RT-PCR in parental cells and spheres treated with 10μM IWR-1 for 96h. **C.** Western blot analysis of Axin2, cyclin D1

and c-Myc protein levels in parental cells and spheres, after IWR-1 treatment for 96h. Numbers below protein bands represent relative protein expression in IWR-1 *versus* DMSO-treated cells after normalization to β-actin (loading control). In **A.** and **C.** bold-italic characters indicate decreased expression compared to control (DMSO) cells (set at 1). The blots used in the figure are cropped. All the gels have been run under the same experimental conditions. **D.** Luciferase reporter assays using pGL4.49[luc2P/TCF-LEF RE/Hygro] reporter plasmid, in osteosarcoma CSCs treated with 100 ng/mL Wnt3A, 10μM IWR-1 or the combination for 24h. Bar graphs represent fold change luciferase activity ± SEM *versus* DMSO-treated cells. . In **B.** and **D.** *p<0.05, **p<0.01, ***p<0.001 compared to respective control cells (N=3, independent samples t-test).

We then analyzed the protein expression levels of Axin2 and of other Wnt signaling β-catenin/TCF downstream targets such as Cyclin D1 and c-Myc, upon IWR-1 treatment for 96h (**Figure 3.5C**). All of them diminished in spheres, with the exception of c-Myc in MNNG-HOS spheres and Axin2 in SJSA-1 spheres, respectively. The down regulation of the cell cycle regulator Cyclin D1 in IWR-1 treated spheres might explain the S and G2/M cell cycle arrest and suppression of cell proliferation, which we observed previously (Figure 3.4**E**). The effects of IWR-1 in parental cells were not as consistent as for spheres, and displayed a mixed-up and down-regulated expression pattern.

To further investigate that Wnt signaling was being suppressed by tankyrase inhibition, and explore whether it could be reactivated in the presence of Wnt3A, a known Wnt/β-catenin activator ligand, we tested MG-63, MNNG-HOS and SJSA-1 spheres with a commercial Wnt reporter containing eight copies of a TCF/LEF response element. The reporter showed a significant increase in luciferase activity in MG-63 and MNNG-HOS cells, but not in SJSA-1 cells, after the addition of Wnt3A alone (100ng/mL). As expected, treatment with IWR-1 alone (10μM) diminished significantly the reporter activity in all the cells. Moreover, IWR-1 in combination with Wnt3A prevented the activation of the Wnt signaling induced by Wnt3A alone, especially in MNNG-HOS cells (**Figure 3.5D**).

3.4.4 Disruption of Wnt/β-catenin signaling impairs osteosarcoma stemness-related traits

Wnt/β-catenin signaling is known to play a prominent role in regulating stem cell traits (Reya and Clevers, 2005). To examine if IWR-1 repressed the stemness properties in osteosarcoma, we used the sphere-forming efficiency and the Aldefluor™ assay as

functional readouts for stem-like cells. Despite that IWR-1 did not prevent first generation sphere formation, treatment of 7-days old first-generation spheres resulted in a significant reduction of secondary sphere-forming efficiency and also spheres size, showing the impairment of self-renewal ability. Overall, IWR-1 inhibited secondary sphere-forming efficiency by approximately 53%, 55% and 44% in MG-63, MNNG-HOS and SJSA-1 cells, respectively (**Figure 3.6A**). Moreover, treatment with IWR-1 diminished the activity of the putative CSC marker Aldefluor™ (**Figure 3.6B, left panel**), an effect that was accompanied by a tendency for a decreased expression of their associated isozymes *ALDH1A1*, *ALDH2* and *ALDH7A1* (**Figure 3.6B, right panel**).

Since Wnt/β-catenin signaling cooperates with transcription factors to regulate pluripotency and self-renewal of embryonic stem cells (Ben-Porath *et al.*, 2008), we tested whether Wnt/β-catenin inhibition using IWR-1 affected the expression of key pluripotency-related genes in spheres. Treatment with IWR-1 diminished significantly *NANOG*, *OCT4* and *SOX2* mRNA expression in spheres (by at least 40% for all the cells). These effects were also statistically significant for some transcription factors in parental MG-63 (*SOX2*) and MNNG-HOS cells (*NANOG*, *OCT4*) (**Figure 3.6C**), probably reflecting the existence of stem-like cells within the whole tumor cell population. IWR-1 also decreased the expression of Sox2 at the protein levels, which was previously found as the most prominently expressed transcription factor in osteosarcoma sphere-forming cells (**Chapter 2**) (**Figure 3.6D**). Indeed, Sox2 expression in untreated spheres was higher than that found in parental cells, as demonstrated by the higher Sox2/β-actin expression ratios in spheres, but IWR-1 decreased this ratio by approximately 75%, 77% and 36% in MG-63, MNNG-HOS and SJSA-1 spheres, whereas its effects were almost negligible in parental cells (**Figure 3.6D**).

Since Wnt/β-catenin signaling is also involved in the regulation of normal skeletal development and osteoblast differentiation (Gaur *et al.*, 2005; Glass II *et al.*, 2005; Mohseny *et al.*, 2012), we explored whether Wnt/β-catenin inhibition decreased the expression of genes involved in osteogenic differentiation, such as *RUNX2*, an osteogenic transcription factor and *SPARC*, a matrix proteoglycan highly expressed by bone cells (Termine *et al.*, 1981; Delany and Hankenson, 2009). mRNA expression levels of these markers were significantly decreased in MG-63 spheres and the same trend was observed in MNNG-HOS and SJSA-1 spheres, although not statistically significant, upon IWR-1 treatment (**Figure 3.6E**). These effects were not significant in IWR-1-treated parental cells, compared to DMSO.

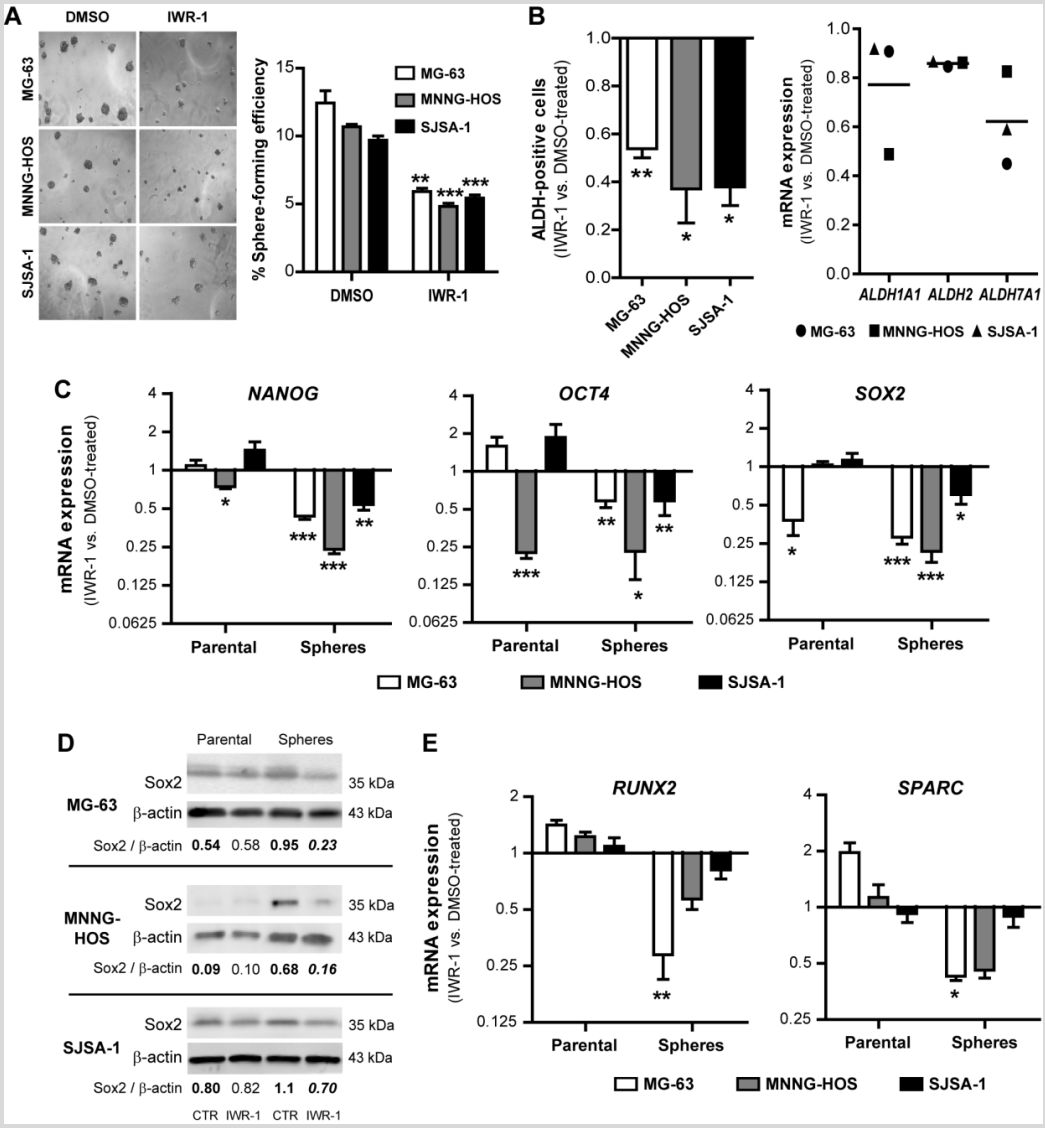

Figure 3.6. IWR-1 reduces osteosarcoma stemness and the expression of several genes involved in stem cell self-renewal and pluripotency. A. Self-renewal efficiency of primary spheres after treatment with IWR-1. **B.** Aldefluor™ activity and mRNA expression of ALDH isozymes in MG-63, MNNG-HOS and SJSA-1 cells after 96h treatment with IWR-1, compared to DMSO-treated cells (set at 1).*p<0.05, **p<0.01 (n=3, Mann-Whitney test). **C, E.** Expression of the pluripotency transcripts (*NANOG*, *OCT4*, *SOX2*) and mesenchymal-related genes (*RUNX2*, *SPARC*) analyzed by quantitative RT-PCR in parental cells and spheres treated with 10μM IWR-1 for 96h. Bar graphs represent fold change mRNA expression ± SEM in IWR-1 *versus* DMSO-treated cells, after normalization of Cq values to three housekeeping genes. *p≤0.05, **p≤0.01, ***p≤0.001 compared to DMSO-treated cells (N=3, independent samples t-test). **D.** Western blot analysis confirms that Wnt/β-catenin inhibition decreases expression of Sox2 stem cell transcription factor. Numbers below protein bands represent relative Sox2 protein expression normalized to β-actin (loading control). Bold-italic characters indicate decreased expression compared to control

(DMSO) cells. The blots used in the figure are cropped. All the gels have been run under the same experimental conditions.

We cannot exclude that other signaling pathways might also be involved in osteosarcoma stem cell self-renewal and acting coordinated with Wnt pathway, such as the hedgehog pathway (Noubissi *et al.*, 2009). To strengthen this hypothesis, we tested the overall activation of hedgehog pathway in osteosarcoma spheres and then whether tankyrase inhibition with IWR-1 down-regulated key molecules involved in hedgehog signaling. mRNA expression studies revealed increased expression of key pathway mediators (receptors *PTCH1* and *SMO*, and transcription regulators *GLI1* and *GLI2*) in spheres, compared to parental cells (**Figure 3.7A**). Moreover, *GLI2*, *PTCH1* and *SMO* expression in spheres was significantly down-regulated in response to Wnt/β-catenin inhibition with IWR-1 (**Figure 3.7B**). These results reinforce the hypotheses that a complex network of signaling pathways coordinate stemness in osteosarcoma and may be modulated by Wnt inhibition.

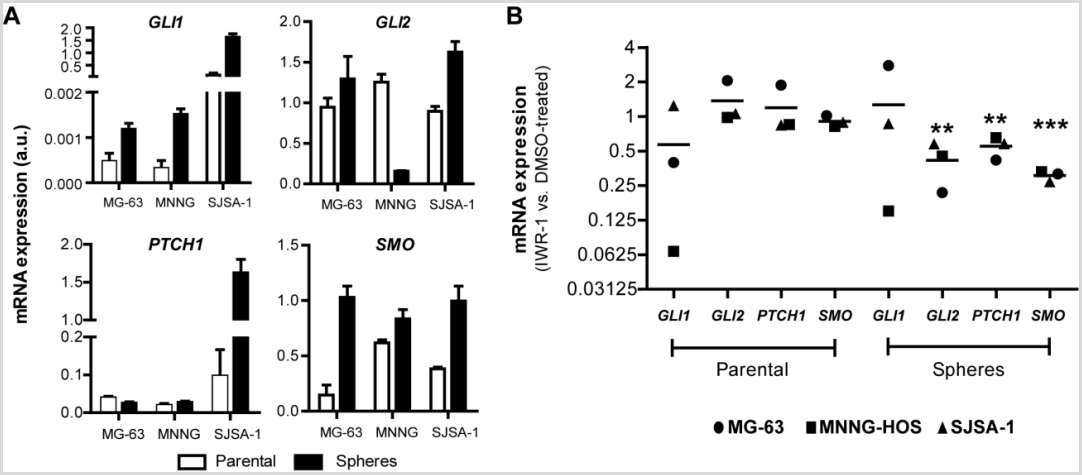

Figure 3.7. Wnt/β-catenin inhibition down-regulates expression of hedgehog-related genes. A. Expression of key transcripts involved in hedgehog signaling is augmented in spheres, compared to their parental cells, as evaluated by quantitative RT-PCR. **B.** Expression of the hedgehog-related genes *GLI1*, *GLI2*, *SMO* and *PTCH1* analyzed by quantitative RT-PCR in the MG-63, MNNG-HOS and SJSA-1 parental cells and spheres treated with 10μM IWR-1 for 96h. IWR-1 decreased the mRNA expression of *GLI2*, *SMO* and *PTCH1*, known to play regulatory roles in stem cell self-renewal. Suppressive effects of IWR-1 were significantly more pronounced in spheres than in parental cells. Values represent mean mRNA expression or fold-difference ± SEM after normalization of Ct values to three housekeeping genes. **p≤0.01, ***p≤0.001 compared to DMSO-treated cells (N=3, independent samples t-test).

3.4.5 Wnt/β-catenin inhibition leads to improved chemosensitivity of osteosarcoma spheres to doxorubicin

After demonstrating the critical role of the Wnt/β-catenin pathway in the maintenance and survival of CSCs, we then tested whether Wnt signaling inhibition with IWR-1 might functionally restore the chemosensitivity of spheres to doxorubicin, which is the central chemotherapeutic used in the treatment of osteosarcoma (Marina *et al.*, 2004). To address this hypothesis, both parental and sphere-forming cells were treated with increasing concentrations of doxorubicin, either alone or in combination with 10μM IWR-1. Cell viability assays revealed that co-treatment with IWR-1 increased substantially the susceptibility of spheres towards doxorubicin (**Figure 3.8A**), an effect that was accomplished by a decreased expression of ABC transporters p-glycoprotein and breast cancer-related protein (Pgp, BCRP, **Figure 3.9**) in spheres, which are established markers of cancer stem-like cells and mediators of resistance to chemotherapeutic agents (Liu *et al.*, 2013). IWR-1 had no significant effects on the cytotoxicity of doxorubicin in parental cells, except MNNG-HOS ($p=0.01$), although not so strong as in the corresponding spheres (**Figure 3.8A**).

To identify the nature of chemosensitizing interactions between IWR-1 and doxorubicin in spheres, we conducted a set of cell cytotoxicity assays using varying concentrations of both drugs in a therapeutic range, as depicted in **Figure 3.8B**. Since there were no significant effects of IWR-1 on the cytotoxicity of doxorubicin in two out of the three parental cell lines, we did not conduct this study in parental cells. The calculation of combination indexes, in the range of selected concentrations, revealed that IWR-1 reversed the resistance of doxorubicin mostly in a synergistic manner, although an antagonistic interaction was noted in SJSA-1-derived spheres for the lowest doxorubicin concentration. Overall, we found that Wnt/β-catenin inhibition by IWR-1 enhances doxorubicin-induced cytotoxicity in spheres.

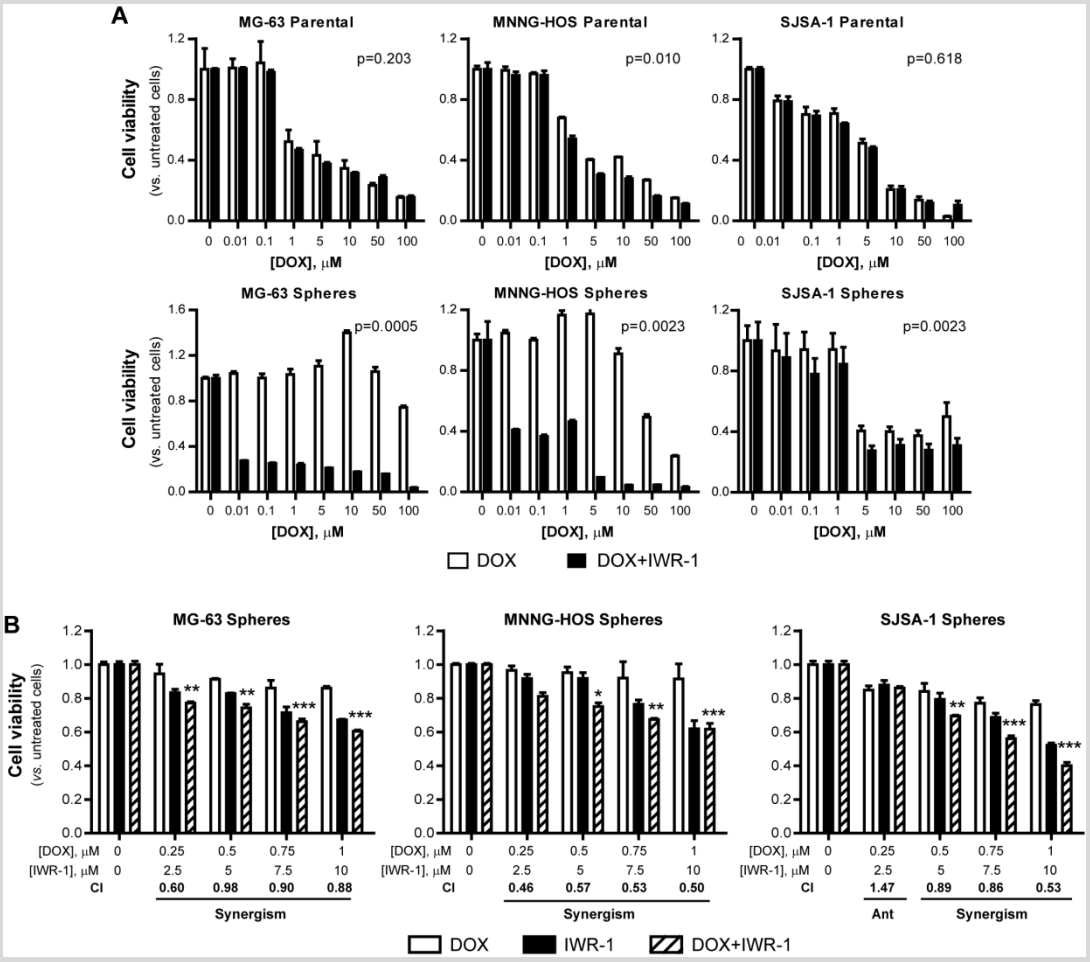

Figure 3.8. Co-treatment with IWR-1 sensitizes osteosarcoma spheres to doxorubicin. A. Viability of MG-63, MNNG-HOS and SJSA-1 parental cells and spheres upon treatment with increasing doses of doxorubicin (0.01-100μM), either alone (DMSO) or in combination with IWR-1 (10μM). Sensitivity to doxorubicin increased significantly in spheres upon IWR-1 treatment, being more pronounced in spheres with β-catenin nuclear-positivity (MG-63 and MNNG-HOS). Cells were treated for 48h with IWR-1 alone and then for further 48h in the presence of doxorubicin and IWR-1. P-values were calculated with paired t-test to compare overall differences between IWR-1 and DMSO-treated groups, using GraphPad Prism 5 software. **B.** Chou-Talalay method was used to estimate synergistic effects between doxorubicin and IWR-1 (concentrations used at a constant ratio of 1:10), measured after 48h of drug exposure with a colorimetric assay. Results are expressed as mean ± SEM of three independent observations. *p≤0.05, **p≤0.01, ***p≤0.001, compared to cells treated with doxorubicin alone (N=6, Two-way ANOVA, with Bonferroni post-test). Abbreviations: *DOX*, doxorubicin; *CI*, combination index; *Ant*, antagonism.

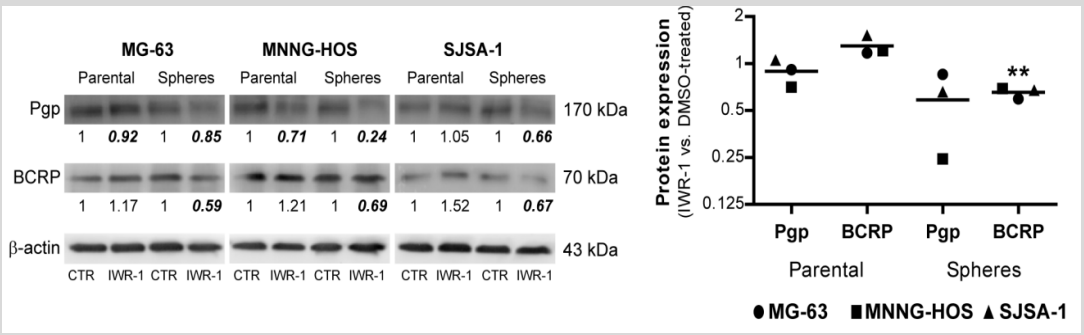

Figure 3.9. Diminished expression of drug-efflux ABC transporters p-glycoprotein (Pgp) and breast cancer-related protein (BCRP) in spheres, after IWR-1 treatment for 96h. Numbers represent relative protein expression in IWR-1 *versus* DMSO-treated cells after normalization to β-actin (loading control). Bold-italic characters indicate decreased expression compared to control (DMSO) cells (set at 1). The blots used in the figure are cropped. All the gels have been run under the same experimental conditions. **p≤0.01 compared to DMSO-treated cells (N=3, independent samples t-test).

3.4.6 IWR-1 demonstrates single anti-tumoral activity and synergizes with doxorubicin in an osteosarcoma mouse model

After demonstrating the key role of Wnt signaling in CSCs self-renewal and the enhanced anti-tumoral activity of IWR-1 in combination with doxorubicin, we then tested the efficacy of the same therapeutic approach on tumor progression using a mouse xenografted model. For this study animals were subcutaneously injected with an osteosarcoma cell line containing CSCs (MNNG-HOS) stably transfected with the pGL4 vector for monitoring Wnt-mediated TCF/LEF transcriptional activity. When tumors reached on average 5mm diameter, tumor-bearing mice were treated each 2 days with 5mg/kg IWR-1 (intra-tumoral) and each 4 days with 8mg/kg doxorubicin (intraperitoneal), for 2 weeks alone or in combination. Treatments were well tolerated as no significant alterations on animal's body weight, fur texture, behavioral activity or signs of gastrointestinal toxicity were observed. The effects of drug combinations were monitored by measuring tumor volumes and TCF/LEF activity.

In line with *in vitro* observations, the administration of IWR-1 induced a marked inhibition of tumor growth as indicated by the slower tumor growth rate and reduction in tumor size by 73% and 71% as compared to control and to doxorubicin-treated groups respectively, at the end of the treatment (**Figure 3.10A,B**). Moreover this drug potentiated the therapeutic efficacy of doxorubicin in relation to doxorubicin-treated animals, as demonstrated by the greater reduction of tumor burden at the end of the treatment in opposite to doxorubicin alone that moderately impacted tumor growth. Importantly, IWR-1 alone and in combination with doxorubicin led to a further attenuation of Wnt/β-catenin signaling in tumors as evidenced by the significantly decreased luciferase reporter activity tested after 10 and 15 days of treatment. In contrary, doxorubicin alone had no significant effects in TCF/LEF activity, in comparison to control animals, suggesting that this drug used in first-line treatment in osteosarcoma was not effective in depleting Wnt/β-catenin active-cells that might survive and sustain tumor growth (**Figure 3.10C,D**).

In fact, mRNA analysis of dissected tumors showed an up-regulation of *AXIN2* and *SOX2* in doxorubicin-treated tumors, an effect that was prevented by co-treatment with IWR-1 (**Figure 3.10E**), suggesting the Wnt/β-catenin signaling is required for the enhanced stem-like phenotype. The residual tumors treated with IWR-1 alone did not show any up-regulation of *AXIN2* or *SOX2* that remained comparable to the basal levels in DMSO-treated tumors.

Immunohistochemical analysis of excised tumors revealed a decreased expression of the key canonical Wnt signaling player β-catenin at nuclear levels in both IWR-1 and doxorubicin+IWR-1 treatment conditions compared to DMSO-treated tumors, which displayed positivity for nuclear β-catenin (**Figure 3.10F**). A few cellular spots also stained positively for nuclear β-catenin in doxorubicin-treated tumors. This emphasizes the specific *in vivo* targeting of Wnt/β-catenin signaling, in line with the decreased TCF/LEF transcriptional activity observed in **Figure 3.10C,D**. Furthermore, the absence of Sox2 staining in tissue sections of tumors treated with IWR-1 alone or in combination with doxorubicin reinforced the role of Wnt signaling as mediator of stemness that occurs upon treatment with doxorubicin (**Figure 3.10F**).

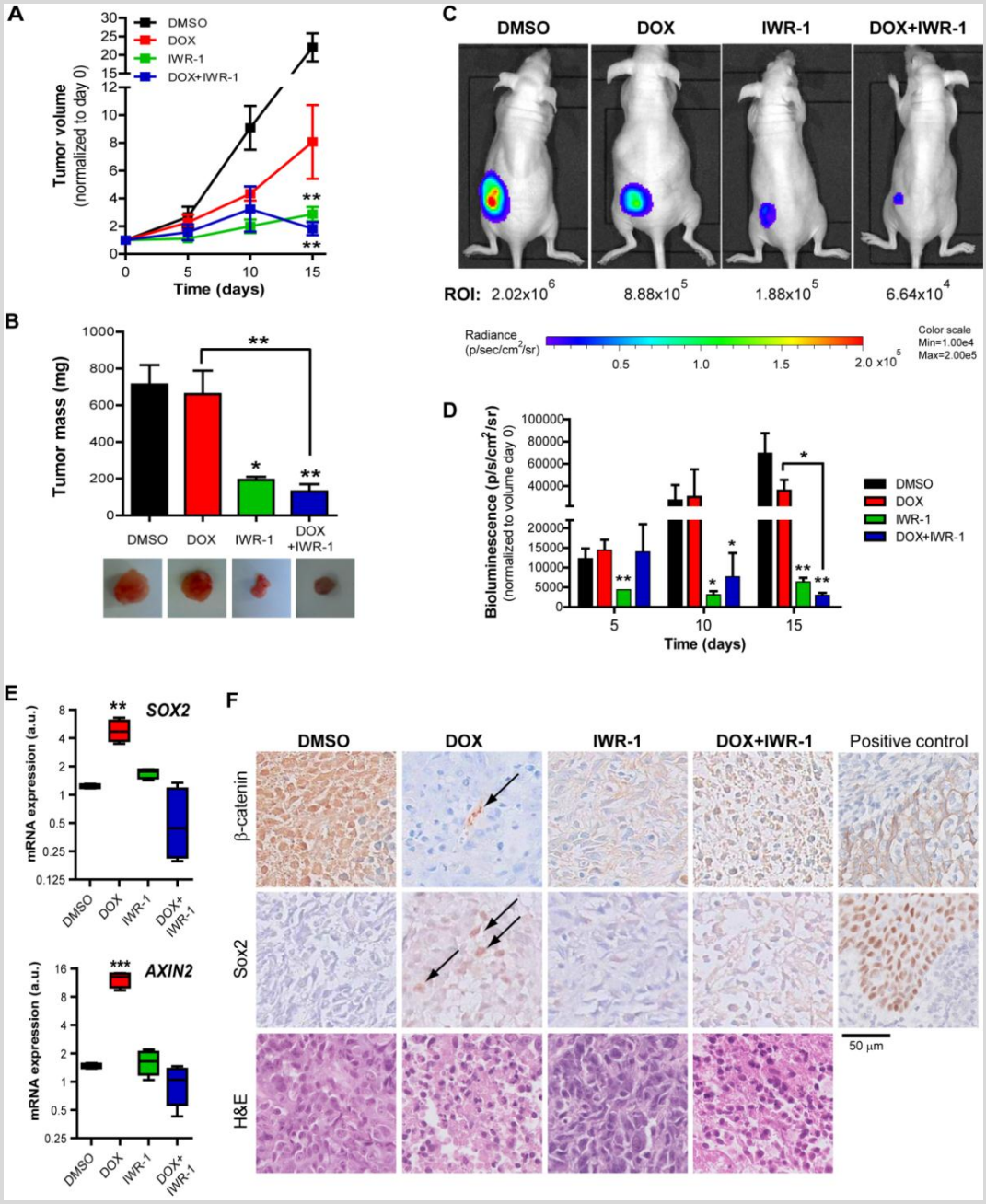

Figure 3.10. IWR-1 inhibits tumor growth and potentiates the anti-tumor efficacy of doxorubicin in an osteosarcoma mouse model. A. Line graph of the mean tumor volumes at the indicated days normalized to day 0 (before starting of treatments) for each treatment group (n=3). **B.** Average mass of excised tumors at day 15 for each treatment group. **C.** Monitoring of Wnt/β-catenin signaling in tumors by measuring TCF/LEF-luciferase activity in tumor-bearing mice after treatments with vehicle, doxorubicin, IWR-1 and IWR-1+doxorubicin. Representative bioluminescence images of Wnt signaling activity in xenografted tumor-bearing mice, after 2 weeks of treatment. **D.** Bioluminescence signals reflecting luciferase activity after treatments normalized to the tumor volume on day 0. Data show mean ± SEM. **E.** Expression of the pluripotency transcript *SOX2* and Wnt target gene *AXIN2* was tested by quantitative RT-PCR in

tumors excised from mice after treatments. Box and whiskers graphs represent absolute mRNA expression, after normalization of Cq values to three housekeeping genes. **F.** Excised tumor tissues were formalin-fixed paraffin-embedded and subjected to immunohistochemical analysis of β-catenin and Sox2 protein expression; bar represents 50μm. Arrows indicate nuclear expression of β-catenin and Sox2. Positive control is normal tonsil for β-catenin and Sox2. In **A.**, **B.**, **D.** and **E.** *p≤0.05, **p≤0.01, ***p≤0.001 compared to control or doxorubicin-only (N=3, One-way ANOVA, with Tukey's post-test).

3.5 Discussion

A number of signaling pathways controlling normal stem cell self-renewal and functions has been implicated in CSCs' regulation. In a previous report, we showed that osteosarcoma cell lines contain a sub-population of CSCs with active Wnt/β-catenin, an important signaling pathway controlling stem cell self-renewal, based on nuclear β-catenin expression and TCF/LEF transcriptional activity (**Chapter 2**). In the present study, we extended the characterization of Wnt/β-catenin signaling activity focusing on the expression of *LRP5/6* co-receptors. The expression of some of these markers has been observed in osteosarcoma and correlated with disease progression (Hoang *et al.*, 2004). However, the presence of transcripts does not necessarily represent active signaling, without evidence that their expression can be modulated by specific signaling pathway inhibition. Therefore, we conducted a set of experiments demonstrating that Wnt/β-catenin inhibition is harmful for osteosarcoma CSCs, impairs their stemness characteristics inducing apoptotic cell death and synergizes with doxorubicin treatment *in vitro* and *in vivo*.

Our data clearly shows that osteosarcoma CSCs were more responsive to Wnt inhibition with IWR-1 than their parental cells, as we observed depletion of nuclear β-catenin, Wnt target genes' expression (especially *AXIN2*), possibly via the down-regulation of TCF/LEF transcriptional activity. TCF/LEF reporter activity was not lowered in transfected cells beyond 70% (MG-63 spheres) and 50% (MNNG-HOS and SJSA-1 spheres) indicating that active feedback loops or alternative mechanisms may exist that prevent complete reduction in reporter activity. This fact is not surprising, especially because IWR-1 is a specific tankyrase inhibitor (Chen *et al.*, 2009), being this inhibition approach upstream of the nuclear TCF/LEF transcript factors location. This may also justify that the observed effects may not be exclusively derived from altered β-catenin levels. Nevertheless, we also observed that IWR-1 decreased the expression of cyclin D1, which is known for its role in controlling cell proliferation by activating G1 kinases

(Baldin *et al.*, 1993). Cyclin D1 down-regulation may in fact correlate with the hampered cell proliferation observed with IWR-1 treatment in osteosarcoma spheres. Moreover, IWR-1 treatment also down-regulated the expression of c-Myc that is involved in the reprogramming of cells to induced pluripotency (Ho *et al.*, 2011). Overall, the effects of Wnt inhibition were more potent in the MG-63 and MNNG-HOS cells than in SJSA-1, which may be explained by the higher magnitude of Wnt activation status in the former cells, based on their higher intensity of nuclear β-catenin as assessed by immunohistochemistry and TCF/LEF activity (**Chapter 2**). Moreover, IWR-1 cytotoxic effects on cell viability were detected at an earlier time-point (48h) in MG-63 and MNNG-HOS spheres than in SJSA-1 spheres.

Since Wnt/β-catenin has been shown to regulate self-renewal and stemness properties in several types of cancer cells, including sarcomas (Jiang *et al.*, 2013; Yan *et al.*, 2001; Vijayakumar *et al.*, 2011; Mao *et al.*, 2014) and associated with the maintenance and survival of CSCs (Reya and Clevers, 2005), we also explored some effects of Wnt inhibition on osteosarcoma stemness. We found that IWR-1 decreased secondary sphere formation, which is used as a functional readout of the impaired self-renewal capacity of CSCs. Moreover, Wnt inhibition was associated with decreased Aldefluor™ activity, expression of classic CSC markers involved in pluripotency, such as Sox2 (Basu-Roy *et al.*, 2012), and markers involved in the osteogenic differentiation potential of mesenchymal cells, such as *RUNX2* (Gaur *et al.*, 2005). These results offer evidence that inhibiting Wnt signaling might be an approach to eliminate CSCs' capacity to regenerate the tumor cell hierarchy and therefore lead to higher degrees of tumor eradication, by compromising their pluripotential and differentiation abilities.

Cancer stem cells are reasonably well-accepted as being the culprits for resistance to conventional chemotherapeutics in osteosarcoma and other tumor types. Therefore, CSCs may serve as potential specific therapeutic targets among tumor cells, providing that specific signaling pathways and biomarkers governing CSCs' functionality can be pharmacology targeted. Our study provides evidence that Wnt inhibition resulted in cell cycle arrest and induction of apoptotic cell death in osteosarcoma spheres via up-regulation of the apoptotic promoters caspases 3/7 and DNA fragmentation in agreement with reports in osteosarcoma (Wessel Stratford *et al.*, 2014) and other cancer models (Huang *et al.*, 2009; Tian *et al.*, 2013), including synovial sarcoma (Barham *et al.*, 2013). Also Dieudonné and colleagues have shown that high Wnt signaling activity seems to repress the pro-apoptotic effects of syndecan-2 protein (Dieudonné *et al.*, 2010), further providing insights for a pathologic role of Wnt signaling in osteosarcoma

via modulation of apoptotic-related signaling pathways. Our results also demonstrate that Wnt inhibition reversed the intrinsic resistance of CSCs to doxorubicin, acting synergistically in the impairment of cell viability. Indeed, other reports support these results, as inhibition of Wnt signaling combined with chemotherapy has been shown to reverse chemoresistance in osteosarcoma *in vitro* and *in vivo* models (Dieudonné *et al.*, 2012; Ma *et al.*, 2013; Scholten *et al.*, 2014). Moreover, an outstanding study using *in vivo* models of several human tumors revealed that targeting the Wnt pathway synergized significantly with classic chemotherapies in decreasing tumor growth (Gurney *et al.*, 2012).

In fact, our *in vivo* results strengthen the assumption that further investigation may be warranted into the potential efficacy of Wnt inhibition in osteosarcoma in parallel with the administration of the regular therapies, as we observed that all animals receiving pGL4-MNNG-HOS cells treated with IWR-1 and doxorubicin resulted in retarded tumor growth, while untreated animals developed large tumor masses. Biochemical alterations consistent with Wnt/β-catenin depletion were further confirmed by genetic and immunohistochemical analysis of sections excised from mouse tumor tissues. Importantly, in addition to the substantial tumor growth inhibition by targeting Wnt-active CSCs, IWR-1 prevented the acquisition of stem-like phenotype induced by doxorubicin if tumor cells are not properly killed, which could lead to survival and expansion of highly tumorigenic cells. Several *in vivo* validated studies indicate that Wnt inhibition exerts anti-tumoral effects by WIF1-mediated (Rubin *et al.*, 2010) or dominant-negative LRP5 receptor-mediated (Guo *et al.*, 2008) down-regulation of matrix metalloproteinases in osteosarcoma. More recently, other groups have shown that Wnt inhibition via TCF inhibition (Dieudonné *et al.*, 2012) or small-molecule compounds that induce stabilization of tankyrases (De Robertis *et al.*, 2014) might be an interesting approach contributing to the clinical management of osteosarcomas.

Earlier results published by our group and others using osteosarcoma cell lines and patient tumor samples revealed that Wnt/β-catenin signaling is inactive in bone cancers (Cai *et al.*, 2010; Du *et al.*, 2014). Herein and in agreement with a recent study (**Chapter 2**), we observed that this pathway is specifically active in CSCs isolated with the sphere assay, but not in the bulk osteosarcoma parental cells. The activation of Wnt/β-catenin signaling in this specific stem-like cell population is not conflicting with the previous observation that this pathway is down-regulated in osteosarcoma, since CSCs represent a minor subset within the bulk tumor without detectable impact on the Wnt activation status due to their relatively small contribution, despite their high intrinsic Wnt activity.

Only in CSC-enriched spheres, the activation of the Wnt pathway can be encountered. Moreover, Wnt inhibition was selectively cytotoxic for CSC-enriched spheres without significant impact in bulk tumor cells, which demonstrates the importance of this regulatory pathway in the self-renewal of osteosarcoma stem cells. The analysis of a public R2 database containing microarray data revealed that the expression of Wnt target genes (*DKK-1* and *c-Myc*) correlates with a poor overall survival in osteosarcoma patients (**Chapter 4**), suggesting that the Wnt/β-catenin activation may represent a new candidate for osteosarcoma therapy targeting CSCs.

The complexity of this pathway has been demonstrated by studies establishing the crosstalk between Wnt signaling and other pathways involved in tumorigenesis such as FOXO1 (Guan *et al.*, 2015), and also with other putative CSC surface markers such as CD133 (Tirinato *et al.*, 2015), which may be both a direct target of TCF/LEF factors (Katoh and Katoh, 2007) as well as a stabilizer of β-catenin via HDAC interaction (Mak *et al.*, 2012). Despite being considered a universal CSC marker, we did not detect expression of CD133 in a large panel of osteosarcoma CSCs including those analyzed in this manuscript (data not shown), suggesting that this marker warrants a broader validation, at least in osteosarcoma. Also, our own data using the Wnt activator SB216763 (GSK3 inhibitor) showed that after 96h this compound did not significantly alter proliferation of MG-63 and SJSA-1 cells, as observed previously by Cai *et al.* (Cai *et al.*, 2010). Altogether, all these studies show that the activation/inactivation status of the pathway should be analyzed at the cellular level (stem *versus* non-stem cell populations) and reveal the complexity of the Wnt pathway in osteosarcomas.

3.6 Conclusion

In summary, our results demonstrate that Wnt/β-catenin signaling is crucial for the maintenance of osteosarcoma stem-like cells, as its pharmacological inhibition impaired key stem cell-related characteristics and induced CSCs apoptosis. Moreover, we suggest that a potential means of improving the poor response to chemotherapy in patients with osteosarcoma would be to consider targeting the Wnt/β-catenin together with the established therapies, since the combination can act synergistically. Last but not least, our *in vivo* results offer pre-clinical evidence that Wnt/β-catenin is a potential therapeutic target for osteosarcoma treatment.

3.7 References

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4 Chapter

Chemotherapy Induces Stemness in Osteosarcoma Cells through Activation of Wnt/β-catenin Signaling

Sara R. Martins-Neves, Daniela I. Paiva-Oliveira, Pauline M. Wijers-Koster, Antero J. Abrunhosa, Carlos Fontes-Ribeiro, Judith V. M. G. Bovée, Anne-Marie Cleton-Jansen*, Célia M. F. Gomes*

(*equal contribution)

Cancer Letters, 370(2):286-295. DOI: 10.1016/j.canlet.2015.11.013

"You can be a victim of cancer, or a survivor of cancer. It's a mindset." – Dave Pelzer

4.1 ABSTRACT

Development of resistance represents a major drawback in osteosarcoma treatment, despite improvements in overall survival. Treatment failure and tumor progression have been attributed to pre-existing drug-resistant clones commonly assigned to a cancer stem-like phenotype. Evidence suggests that non stem-like cells, when submitted to certain microenvironmental stimuli, can acquire a stemness phenotype thereby strengthening their capacity to handle with stressful conditions.

Here, using osteosarcoma cell lines and a mouse xenograft model, we show that exposure to conventional chemotherapeutics induces a phenotypic cell transition towards a stem-like phenotype. This associates with activation of Wnt/β-catenin signaling, up-regulation of pluripotency factors and detoxification systems (ABC transporters and Aldefluor activity) that ultimately leads to chemotherapy failure. Wnt/β-catenin inhibition combined with doxorubicin in the MNNG-HOS cells prevented the up-regulation of factors linked to transition into a stem-like state and can be envisaged as a way to overcome adaptive resistance. Finally, the analysis of the public R2 database, containing microarray data information from diverse osteosarcoma tissues, revealed a correlation between expression of stemness markers and a worse response to chemotherapy, which provides evidence for drug-induced phenotypic stem cell state transitions in osteosarcoma.

Keywords: osteosarcoma; stemness; aldehyde dehydrogenase; pluripotency; Wnt/β-catenin

4.2 INTRODUCTION

Osteosarcoma is the most common primary malignant bone tumor affecting children and adolescents. Therapeutic management relies on multimodal conventional therapies, consisting essentially of doxorubicin, cisplatin and methotrexate. This regimen improved overall survival to approximately 65%, but still nearly 45% of patients develop distant metastasis (Pakos *et al.*, 2009). Drug resistance almost invariably occurs, limiting treatment effectiveness. Chemotherapy shrinks the tumor mass, but may also exert a selective pressure on tumor cells leading to the outgrowth of the fittest surviving clones.

Recently, the ability of tumor cells to adapt to specific microenvironmental stimuli by changing their phenotype is believed to constitute a defense mechanism. This phenotypic switching allows tumor cells to evade surveillance of the immune system, survive unfavorable conditions, and escape therapies. The development of a multidrug-resistance phenotype has been studied in several tumors and associated, among others, with the overexpression or activation of drug efflux pumps (Gottesman *et al.*, 2002; Gomes *et al.*, 2006), improved DNA-repair capacity (Oliver *et al.*, 2010), over-activation of the anti-apoptotic machinery and with the epithelial-mesenchymal transition (Piskareva *et al.*, 2015; Haslehurst *et al.*, 2012).

Osteosarcoma contains a subset of cells with attributes of cancer stem cells (CSCs) also designated tumor-initiating cells (TICs). These cells are endowed with self-renewal abilities and can drive tumor growth, dissemination and recurrence after chemotherapy. We have previously shown that cells with this phenotype display increased self-defense mechanisms against chemotherapy linked to quiescence, enhanced expression and activity of drug-efflux transporters and detoxifying systems (**Complementary Results, Figure C7.1**). Furthermore osteosarcoma CSCs express genes involved in self-renewal and regulators of the Wnt/β-catenin pathway (Martins-Neves *et al.*, 2012; **Chapter 2**).

Evidence supports the hypothesis that CSCs can result from the oncogenic transformation of normal stem cells or the acquisition of stemness-related properties by non-stem cancer cells in response to microenvironmental signals (Friedmann-Morvinski and Verma, 2014). In fact, cancer cells' exposure to conventional therapies can be a novel potential source of plasticity and stemness within tumors (Pisco and Huang, 2015). Moreover, several studies suggest that chemotherapy (Hu *et al.*, 2012; Abubaker

et al., 2013) can promote or enhance a stem cell-related phenotype in previously differentiated tumor cells (Auffinger *et al.*, 2014; Lagadec *et al.*, 2013).

The enrichment of stem-like cells induced by therapy has been subject of research (Tang *et al.*, 2011), but the exact mechanisms underlying this phenomenon and whether stemness-related markers are correlated with poor outcome in osteosarcoma are still open questions. We investigated whether low concentrations of the chemotherapeutics most used in osteosarcoma had the ability to induce a stem-like phenotype on established cell lines, representative of two histological subtypes of high-grade osteosarcoma. We found increased expression of key components involved in Aldefluor™ activity, multidrug resistance and embryonic stem cell (ESC)-related pluripotency, and activation of the Wnt/β-catenin pathway. These results add new valuable information regarding the reprogramming of stemness networks in osteosarcoma that may contribute to chemoresistance, and also explain therapy failures, which eventually occur after initially well-succeeded therapeutic approaches.

4.3 Material and Methods

4.3.1 Cell culture and treatments

Human osteosarcoma cell lines derived from fibroblastic (HOS, MG-63, MHM, MNNG-HOS) or osteoblastic (OHS, U2OS) high-grade osteosarcoma were cultured in RPMI-1640 medium (Invitrogen, Karlsruhe, Germany) supplemented with 10% v/v heat-inactivated fetal bovine serum (Invitrogen) and 1% v/v penicillin/streptomycin (Invitrogen), under adherent conditions at 37°C in a humidified incubator with 5% CO_2/95% air. Cell lines were obtained from the American Type Culture Collection (HOS, MG-63, MNNG-HOS, U2OS) or from the EuroBoNeT panel (MHM, OHS) (Ottaviano *et al.*, 2010). Cell line authentication was performed using the GenePrint 10 system (Promega). Mycoplasm infections were monitored bi-monthly with a PCR-based assay (van Kuppeveld *et al.*, 1992).

Cells were treated with increasing concentrations of doxorubicin (0-100µM, DOXO-cell®, Portugal), cisplatin (0-100µM, Teva Pharma, Portugal) and methotrexate (0-100µM, Teva Pharma) during 48h. Cell viability was assessed using the MTT assay and sigmoidal dose-response curves were generated to calculate the mean IC_{50} values (**Table 2.4**) of each drug that were used in subsequent studies, using Origin Pro 8 software.

MNNG-HOS cells were transfected with pGL4.49[luc2P/TCF-LEF RE/Hygro] Vector (Promega) as described in **Chapter 2, section 2.3.4,** and treated with the IC_{50} concentrations of each drug during 24h. The Wnt/β-catenin inhibitor IWR-1 (Sigma-Aldrich®, St Louis, USA) was used at a concentration of 10μM. Luciferase activity was measured using the D-luciferin (Caliper Life Sciences Inc., PerkinElmer, Massachusetts, USA, 30 mg/mL) as substrate and the Living Image software in IVIS® Lumina XR (Caliper). Bioluminescence units represent radiance (photons/s/cm^2/sr). Cells were treated with doxorubicin 0.5μM, cisplatin 5μM and methotrexate 0.05μM, except where indicated.

4.3.2 Aldefluor™ assay

Chemotherapy-enrichment of cells with stem-like properties was assessed by testing Aldefluor™ activity (Stem Cell Technologies) according to the manufacturer instructions, following 24h treatment with a single dose of doxorubicin, cisplatin or methotrexate. Chemotherapy-treated cells were collected and analyzed using a BD™ LSR II flow cytometer and BD FACSDiva™ software (Becton Dickinson Biosciences). Aldefluor™ data were analyzed using WinList™ 3D 7.1 software (Verity Software House, Topsham, ME). Further details were given in **Chapter 2, section 2.3.11**.

4.3.3 Analysis of mRNA expression

Total RNA was isolated using TRIzol® reagent from osteosarcoma cells treated with doxorubicin, cisplatin or methotrexate, and from homogenized tumor tissues excised from untreated and animals treated with doxorubicin alone or in combination with IWR-1 for 2 weeks. Complementary DNA was synthesized using GRS RT-PCR Kit (#GK14.0100, GRiSP Research Solutions, Porto, Portugal). Analysis of mRNA expression was performed as described in **Chapter 2, section 2.3.9**, using the same instrumentation and software analysis. PCR-primers used were already published: *ALDH1A1* (Feldmann *et al.*, 2008), *ALDH2* and *ALDH7A1* (van den Hoogen *et al.*, 2010) (oligos from NZYTech, Lisbon, Portugal), *ABCG2*, *ABCB1* (Gomes *et al.*, 2006), *SOX2* (Park *et al.*, 2012), *KLF4*, *NANOG*, *OCT4* (**Chapter 2**), and *AXIN2* (Cai *et al.*, 2010) (oligos from Eurofins Genomics, Ebersberg, Germany). Housekeeper genes used for data normalization were *CAPNS1* and *SRPR*, which are stably expressed in osteosarcoma (Gomes *et al.*, 2006).

This analysis was performed on the six cell lines, after treatment with doxorubicin, cisplatin or methotrexate for 24h and 96h. The first-time point is intended to assess

drug effects on the induction of stemness-related gene expression, while the last is dedicated to evaluate the long-term drug effects in the selection of a stem-like cell population that survived chemotherapy. Analysis of *ALDH*-related isozymes was only tested in the cell lines that were subjected to ALDH activity analysis (HOS, MNNG-HOS and U2OS).

4.3.4 Western blotting

Western blotting procedures were carried out as previously described (Martins-Neves *et al.*, 2012). Protein samples were collected from chemotherapy-treated cells after 48h. Expression of BCRP, P-glycoprotein, Klf4, Nanog, Oct4 and Sox2 was tested in total protein lysates (prepared in a RIPA buffer) from HOS, MNNG-HOS and U2OS cells. Expression of β-catenin was analyzed in nuclear lysates from MNNG-HOS cells. Antibody details are given in **Table 4.1**.

Nuclear lysates were extracted from HOS, MNNG-HOS and U2OS cell lines treated with 0.5μM doxorubicin, 5μM cisplatin and 0.05μM methotrexate for 48h. After washing twice with cold PBS, cells were scraped with a lysis buffer (10mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, protease inhibitors, 0.4% NP-40 and 1mM DTT) and transferred to 1.5mL tubes. After 30min on ice with occasional vortexing, lysates were centrifuged at 4°C, 13,000RPM for 7min. Pelleted fraction was then washed once with cold PBS and submitted to another centrifugation step. To collect nuclear protein fraction, pellets were ressuspended and mechanically dissociated in a nuclear lysis buffer containing 20mM HEPES, pH 7.9, 0.4 mM NaCl, 1 mM EDTA, protease inhibitors, 10% glycerol and 1mM DTT. This mixture was then placed on ice for 1h, with vortex each 20min, sonicated thrice for 1s at a pulse of 40V and centrifuged as detailed before. The supernatant was kept at -20°C until use.

Table 4.1. Antibodies used in western blotting.

Antibody	Dilution	Source	Company
BCRP	1:250	Mouse	clone BXP-21, Chemicon Int., Temecula, CA, USA
P-glycoprotein	1:100	Mouse	clone C219, Calbiochem®, Millipore, Germany
Klf4	1:500	Rabbit	#4038 Cell Signaling Technology, Danvers, MA, USA
Nanog	1:1,000	Rabbit	#3580 Cell Signaling Technology, Danvers, MA, USA
Oct4	1:1,000	Rabbit	#2750 Cell Signaling Technology, Danvers, MA, USA
Sox2	1:1,000	Rabbit	#3579 Cell Signaling Technology, Danvers, MA, USA
β-catenin	1:500	Mouse	Cat. 610153, BD Transduction Laboratories™
Lamin A	1:1,000	Rabbit	#2032 Cell Signaling Technology, Danvers, MA, USA
β-actin	1:5,000	Mouse	Sigma-Aldrich®, St Louis, USA

4.3.5 Animal studies and ethics statement

Animal studies were conducted according to the EU Directive 2010/63/EU for animal experiments and approved by the Institutional Ethics Committee of the Faculty of Medicine of University of Coimbra (Approval ID:38-CE-2011).

Two million MNNG-HOS cells in sterile PBS were injected subcutaneously in immunocompromised Swiss nude mice (6-8 weeks, Charles River Laboratories). Animals (n=3 per group) were left untreated (control), or treated with doxorubicin 8mg/kg alone or in combination with 5mg/kg IWR-1, at each two days for 2 weeks. Tumor growth was monitored using a caliper and volume estimated using the formula $(LxW^2)x0.5$. Animals were sacrificed by cervical dislocation when tumors reached a maximum of $500mm^3$.

4.3.6 Kaplan-Meier survival analysis

For the generation of Kaplan-Meier survival curves we explored the R2 database ('R2: Genomics Analysis and Visualization Platform - http://r2.amc.nl') which contains genome-wide gene expression data of high-grade osteosarcoma patient samples (dataset: Mixed Osteosarcoma - Kuijjer - 127 - vst - ilmnhwg6v2). We selected either the total samples in the database ("No filter") or samples with a poor response to chemotherapy, which corresponds to less than 90% necrosis after neoadjuvant chemotherapy ("Poor response") for the analysis shown in **Table 4.3**. High-grade osteosarcoma diagnostic biopsies and resections were included in the "No filter" group (88 samples) and "Poor response" group (18 samples).

4.3.7 Statistical analysis

Graphical artwork and statistical analyses were computed with GraphPad Prism version 5.04 (GraphPad Software, San Diego, CA), except Kaplan-Meier curves. Significance was set at $p<0.05$.

4.4 Results

4.4.1 Conventional chemotherapeutics increase aldehyde dehydrogenase signaling

To study whether conventional chemotherapy may promote stem-like properties in osteosarcoma cells, we generated dose-response curves (at 48h) with increasing drug concentrations to determine the mean IC_{50} of each drug, which were used in subsequent studies (**Table 4.2** and **Figure 4.1**). All cell lines displayed a sigmoidal dose-response curve, in response to the increasing doses of drugs, independently of the histological subtype of the tumors from which they were derived (fibroblastic or osteoblastic). The average IC_{50} values of doxorubicin, cisplatin and methotrexate were 0.5μM (0.31 – 0.92μM), 5μM (3.3 – 15.8 μM) and 0.05μM (0.005 - 0.02 μM), respectively, and were used in further experiments. The differences on the drug IC_{50} values between cell lines were not correlated with the basal expression levels of any of the stem cell-related markers that were analyzed throughout this study (**Figure 4.2**).

Table 4.2. IC_{50} values of doxorubicin, cisplatin and methotrexate in osteosarcoma cell lines.

IC_{50} (μM)	HOS	MG-63	MHM	MNNG	OHS	U2OS
Doxorubicin	0.91	0.31	0.92	0.46	0.52	0.39
Cisplatin	3.72	7.55	15.87	3.30	4.17	12.48
Methotrexate	0.008	0.020	0.022	0.022	0.005	0.007

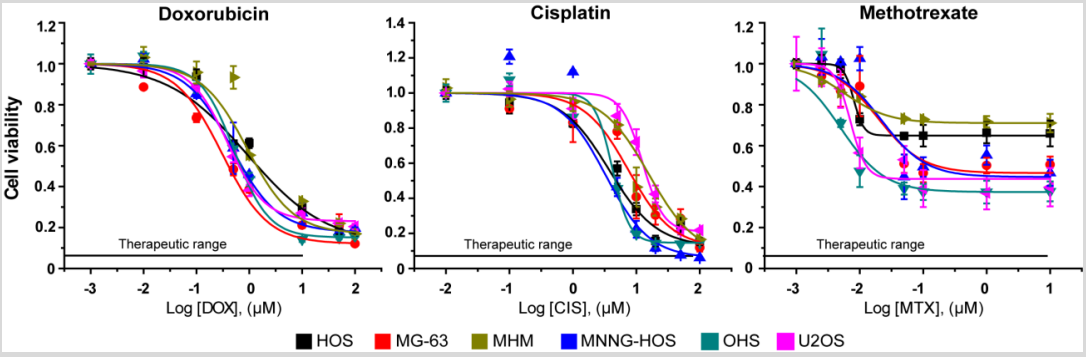
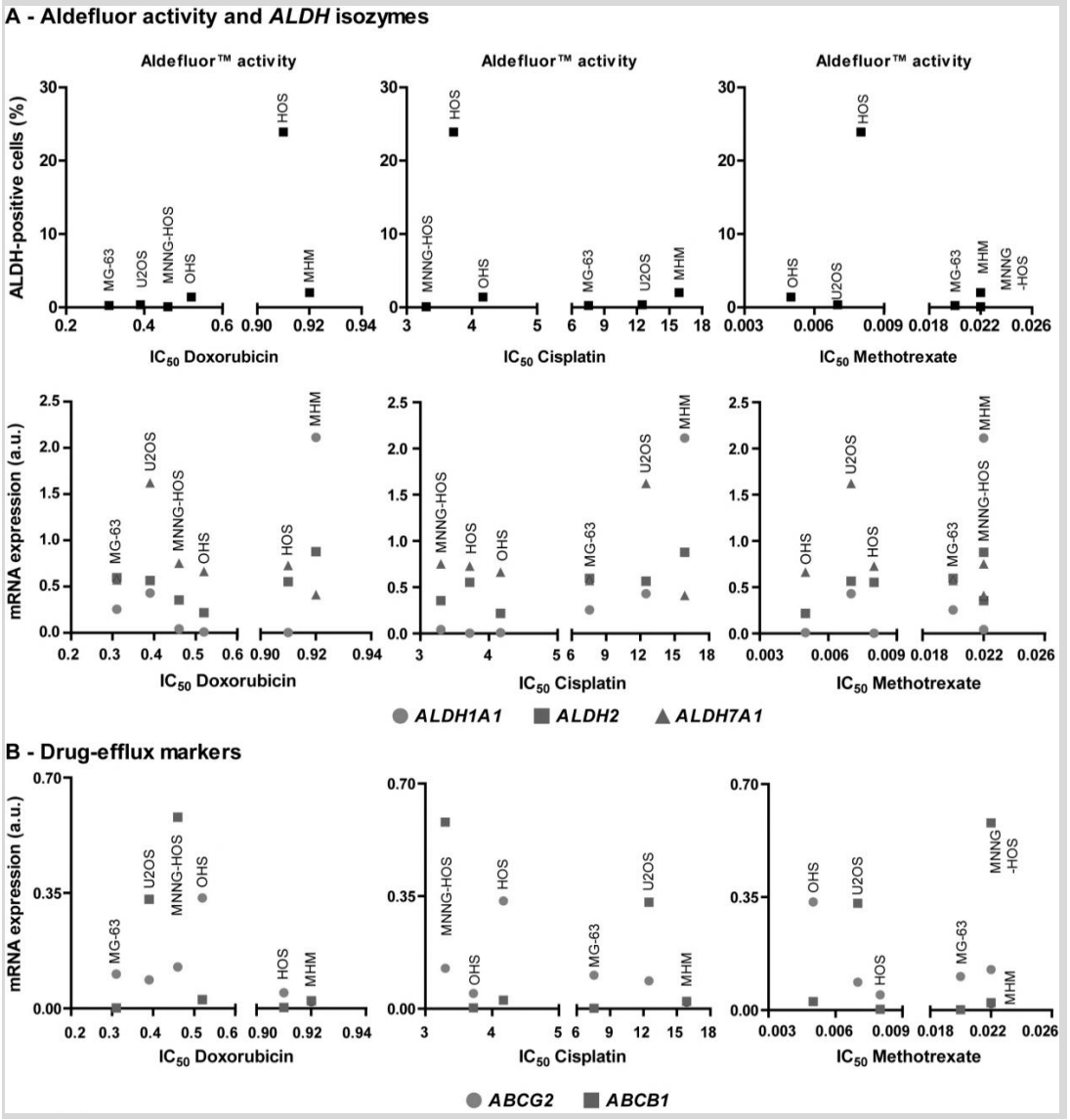


Figure 4.1. Dose-response curves to doxorubicin (DOX), cisplatin (CIS) and methotrexate (MTX) of several osteosarcoma cell lines. See also **Table 4.2** for IC_{50} values.


A - Aldefluor activity and ALDH isozymes
Aldefluor™ activity
ALDH-positive cells (%)
MG-63
U2OS
MNNG-HOS
OHS
HOS
MHM
IC50 Doxorubicin
IC50 Cisplatin
IC50 Methotrexate
mRNA expression (a.u.)
ALDH1A1
ALDH2
ALDH7A1
B - Drug-efflux markers
ABCG2
ABCB1

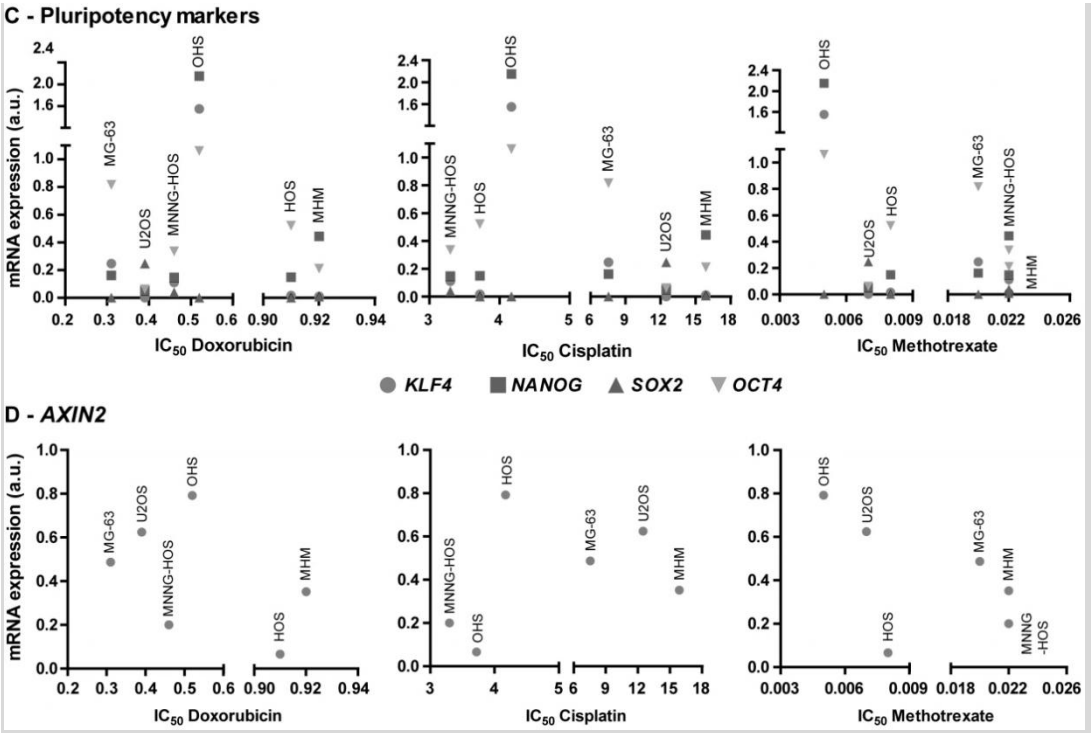


Figure 4.2. Scatter-plots displaying the correlation between the constitutive mRNA expression of stem cell-related markers analyzed in this study and the IC_{50} values of doxorubicin, cisplatin and methotrexate. There is no particular linearity or correlation between the constitutive mRNA expression of stem cell-related markers and the IC_{50} values of doxorubicin, cisplatin and methotrexate.

We explored whether drug treatment induced an increase in Aldefluor™ activity, a functional CSC marker. This study was performed in three cell lines randomly selected from our panel, HOS, MNNG-HOS and U2OS, which were used in most subsequent studies. Cells were treated for 24h and then assayed with the Aldefluor™ kit, using 4-diethylaminobenzaldehyde (DEAB) as negative control (**Figure 4.3A**).

Relative to non-treated cells, HOS, MNNG-HOS and U2OS cells had significantly increased levels of Aldefluor™-positive cells, displaying on average 3.35-, 7.35- and 6.61-fold increases, after exposure to doxorubicin, cisplatin and methotrexate, respectively (**Figure 4.3B**). We also measured Aldefluor™ activity after 96h, and observed the same tendency, although not significant (data not shown), probably due to the higher extent of cellular death. This may reflect the selection of slower proliferating cells, which are spared by the drugs that mainly kill the faster proliferating ones. Interestingly, HOS cells showed on average 3-fold more Aldefluor™ activity after chemotherapy exposure, despite that they already display the highest constitutive Aldefluor™ activity (≈25%), compared to MNNG-HOS (0.09%) and U2OS cells (0.37%).

Additionally, we measured the mRNA expression of several gene transcripts encoding three ALDH isoforms: *ALDH1A1* which is the primary isoform contributing to the Aldefluor™-positive profile, a classic marker for stem cells and chemoresistant properties in tumors, *ALDH2* also a major contributor to Aldefluor™ activity and *ALDH7A1* recently described in CSCs of several tumors (Shi *et al.*, 2011; Ferreira-Teixeira *et al.*, 2015). Gene expression of *ALDH1A1* showed the highest fold-change compared to untreated cells (doxorubicin 43.22-fold, cisplatin 10.45-fold, methotrexate 11.04-fold). The two other isoforms *ALDH2*, and *ALDH7A1* that also contribute to ALDH activity, showed a tendency for increased gene expression, although less robust than that of *ALDH1A1*, being on average below 2-fold for all the treatments tested (**Figure 4.3C**).

4.4.2 Chemotherapeutics up-regulate multidrug resistance-related transporters

To investigate whether chemotherapy enhanced ABC transporters, we measured the expression of *ABCG2*/BCRP and *ABCB1*/P-glycoprotein. mRNA expression increased significantly upon 24h and 96h of exposure to doxorubicin, which demonstrated to be the most potent inductor of drug transporters. Compared to cisplatin and methotrexate, doxorubicin showed the highest capacity to induce and maintain the concomitant up-regulation of *ABCG2* and *ABCB1* at least during 96h. Cisplatin and methotrexate only increased significantly the expression of *ABCG2* at 24h (**Figure 4.3D**). This may be due to the fact that methotrexate is preferentially discarded enzymatically by dihydrofolate reductase activity (Scionti *et al.*, 2008) and cisplatin is not a transport substrate of P-glycoprotein or BCRP (Shi *et al.*, 2011).

We then examined the protein expression of these markers in the HOS, MNNG-HOS and U2OS cells following treatment with the same concentrations of chemotherapy used for mRNA analysis, but during 48h. Drugs induced a differential overexpression of BCRP and P-glycoprotein in these particular cell lines. Doxorubicin induced an up-regulation of both transporters in MNNG-HOS cells, but not in U2OS or HOS cells. Cisplatin and methotrexate up-regulated both transporters in the U2OS cells and P-glycoprotein in the MNNG-HOS cells (**Figure 4.3E**). Interestingly, none of the three drugs was able to induce an up-regulation of BCRP or P-glycoprotein in HOS cells. This may be due to the fact that HOS cells have the highest constitutive Aldefluor™ activity (**Chapter 2, section 2.4.7**), which can be interpreted as an intrinsic self-defensive mechanism to evade drug-induced cytotoxicity.

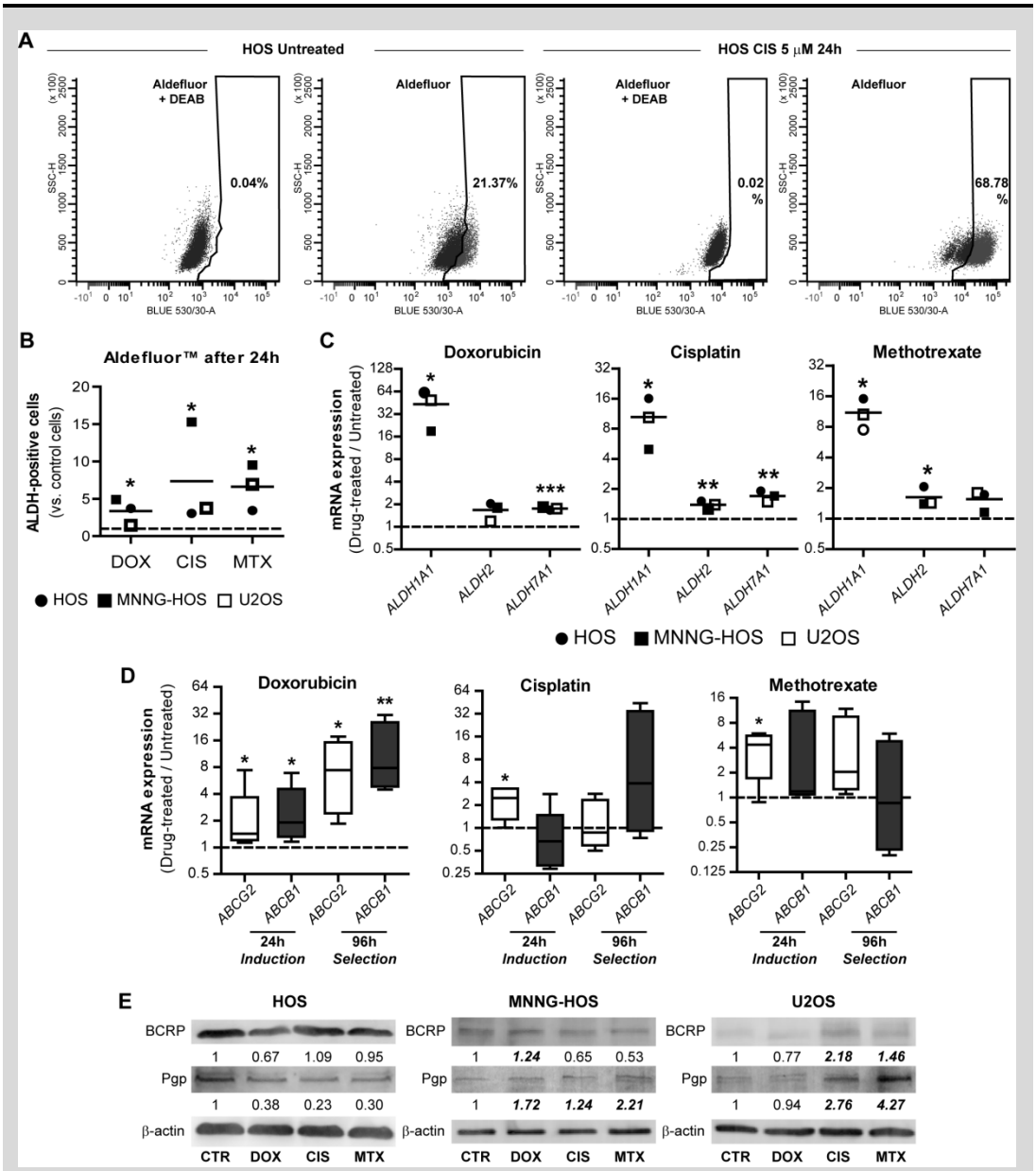


Figure 4.3. Chemotherapeutics enhance the fraction of Aldefluor™-positive cells and up-regulate the expression of multidrug resistance-related proteins in osteosarcoma cells. **A.** Representative flow-cytometry dotplots showing the gating strategy used to detect ALDH-positive events in HOS cells treated with cisplatin 5μM. Diethylaminobenzaldehyde (DEAB) served as a negative control. Aldefluor™ green fluorescence signal was collected on the blue 530/30 channel. **B.** Osteosarcoma cell lines have significantly increased Aldefluor™ activity, after 24h of exposure to doxorubicin (DOX), cisplatin (CIS) or methotrexate (MTX), compared to untreated cells (set at 1, represented by the dashed line). **C.** Expression of *ALDH*-related gene isoforms is significantly increased after 24h of drug exposure, in relation to untreated cells (set at 1). Data represents relative mRNA expression of drug-treated cells (HOS, MNNG-HOS and U2OS) in comparison to untreated cells, after Ct values normalization using two housekeeping genes. *P<0.05, **P<0.01, Mann-Whitney test. In panels (**B-C**) HOS, MNNG-HOS and U2OS cells were

tested and each dot represents a cell line. **D.** Relative mRNA levels of *ABCG2* and *ABCB1* of drug-treated cells normalized to untreated cells. Six osteosarcoma cell lines described in the Methods section were treated with DOX (0.5μM), CIS (5μM) or MTX (0.05μM), for 24 and 96h. **E.** Expression of BCRP and P-glycoprotein (Pgp) in total protein extracts from HOS, MNNG-HOS and U2OS cells after exposure to the same drug concentrations for 48h. Numbers below protein bands represent relative protein expression in treated *versus* untreated control (CTR) cells after normalization to β-actin (loading control). Bold-italic characters indicate increased expression compared to CTR cells (set at 1).

4.4.3 Chemotherapeutics up-regulate pluripotency-related markers expression

The ESC-associated genes *KLF4*, *NANOG*, *OCT4*, and *SOX2*, known to play a prominent role in self-renewal and pluripotency, are up-regulated in sphere-forming cells isolated from osteosarcoma cell lines, without any previous exposure to chemotherapy (**Chapter 2**). We tested whether drugs could induce stemness in osteosarcoma cells, by measuring changes in the mRNA expression levels of these transcripts following drugs exposure. This assay was performed in all cell lines enrolled in this study. In general, all drugs induced pluripotency in osteosarcoma cells through a variable up-regulation of transcription factors in relation to untreated cells (**Figure 4.4A**). Doxorubicin significantly increased the average gene expression levels of *KLF4*, *NANOG* and *SOX2*, but not of *OCT4*, at both 24h and 96h. Cisplatin was also equally effective in inducing up-regulation of these genes, except *SOX2* at 96h. Methotrexate had similar significant effects, except for *NANOG* and *SOX2* at 96h (**Figure 4.4A**).

Afterwards, we validated these results in total protein lysates from HOS, MNNG-HOS and U2OS cells (**Figure 4.4B**) by Western blot analysis and found that each drug per se induced a significant up-regulation of at least two transcription factors in each of the cell lines. The discrepancy between gene and protein expression observed for some genes and treatment conditions might be explained by the fact that post-transcriptional and post-translation modifications may occur. In fact, some studies suggest that the correlation between mRNA expression and protein levels can be as little as 40% depending on the system (Vogel and Marcotte, 2012).

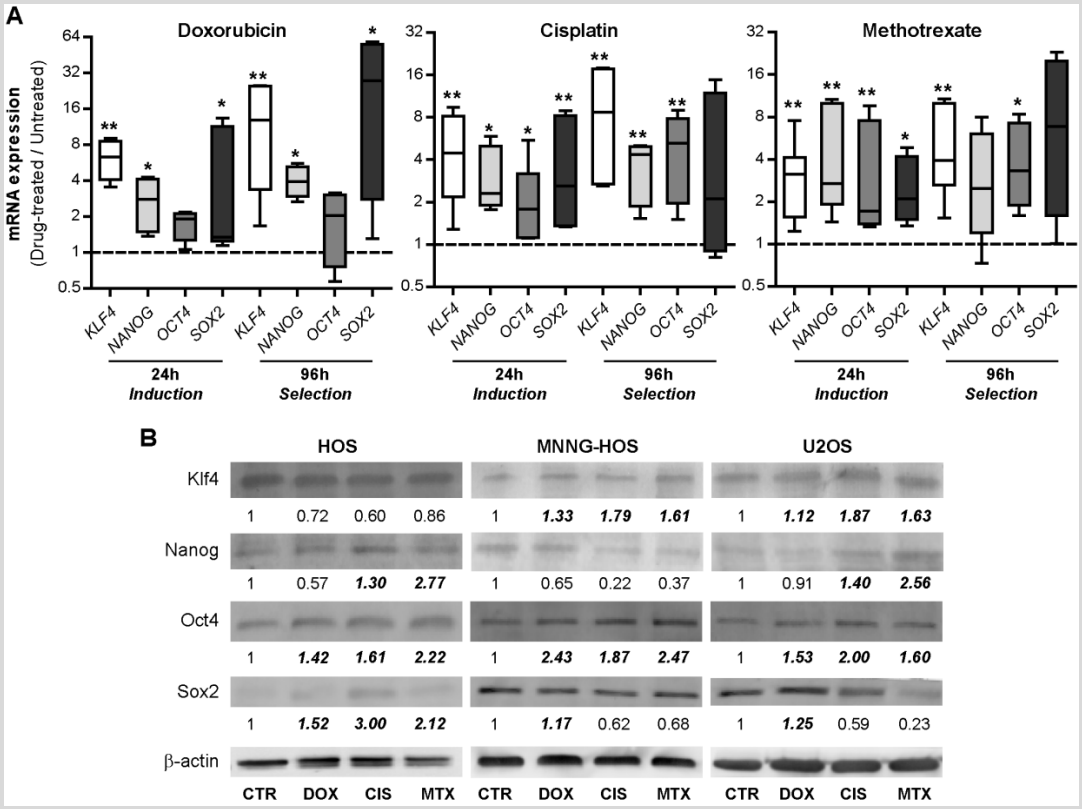


Figure 4.4. Chemotherapeutics significantly up-regulate the expression of pluripotency-related genes in osteosarcoma cells. A. Osteosarcoma cell lines were treated as monolayer cultures with doxorubicin (DOX), cisplatin (CIS) or methotrexate (MTX), for 24 or 96h. Data represents relative mRNA expression of drug-treated cells in comparison to control (CTR) cells from the panel of six cell lines described in the Methods section, after Ct values normalization using two housekeeping genes. *P≤0.05, **P≤0.01, ***P≤0.001, compared to CTR untreated cells, Mann-Whitney test. **B.** Protein expression levels of pluripotency-related markers in total protein extracts from the indicated cell lines after 48h drug exposure. Numbers below protein bands represent relative protein expression in treated *versus* untreated cells after normalization to β-actin. Bold-italic characters indicate increased expression compared to untreated cells (set at 1).

4.4.4 Wnt/β-catenin signaling is activated after chemotherapy

We checked the activation status of the developmental Wnt/β-catenin pathway in drug-treated cells, by testing the levels of *AXIN2*, a specific Wnt/β-catenin signaling target gene (Jho *et al.*, 2002). This signaling pathway appears to be specifically activated in osteosarcoma spheres displaying stem cell properties, but not in differentiated cells (**Chapter 2, section 2.4.4**). An up-regulation of *AXIN2* was observed after exposure to

all drugs, being statistically significant at 24h (doxorubicin and methotrexate) and 96h (cisplatin and methotrexate) (**Figure 4.5A**).

Next, we tested whether chemotherapy leads to an increase in the Wnt/β-catenin signaling activity through β-catenin-TCF/LEF-mediated transcriptional activity. This study was performed in the MNNG-HOS cell line, since these cells display the highest up-regulation of *AXIN2* after drug treatment. As shown in **Figure 4.5B**, TCF/LEF transcriptional activity increased significantly after treatment with doxorubicin (3.33-fold at 0.5 μM), cisplatin (3.30-fold at 5 μM) and methotrexate (2.27-fold at 0.05 μM), indicating that chemotherapy up-regulated the canonical Wnt signaling in these cells.

To further explore if Wnt/β-catenin activation could be prevented by specific Wnt inhibition, we treated pGL4-transfected MNNG-HOS cells with doxorubicin in combination with IWR-1 (a selective tankyrase inhibitor antagonist of the Wnt/β-catenin pathway). The combination of IWR-1 with doxorubicin decreased TCF/LEF reporter activity (**Figure 4.5C**) and prevented nuclear β-catenin accumulation (**Figure 4.5D**) induced by doxorubicin treatment alone. Levels of total β-catenin protein were slightly increased by doxorubicin treatment, but the combination with IWR-1 induced a reduction in total β-catenin, consistent with the expected mechanism of action of this tankyrase inhibitor, which leads to β-catenin destruction at the cytoplasmic level (**Figure 4.5D**). IWR-1 counteracted the stemness-related phenotype induced by doxorubicin, as in the presence of IWR-1, expression of *ALDH1A1*, *ABCG2*, *OCT4* and *AXIN2* transcripts was significantly decreased, compared to doxorubicin-only treated cells (**Figure 4.5E**). A similar tendency, although not significant, was observed for *ABCB1*, *KLF4*, *NANOG* and *SOX2*. Furthermore, we explored whether inhibition of Wnt stemness-related pathway in MNNG-HOS cells correlated with increased cell sensitivity to doxorubicin, as resistance to chemotherapy is a key characteristic of stem-like cells. Cells were treated with increasing concentrations of doxorubicin, with or without IWR-1. Wnt inhibition in fact elicited a significant decrease in cell viability, providing evidence that the inhibition of Wnt pathway can sensitize osteosarcoma cells to doxorubicin in parallel with blocking the acquisition of stemness features by tumor cells (**Figure 4.5F**).

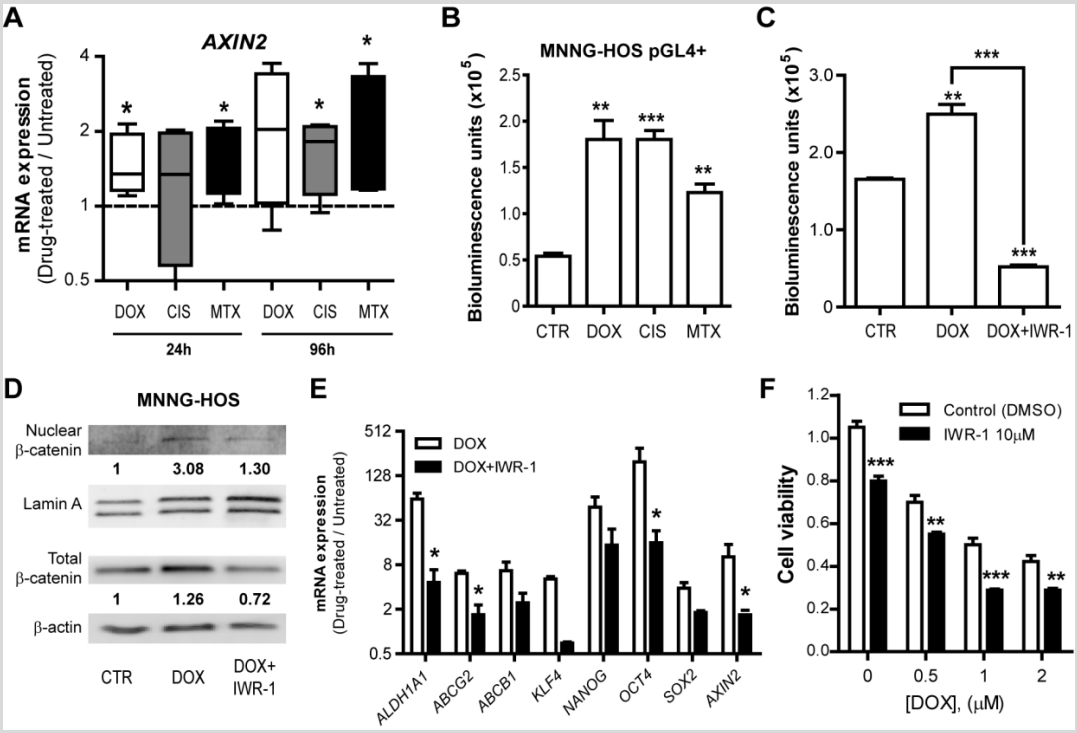


Figure 4.5. Chemotherapeutics activate the Wnt/β-catenin signaling in osteosarcoma cells. **A.** Six osteosarcoma cell lines were treated as monolayer cultures with doxorubicin (DOX), cisplatin (CIS) or methotrexate (MTX), for 24 or 96h, and tested for *AXIN2* mRNA expression. **B-C.** Luciferase reporter assays using pGL4.49[luc2P/TCF-LEF RE/Hygro] reporter plasmid in MNNG-HOS cells treated with DOX, CIS or MTX (**B**), or combination of DOX with the Wnt inhibitor IWR-1 (10 μM) for 24h (**C**). Bar graphs represent luciferase activity ± SEM (photons/s/cm^2/sr) in drug-treated *versus* untreated CTR cells, and **P≤0.01, **P≤0.001, independent samples t-test. **D.** Nuclear β-catenin protein in MNNG-HOS cells treated with DOX or the combination DOX+IWR-1. Numbers below protein bands represent relative protein expression in treated *versus* untreated cells after normalization to lamin A, used as a nuclear loading control. Levels of total β-catenin were normalized to β-actin. **E.** Effects of Wnt inhibition on the expression of stemness-related genes induced by DOX, in MNNG-HOS cells. *P≤0.05, compared to DOX-treated cells, Mann-Whitney test. **F.** Effects of Wnt/β-catenin inhibition on MNNG-HOS cell survival following treatment with DOX. Cell viability was estimated using MTT assay after 48h drug exposure. Data represents mean ± SEM (N=3). **P≤0.01, ***P≤0.001, compared to cells treated with DOX alone; Two-way ANOVA, with Bonferroni post-test. In **A.** and **E.** data represents relative mRNA expression of drug-treated cells in comparison to control (CTR) cells, after Ct values normalization using two housekeeping genes and, *P≤0.05, **P≤0.01, ***P≤0.001, compared to CTR untreated cells, Mann-Whitney test.

4.4.5 Doxorubicin up-regulates stemness markers expression in an osteosarcoma mouse xenograft model

We validated the results observed *in vitro* using an *in vivo* osteosarcoma subcutaneous model. Doxorubicin alone and combined with IWR-1 caused a decrease in the tumor growth rate as compared to the control group (**Figure 4.6A**). However, the combination of both drugs elicited a higher inhibitory effect in tumor growth comparatively to doxorubicin-treated animals, as indicated by the average mass of tumors excised from mice in control (780±125 mg) and treated groups (doxorubicin: 535±21 mg; doxorubicin+IWR1: 130±70 mg) (**Figure 4.6B**). Body-weight remained stable and did not differ significantly between the three groups during the 2-week period (**Figure 4.6C**).

Excised tumors were tested for the expression of the transcripts previously analyzed *in vitro* (**Figure 4.6D**). We found a marked tendency for increased expression of all the aldehyde dehydrogenase-related transcripts analyzed being 84.16-, 9.66- and 9.48-fold higher than the average expression in control group for *ALDH1A1*, ALDH2 and ALDH7A1 isoforms, respectively. A significantly increased expression of *ABCG2* (2.77-fold *versus* control) and *ABCB1* (5.96-fold) was observed in doxorubicin-treated tumors. Moreover, all the pluripotency-related genes tested, except *KLF4* (0.45-fold *versus* control), were also increased (27.4-fold *OCT4*, 11.42-fold *NANOG* and 3.13-fold *SOX2*) in doxorubicin-treated tumors. Doxorubicin also increased the expression of the Wnt specific target *AXIN2* on average 8.66-fold, an effect that was prevented by IWR-1. IWR-1 also counteracted the doxorubicin-induced expression for both *ALDH* isoforms and drug transporters and induced a decreasing tendency of *OCT4* and *SOX2* overexpression (**Figure 4.6D**).

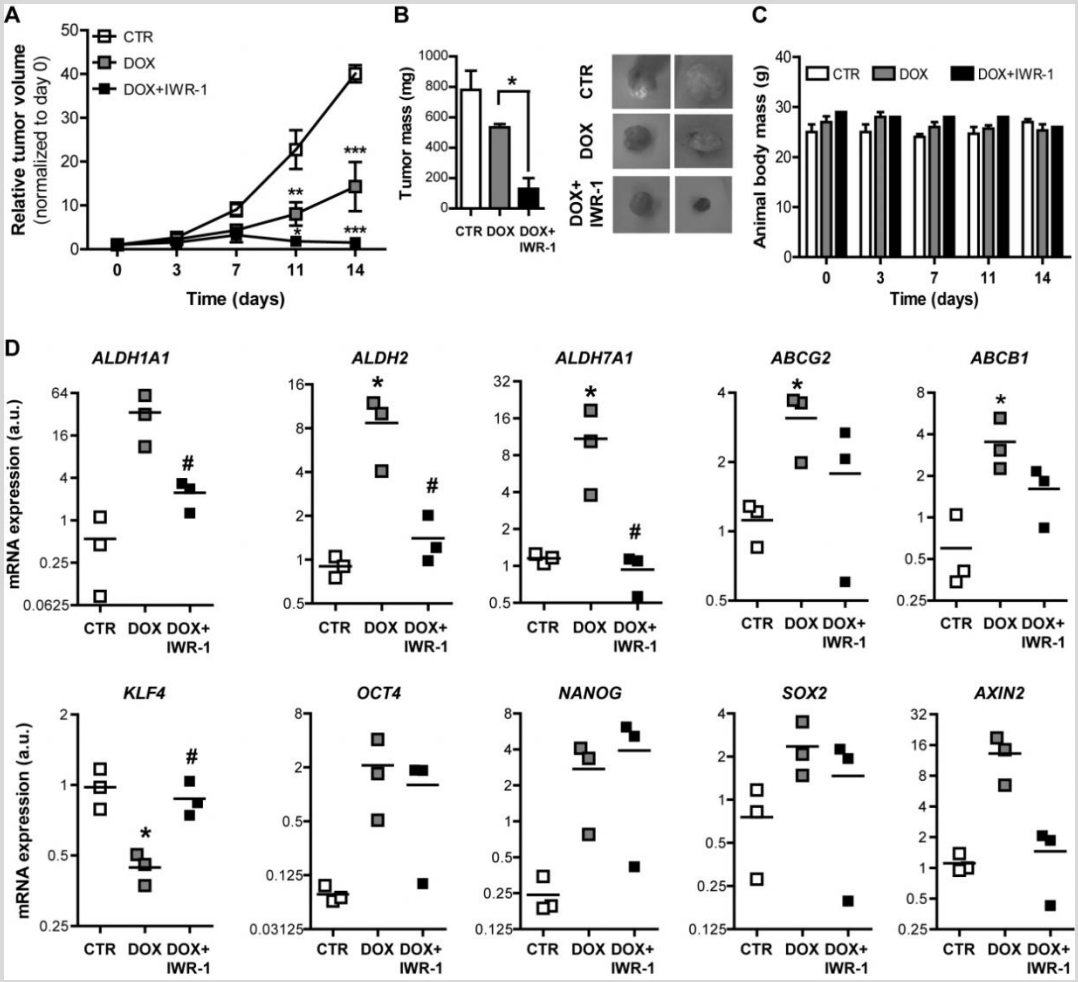


Figure 4.6. Combination of IWR-1 and doxorubicin suppresses tumor growth rate and prevents the up-regulation of stemness-related markers. A. Subcutaneous tumor volumes at the indicated time-point normalized to the tumor volume when treatments were initiated. Data shows mean ± SEM of three independent tumor samples; **P≤0.01, ***P≤0.001, two-way ANOVA, compared to control (CTR) for the same time-point. **B.** Tumor masses and representative images of excised tumors from the control and treated animals. *P≤0.05, Mann-Whitney test. **C.** Animal body mass showed no differences between CTR, DOX and DOX+IWR-1 treatment over the total time-period tested. Bar graphs represent mean ± SEM of three independent tumor samples. **D.** mRNA expression of *ALDH* isoforms, drug-efflux transporters, pluripotency-related markers and Wnt target gene *AXIN2*. All genes with the exception of *KLF4* are up-regulated in the DOX-treated tumors compared to CTR group (N=3 per group). *P≤0.05 compared to CTR group, #P≤0.05 compared to DOX-treated group, Mann-Whitney test.

4.4.6 Expression of stemness markers correlates with worse overall survival in osteosarcoma patients with a poor response to chemotherapy

To reinforce that expression of key markers associated with stemness characteristics might be enhanced upon chemotherapy and convey clinical significance to our study, we explored data from the R2 bioinformatic tool. Kaplan-Meier analysis extracted from the database revealed that a high expression of *ABCG2*, but a low expression of *ABCB1*, showed a tendency to correlate with worse overall survival in patients with a poor response to chemotherapy ("Poor response" group) (**Table 4.3** and **Figure 4.7**). Also, high *ABCG2* expression, but not *ABCB1*, correlated with worse metastasis-free survival in the same set of samples (**Table 4.3**). We found that high expression of *KLF4*, *NANOG* and *SOX2*, but not of *OCT4*, correlate with worse overall survival, in the set of samples classified as "Poor response" (**Figure 4.7B**). Moreover, in this set of samples, *KLF4* and *SOX2* expression also correlates with worse metastasis-free survival (**Table 4.3**).

Table 4.3. Overall survival and metastasis-free survival (considering the worst prognosis) in total samples and samples filtered in "Poor response" (Kuijjer R2 database, see **Material and Methods, section 4.3.6**) according to the levels of mRNA expression (high or low) of the indicated transcripts.

	No filter		Filter: Poor response	
	Worse overall survival	**Worse metastasis-free survival**	**Worse overall survival**	**Worse metastasis-free survival**
Pluripotency transcription factors				
KLF4	**High**	**High**	**High**	**High**
NANOG	Low	Low	**High**	Low
OCT4 (POU5F1)	Low	Low	**High**	Low
SOX2	Low	Low	**High**	**High**
Drug-efflux transporters				
ABCB1	Low	**High**	Low	Low
ABCG2	Low	**High**	**High**	**High**
Wnt target genes				
AXIN2	Low	**High**	Low	Low
CTNNB1	**High**	**High**	Low	Low
DKK-1	**High**	**High**	**High**	**High**
MYC	**High**	**High**	**High**	**High**

We also analyzed the expression of *AXIN2* and *CTNNB1* (β-catenin). A low expression of both transcripts correlated with worse overall survival in the "Poor response" filtered samples of the database (**Figure 4.7C**). However, we found that low expression of *AXIN2* correlated with worse overall survival in the total samples available on the dataset, but a high expression of the oncogene and Wnt mediator *CTNNB1* correlated with worse overall survival (**Figure 4.7D**). Moreover, analysis of the expression of other Wnt/β-catenin targets, such as *DKK-1* and *MYC* revealed that a high expression of these genes correlates with worse overall survival in both groups of samples analyzed ("No filter" and "Poor response") (**Table 4.3**).

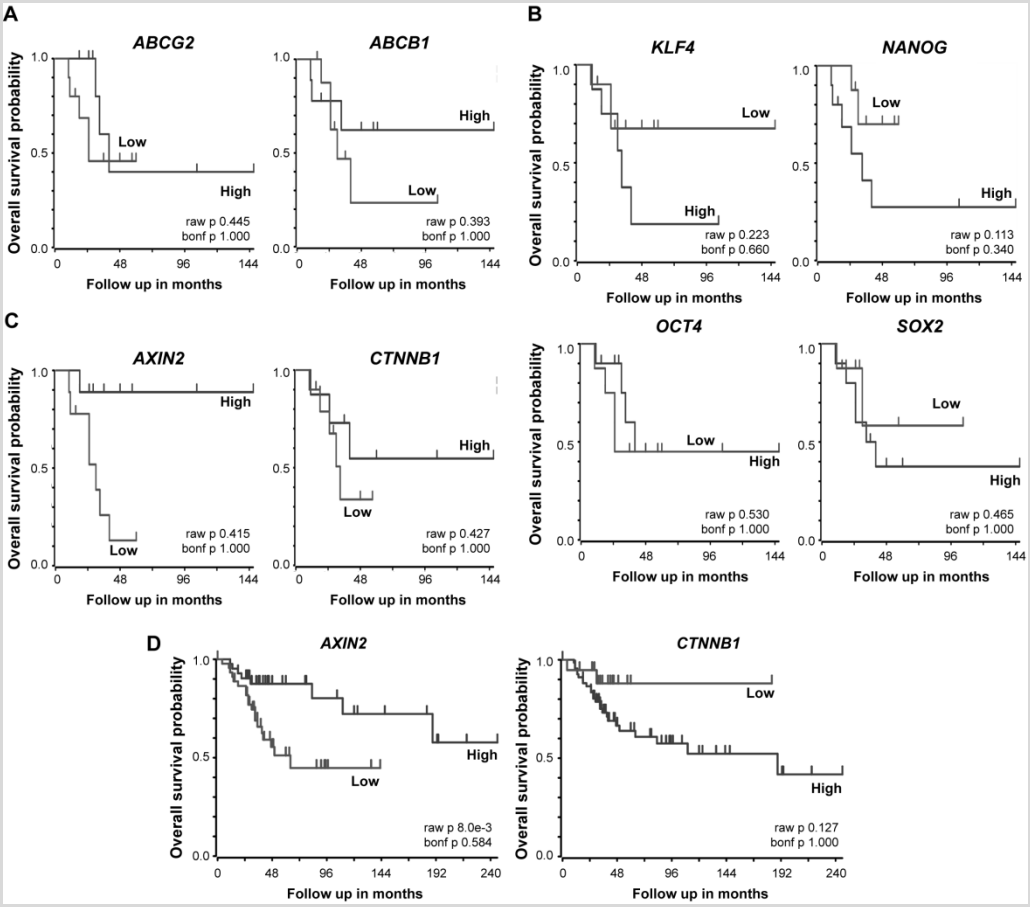


Figure 4.7. Kaplan-Meier analysis of osteosarcoma patient data available in the R2 bioinformatic tool, in patients with poor response to chemotherapy ("Poor response") (A-C) and in the non-filtered samples present in the database (D). A. Data shows that high *ABCG2* (but not *ABCB1*) mRNA expression levels correlate with worse overall survival in "Poor response" samples. **B.** Moreover, high *KLF4*, *NANOG* and *SOX2* (but not *OCT4*) transcript expression levels correlate with worse overall survival in the same set of samples. **C-D.** The same type of analysis reveals that low *AXIN2* and *CTNNB1* expression correlate with worse survival in "Poor response" samples; high *CTNNB1* (but not *AXIN2*) expression levels correlate with worse overall survival in the total set of samples of the R2 database.

4.5 Discussion

Cancer cell populations displaying stemness features and enhanced survival capacity seem to mediate chemoresistance. However, whether chemotherapy can potentiate stem-like characteristics in osteosarcoma non-stem cell populations is still unknown. Here we provide evidence that conventional chemotherapeutics used in osteosarcoma treatment induce stemness properties in differentiated osteosarcoma cells through activation of the Wnt/β-catenin pathway, as summarized in **Figure 4.8**.

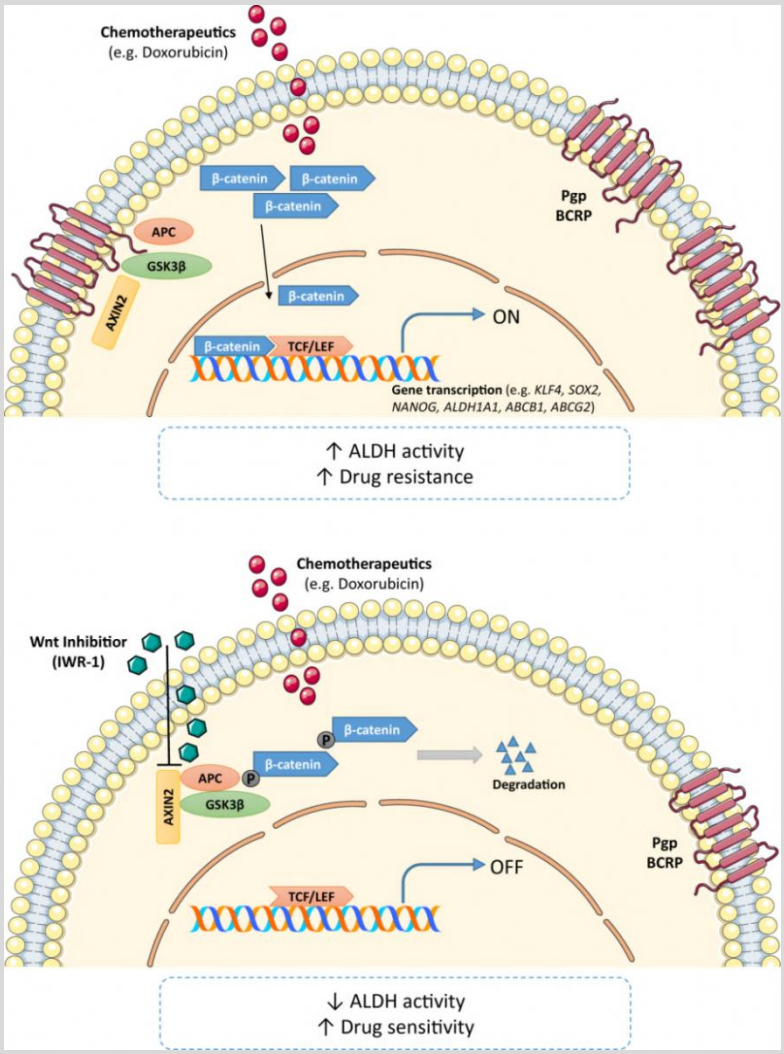


Figure 4.8. Schematic representation of the mechanism underlying the chemotherapy-induced stemness in osteosarcoma. Chemotherapeutics, such as doxorubicin, activate the self-renewal related Wnt/β-catenin signaling pathway, which modulates the activity of stemness-related networks, pluripotency transcription factors and detoxification-related pathways (ALDH and ABC transporters). Inhibiting Wnt signaling with IWR-1 counteracts the doxorubicin-induced stemness phenotype in osteosarcoma and increases cells' susceptibility to drug effects.

Aldehyde dehydrogenases play an important role in chemoresistance and have been considered an integral part of the stem cell signature in some tumors (Zhuang *et al.*, 2012). We observed a significant increase of the Aldefluor™ activity after cells' exposure to doxorubicin, cisplatin and methotrexate. Induction of this phenotype occurred immediately after 24h as an adaptive trait to stress-induced chemotherapy, and was maintained to at least 96h suggesting that a subset of cells with an intrinsic capacity to survive the harmful drug effects was enriched after treatment and concurrently displayed a pronounced capacity for drug detoxification. This phenotype was accompanied by a significant increase of the *ALDH1A1*, *ALDH2* and *ALDH7A1* isozymes, suggesting they can contribute to Aldefluor™ positivity (Le Magnen *et al.*, 2013; Landen *et al.*, 2010) but also for further development of chemoresistance (Moreb *et al.*, 2012) and associated invasive phenotypes (van den Hoogen *et al.*, 2011). We cannot exclude that other ALDH isoforms can contribute to the Aldefluor™ activity observed in our panel of cell lines, particularly *ALDH1A3*, which has been found in ALDH-positive cells of some tumors (Saw *et al.*, 2012; Marcato *et al.*, 2011). Doxorubicin induced an increase in the mRNA expression of the previous *ALDH* isozymes in the xenograft model. Our results are in line with several reports demonstrating an increase in ALDH-positive cells after chemotherapy. Some of them showed that high ALDH1 expression in post-treatment species is a predictor of poor prognosis in several cancer types (Ginestier *et al.*, 2007). However, an analysis using the R2 bioinformatic tool allowed us to conclude that, at least at genomic levels, the high expression of ALDH-related isozymes *ALDH1A1*, *ALDH2* and *ALDH7A1* in high-grade osteosarcoma tissue samples does not correlate with worse overall survival or metastasis-free survival in the groups of samples selected for analysis (data not shown), suggesting that in osteosarcomas these enzymes do not necessarily have a predictive value in terms of outcome.

Several studies demonstrated that stem-like cells express high levels of ABC transporters that actively contribute to their enhanced chemoresistance (Martins-Neves *et al.*, 2012; Lou and Dean, 2007). Augmented expression of *ABCG2* and *ABCB1* was consistently observed after doxorubicin exposure overtime but the same tendency was not so clearly seen after cisplatin or methotrexate treatment. Doxorubicin is a substrate for both *ABCB1* and *ABCG2* drug efflux transporters. Even though we observed a tendency of increased *ABCB1* expression after exposure to methotrexate for 24h, the same tendency was not maintained after 96h. Methotrexate is a chemotherapeutic drug generally not regarded as a substrate for *ABCB1* (Gregers *et al.*, 2015). Moreover,

methotrexate is preferentially discarded by dihydrofolate reductase activity in cancer cells (Shi *et al.*, 2011). These two facts may contribute to the differences observed between doxorubicin and methotrexate in the induction of *ABCB1* expression. Cisplatin did not induce significant increases of *ABCB1* and *ABCG2* transporters for both time-points tested, since this drug is not a substrate for *ABCB1* and *ABCG2* (Shi *et al.*, 2011). Nevertheless, we also observed increased expression of both ABC transporters in 2 (MNNG-HOS and U2OS) out of 3 cell lines, with HOS being the exception. This may be due to their exceptionally higher Aldefluor™ activity, which may supply HOS cells sufficient protection from cytotoxic compounds avoiding cells' necessity to explore other detoxification-related mechanisms. We did not explore the existence of a side-population fraction, but the increased *ABCG2* expression indicates that this functional characteristic may also occur in osteosarcoma cells after doxorubicin treatment. This is a plausible hypothesis since a direct correlation seems to exist between the side-population fraction and *ABCG2* mRNA expression in osteosarcoma cell lines (**Chapter 2, section 2.4.6**). Overall, these results also corroborate the recent hypothesis that stem cell subsets might be heterogeneous in nature, and respond differentially to microenvironmental stimuli such as chemotherapeutic insults (Chen *et al.*, 2012).

The interplay of signaling pathways coordinating pluripotency and self-renewal is a well-described feature in human cancers (Atlasi *et al.*, 2014). We observed significant increases in the expression of ESC-related factors at both gene and protein levels after drug treatment *in vitro*. Similar results were observed in our doxorubicin-treated *in vivo* model, with the exception of *KLF4*. Moreover, also in patient tissue samples with a poor response to chemotherapy ("Poor response"), the mRNA expression of all the previously mentioned ESC transcripts correlated with poor overall survival (**Table 4.3**). These results are in line with previous reports showing that chemotherapeutics can modulate the expression of such stemness-related markers leading to survival and expansion of highly tumorigenic cells in several tumors (Tsuchida *et al.*, 2008).

Our results showed an up-regulation of *AXIN2* expression in the panel of osteosarcoma cells lines after treatment with the three drugs, and also in the excised tumors following treatment with doxorubicin. Since *AXIN2* expression might also be modulated by other signaling pathways (Hughes and Brady, 2005), we measured the TCF/LEF-luciferase transcriptional activity in MNNG-HOS cells. All drugs increased luciferase activity, demonstrating specific activation of Wnt signaling in this cell line. Importantly, Wnt inhibition using IWR-1 prevented the doxorubicin-induced reporter

activation and expression of stemness markers, suggesting a role for the Wnt signaling in the positive regulation of stem cell-related markers. In fact, Wnt/β-catenin signaling has been shown to regulate ESC differentiation and self-renewal via β-catenin regulation (Kielman *et al.*, 2002; Cheng *et al.*, 2013) and to sustain the expression of specific pluripotency transcription factors (Sato *et al.*, 2004; ten Berge *et al.*, 2011). Moreover, Wnt/β-catenin seems to regulate the transcription of *ABCG2*/*ABCB1* in osteosarcoma. Here we showed that doxorubicin-induced expression is counteracted by Wnt inhibition, emphasizing that the regulation of key players involved in therapy resistance is under the control of Wnt signaling, as in other tumors (Yamada *et al.*, 2000; Stein *et al.*, 2012; Corrêa *et al.*, 2012), a fact that may contribute to tumor progression (Kim *et al.*, 2012). In fact, we observed that Wnt/β-catenin inhibition using IWR-1 prevented the doxorubicin-induced expression of all the transcripts we tested *in vitro*. Importantly, such effect was also verified *in vivo* in the osteosarcoma model. Co-treatment of IWR-1 with doxorubicin significantly reduced the doxorubicin-induced expression of all ALDH isoforms and a similar trend was observed for ABC transporters, *OCT4*, *SOX2* and *AXIN2*. These results corroborate previous findings of Xu *et al.* in breast cancer *in vitro* models, where β-catenin silencing reduced the percentage of Aldefluor™-positive cells as well as the expression of stem-cell related genes Bmi-1 and c-Myc (Xu *et al.*, 2015). Moreover, β-catenin has also been proposed to modulate the transcriptional activity of *ABCG2*, via Twist-1 binding to β-catenin in lung cancer (Chang *et al.*, 2015) and also the *ABCB1* mRNA expression in chronic myeloid leukemia (Corrêa *et al.*, 2012). This effect appears to be mediated via TCF/LEF binding sites within the proximal promoter regions of ABC transporters.

Importantly, data extracted from the R2 database revealed that increased expression of the oncogene *CTNNB1* and of *DKK-1* and *MYC* Wnt targets correlates with poor overall survival in osteosarcoma patient samples, further suggesting that Wnt/β-catenin activation might serve as a predictive marker of poor prognosis in osteosarcoma and deserves investigation.

In summary, chemotherapeutic drugs appear to promote a stem-like phenotype through activation of the Wnt/β-catenin pathway in osteosarcoma. This may occur by induction of a stemness phenotype that takes place immediately at 24h, a time-point at which extensive cellular death was not observed (**Complementary Figure C7.1**) followed by a selective enrichment of pre-existing cancer stem-like cells and those that acquired this phenotype and survived therapy. This agrees with evidence that tumor cells might acquire phenotypic plasticity and explore genetic programs that convene extra resilience

and stemness features. Our results open the hypothesis that conventional therapies are unlikely to lead to meaningful disease remissions and survival benefits if stem-like cells are not targeted. Specifically, we give evidence that targeting self-renewal Wnt/β-catenin pathway might be an effective approach to overcome the stemness plasticity that non-stem cells might acquire after cancer treatments in osteosarcoma.

4.6 References

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5 Chapter

General Discussion and Main Conclusions

"What does not kill us makes us stronger." – Friedrich Nietzsche

5.1 General discussion

The development of local recurrence and metastatic disease most probably attributable to poor response to standard therapy still constitute the major clinical problem preventing the cure of high-grade osteosarcoma patients. Despite progress in the research of new therapeutic targets and compounds, resistant cells displaying stem-like properties seem to play a leading role in therapeutic failures and to be the culprit cells responsible for associated tumor recurrence. From a biological standpoint, the co-occurrence of CSCs with distinct properties of self-renewal, tumorigenicity, clonogenicity and resistance to chemotherapies, within an individual tumor, is no longer questionable, but CSC-targeted therapies are not yet established for the treatment of osteosarcoma as well as to other malignancies. One of the reasons is the technical issues concerning the isolation of this rare cell subset and the difficulties in unequivocally identify specific markers that can substantiate the stem cell-like phenotype in osteosarcoma and the histological validation in patient clinical samples. Moreover, the complex genetic and phenotypic landscape underlying osteosarcomagenesis hampers the identification of unique causative disease markers that may be used as therapeutic targets.

The studies presented in this thesis aimed to identify these CSCs, characterize functionally their stemness profile and tumorigenic properties using established human cell lines and *in vivo* models, and also explore potential targets that might be used for the development of CSC-specific therapy in osteosarcoma. Our conclusions provide some aspects with clinical relevance in osteosarcoma stem cell research.

In the first part of this study we characterized the stemness profile of osteosarcoma CSCs and showed that it contains heterogeneous stem-like populations. We observed that cell lines derived from fibroblastic high-grade osteosarcoma formed spherical colonies, enriched for CSCs, more easily than cell lines derived from osteoblastic osteosarcoma, which may be explained by the higher degree of differentiation of osteoblastic tumors in comparison to fibroblastic tumors. Moreover, previous studies using gene expression profiling showed that the histological subtype is retained in cell lines (Kuijjer *et al.*, 2011), thereby showing that this phenomenon may be not restricted to cell lines but also present in primary tumor samples. We also noted that spheres derived from fibroblastic osteosarcoma cell lines partially overlapped with Aldefluor-positive cells. Moreover, complementary data shows that quiescent and tumorigenic spheres, also overexpress ALDH-related transcripts such as *ALDH1A1*, *ALDH2* and

ALDH7A1 and transcripts involved in drug efflux, such as *ABCG2* and *ABCB1* (**Complementary Results, Figure C7.2**), which overall may contribute to the heightened chemoresistance of osteosarcoma spheres. Indeed, chemoresistance is a well-documented feature of osteosarcoma CSCs, as we observed that osteosarcoma spheres are more resistant to conventional chemotherapeutics, such as doxorubicin, cisplatin and methotrexate, than their more differentiated and proliferative counterparts (**Complementary Results, Figure C7.3, Table C7.2**) and their cell cycle profile is nearly unaltered after doxorubicin treatment (**Complementary Results, Figure C7.4**).

Our results support the notion that diverse and phenotypically distinct CSCs exist within osteosarcoma that cannot be prospectively isolated based on a single marker approach. In fact, some studies have demonstrated that some degree of heterogeneity exists concerning the expression levels of stemness markers in CSC sub-populations. In fact, not all CSCs consistently overexpress the core group of e.g. pluripotency transcription factors Oct4, Nanog and Sox2 (Basu-Roy *et al.*, 2015), or express classical CSC surface markers such as CD133 (Shmelkov *et al.*, 2008). In our own set of osteosarcoma cell lines, we found consistent overexpression of Sox2 and Klf4 in spheres, but did not detect augmented expression of *OCT4* and *NANOG* in all of them, compared to corresponding parental cells (**Chapter 2, Figure 2.2**). This suggests that a variable repertoire of several ESC-like genes, other than those directly involved in pluripotency characterizes osteosarcoma stem-like cells. Complementary results showed that osteosarcoma spheres also possess enhanced capacity to undergo mesenchymal multilineage differentiation when compared to parental cells, which further substantiates their stem cell nature (**Complementary Results, Figure C7.5, Table C7.3**). Importantly, we found that osteosarcoma spheres specifically have constitutively activated the Wnt/β-catenin signaling, an important pathway regulating CSCs self-renewal, as demonstrated by nuclear β-catenin staining and TCF/LEF transcriptional activation (**Chapter 2, Figure 2.7**).

Several studies suggest that a promising strategy to circumvent chemoresistance resides in the hypothesis of inhibiting vital signaling pathways supporting CSCs' survival and stemness maintenance. We demonstrated that in osteosarcoma, the inhibition of canonical Wnt signaling compromised CSCs' survival as the tankyrase inhibitor IWR-1 elicited specific cytotoxicity towards this cell sub-population and arrested their cell cycle progression, paralleled by apoptotic cell death (**Chapter 3, Figures 3.1, 3.3, 3.4**).

Abnormal activity of pathways that control stem-cell self-renewal is crucial to the tumorigenicity of CSCs. Moreover, several studies demonstrate that these embryonic pathways may crosstalk and interact with other signaling pathways important for differentiation, pluripotentiality and survival of CSCs (Cole *et al.*, 2008; Zhang *et al.*, 2013; Sunayama *et al.*, 2010; Brechbiel *et al.*, 2014). We observed a specific down-regulation of canonical Wnt signaling in osteosarcoma CSCs elicited by the tankyrase inhibitor IWR-1 (**Chapter 3, Figure 3.5**). IWR-1 diminished the transcriptional expression of target genes such as the pathway receptors *LRP5*, *LRP5* and negative regulators *AXIN2* and *DKK-1*, and decreased the protein levels of Axin2, which participates in the cytoplasmic β-catenin destruction complex, of Cyclin D1 that regulates cell cycle progression, and of c-Myc, which is a well-known oncogenic transcription factor involved in e.g. cell apoptosis and metabolism. Overall, these effects were more pronounced in the osteosarcoma spheres as indicated by the decrease in the β-catenin nuclear-to-cytoplasmic ratios, in comparison to that in untreated spheres. The reduction of nuclear levels of β-catenin resulted in decrease of TCF/LEF transcriptional activity with subsequent down-regulation of Wnt target genes. Although a recent report suggests that inhibiting Wnt antagonists such as DKK-1 (Goldstein *et al.*, 2016) may slow the growth of osteosarcoma xenografts and inhibit metastasis, we herein demonstrate that the activity of Wnt signaling in osteosarcoma should be carefully analyzed, if possible at the single cell levels, as the most aggressive CSCs seem to be the ones with pathway activation, while bulk parental cells show inactive signaling (**Chapter 2, Figure 2.7**), as previously reported (Cai *et al.*, 2010) and are not as responsive to pathway inhibition as osteosarcoma CSCs (**Chapter 3, Figure 3.1-3.4**). Previous results in fact suggested that the Wnt/β-catenin signaling is downregulated in osteosarcoma biopsy samples compared to normal osteoblasts (Cai *et al.*, 2010). However, Wnt/β-catenin is activated in the small stem cell-like population, consistent with Wnt's role in stem cell maintenance. Therefore, instead of being considered a prognostic marker in osteosarcoma, Wnt activation should be envisaged as an important target for therapy to eradicate chemoresistant CSCs. Indeed, osteosarcoma spheres, which are constitutively resistant to doxorubicin, cisplatin and methotrexate (**Complementary Results, Figure C7.3, Table C7.3**) were sensitized to the cytotoxic effects of doxorubicin by IWR-1 treatment (**Chapter 3, Figure 3.8**).

We also observed that Wnt inhibition with IWR-1 compromised spheres self-renewal, an effect that was accompanied by the down-regulation of key pluripotency markers, specially Sox2, previously shown as a promising osteosarcoma CSC marker (**Chapter 2,**

Figure 2.2) (Basu-Roy *et al.*, 2012), *in vitro* and in the *in vivo* xenograft model (**Chapter 3, Figure 3.10**). Wnt inhibition with IWR-1 also induced a down-regulation of Aldefluor activity and the expression of Hedgehog-related markers (*GLI2*, *SMO* and *PTCH1*), which emphasizes the crosstalk between canonical Wnt signaling and other signaling pathways crucial for CSCs survival and self-renewal.

Our data is corroborated by results observed in breast cancer cells, in which inhibition of Wnt signaling also decreased levels of Aldefluor-positive cells (Xu *et al.*, 2015) and embryonic stem cell markers, *ALDH1*, *ABCG2* and also members of Hedgehog signaling *GLI1* and *GLI2* (Xu *et al.*, 2016). In our set of samples, IWR-1 also downregulated expression of *RUNX2* and *SPARC*, which are well-known mediators of the osteogenic differentiation cascade of MSCs and known to interact with canonical Wnt signaling (Gaur *et al.*, 2005; Nie and Sage, 2009; van der Deen *et al.*, 2012). Our results show that there exists crosstalk between Wnt/β-catenin signaling and pathways controlling self-renewal and osteogenic differentiation.

We also found that IWR-1 treatment did not affect cell viability of normal human MSCs (**Chapter 3, Figure 3.1**), which poses an interesting challenge for the design of drugs aimed at depleting the CSCs while sparing the function of normal cells. The *in vivo* anti-tumor capacity of Wnt/β-catenin inhibition was demonstrated in xenografted osteosarcoma animal models (**Chapter 3, Figure 3.10**). The massive decrease in tumor burden observed in the subcutaneous model after IWR-1 treatment, either alone or in combination with doxorubicin, reveals the crucial role of the canonical Wnt signaling in mediating the survival and proliferative capacity of stem-like tumor-initiating cell populations. In fact, we observed that the reduced tumor growth elicited by IWR-1 and doxorubicin combined treatment was associated with a down-regulation of TCF/LEF transcriptional activity and expression of *AXIN2*, Sox2 and nuclear β-catenin. Other groups also observed that Wnt inhibition compromised typical features of tumor aggressiveness, such as cellular invasion, clonogenicity and *in vivo* tumor development, in murine and human osteosarcoma cells (Brun *et al.*, 2013; Li *et al.*, 2014) and for instance in colon cancer (Lau *et al.*, 2013). A combinatorial treatment approach must be considered for osteosarcoma patients, being advantageous and synergistic in killing both CSC and non-CSC populations within the tumors, as recently observed in other tumors (Arqués *et al.*, 2016).

Altogether these results showed that blockade of Wnt/β-catenin signaling suppressed the growth and typical phenotypic characteristics of osteosarcoma CSCs,

and suggest that Wnt/β-catenin signaling inhibition constitutes an important CSC-targeted therapeutic approach in osteosarcoma.

Conventional chemotherapy has undoubtedly promoted significant survival in patients suffering from osteosarcoma in the last decades. However, of particular interest for tumor progression associated with therapy failures, relapses and metastasis is the stress inflicted onto the residual non-killed cancer cells and the resultant cell state phenotypic transition. It is possible that cell stress imparted by cytotoxic agents actively induces a state transition specifically into a stem-like state, which actually is a stress-response state. In fact, we proved that short-term exposure to chemotherapeutic agents such as doxorubicin, cisplatin and methotrexate induced stem-like cell phenotypic transition in differentiated osteosarcoma cells, as demonstrated by the increased expression of pluripotency markers and ALDH-related activity and isozymes, and also of drug efflux transporters P-glycoproteins and BCRP, an effect that was mediated by activation of the Wnt/β-catenin pathway. Expression of these markers was particularly enhanced by doxorubicin treatment. These results primarily observed *in vitro* were confirmed *in vivo* in a doxorubicin-treated tumor model.

A variable repertoire of ESC-related markers is found in osteosarcoma CSCs and these markers are probably important contributors for the aggressive phenotype of osteosarcoma, as their high expression correlates with poor response to therapy (**Chapter 4, Figure 4.7**). For instance, Zheng *et al.* found that only *SOX2* expression was augmented upon doxorubicin treatment, but not *OCT4* or *NANOG* (Zheng *et al.*, 2013). These results emphasize the fact that chemotherapy may not alter the expression of certain stemness genes, which mechanistically operate together in normal embryonic stem cells (Kashyap *et al.*, 2009) and that were previously well-established as CSC markers. Moreover, they enlighten for both the existence of native heterogeneous CSC populations among tumors and also for the occurrence of genotypic/phenotypic transitions that may occur after systemic chemotherapies, which can also be dependent on the temporal therapeutic window (Goldman *et al.*, 2015) and the tumor microenvironment. In fact, in the study described in **Chapter 4**, we provide evidence corroborating this assumption as the expression of *KLF4*, indicated as a candidate osteosarcoma CSC marker (**Chapter 2**) is increased *in vitro*, but not *in vivo*, after doxorubicin treatment (**Chapter 4, Figures 4.4, 4.6**).

Inhibiting Wnt with IWR-1, however, prevented the altered cell state dynamics induced by chemotherapeutic stress *in vitro* and *in vivo* (**Chapter 4**). These results pose

the question that drug-tolerant cells support the reprogramming of stemness networks which then contribute to the plastic heterogeneity of tumor cells subjected to stressful conditions and influence the overall response to treatment. Moreover, to convey some clinical relevance to this hypothesis, analysis of microarray data from the publicly available R2 database showed that expression of some pluripotency transcripts correlates with worse overall survival in patients responding poorly to chemotherapy (**Chapter 4, Table 4.3, Figure 4.7**).

5.2 Main Conclusions

From the results described in this thesis, we presented evidence that

- different techniques employed in CSC isolation seem to enrich for molecularly heterogeneous populations;
- osteosarcomas contain a small subset of heterogeneous stem-like cell populations with overexpression of Sox2 and Klf4 pluripotency-markers;
- osteosarcoma cell lines have not completely overlapping cell subsets with enhanced Aldefluor-activity as well as a side-population subset;
- tumorigenic osteosarcoma spheres display activated Wnt/ß-catenin signaling;
- inhibiting canonical Wnt signaling in osteosarcoma CSCs with IWR-1 impaired self-renewal, Wnt activity and the expression of important stemness-related genes;
- IWR-1 induced apoptosis of osteosarcoma CSCs and in combination with doxorubicin treatment elicited synergistic cytotoxicity;
- *in vivo*, IWR-1 alone and in synergy with doxorubicin significantly decreased tumor progression, associated with down-regulation of TCF/LEF transcriptional activity, nuclear β-catenin and expression of the putative CSC marker Sox2;
- chemotherapeutic agents induced a phenotypic stem-like cell transition in osteosarcoma, by inducing ALDH activity and expression of pluripotency genes and ABC transporters;
- doxorubicin up-regulated stemness markers by activation of Wnt/β-catenin and pathway inhibition prevented the doxorubicin-induced stem-like phenotype;
- expression of stemness-related markers correlated with worst survival in osteosarcoma patients.

Altogether, our results suggest that phenotypic heterogeneity exists among osteosarcoma stem-like cells and depends on the microenvironmental status, and that the Wnt/β-catenin is a critical target for therapeutic intervention in osteosarcoma cancer stem cell populations.

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6

ANNEX I
METHODS TO ISOLATE CANCER STEM CELLS

Despite intensive research in the past decade, owing to CSCs' rarity and absence of established markers, the definition of CSCs has been done mostly based on functional criteria. In fact, the discovery of a putative population of stem cells in a certain tumor tissue is mostly contingent to the application of rigorous in vitro culture techniques. In this section, we highlight several of the *in vitro* assays currently used to identify and isolate CSCs, emphasizing their principles, utility and potential pitfalls in CSC research.

6.1 SPHERE-FORMATION ASSAY

The identification of CSCs based on the sphere-forming assay was firstly reported in human brain tumors (Singh *et al.*, 2003), a study that actually was subsequent to the very initial use of the sphere assay. In fact, Reynolds and Weiss were the first to successfully isolate normal neural stem cells using the historical neurosphere assay (Reynolds and Weiss, 1992). These facts show that CSC research is sometimes based on the transferring of knowledge and technical principles used in normal stem cell research. In the particular case of osteosarcoma, one decade ago Gibbs and colleagues were the first to successfully report the identification and isolation of osteosarcoma CSCs (Gibbs CP *et al.*, 2005). Since then, several studies have employed the sphere assay as proposed, including our own group (Martins-Neves *et al.*, 2012; Gonçalves *et al.*, 2015). The protocol that Gibbs and co-workers optimized has been consistently used throughout our previously published work and in the studies described in this thesis.

The basic principle underlying the sphere assay (**Figure C6.1**) relies on the capacity of the most primitive, undifferentiated and resilient cells to survive the harsh conditions of this assay, which do not support the survival and long-term expansion of the majority of cells that eventually die by e.g. anoikis, that is the lack of a substrate where the cells can attach.

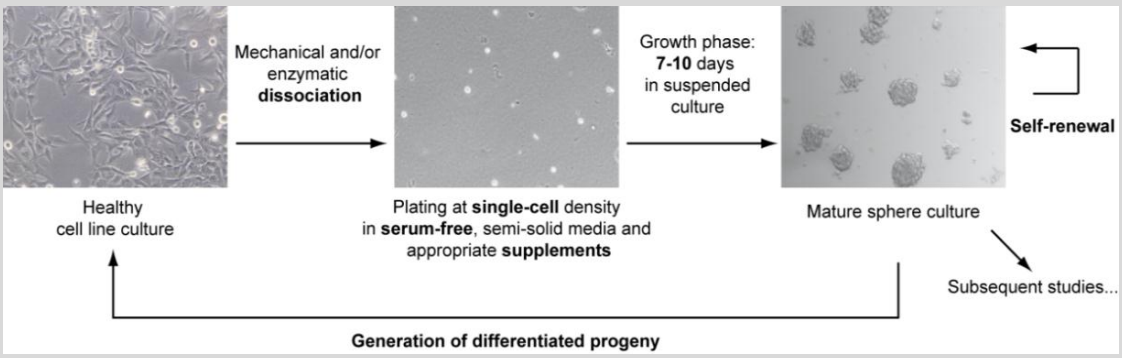

Figure C6.1. Schema explaining the procedures involved in the sphere-forming assay. Diagram depicting the fundamental steps and parameters involved in the sphere assay protocol. Pictures represent the MG-63 osteosarcoma cell line, used in the studies described in this thesis.

The culture conditions employed in the sphere assay usually include:

▪**Culture of cells in serum-free medium and non-adherent surfaces**. Serum-supplemented media and adherence are both known to promote cancer cell differentiated state (Vescovi *et al.*, 2006). Therefore, the elimination of the unknown serum-derived factors and adherence facilitate the selection of the more undifferentiated cells. The serum starvation of the culture system and the absence of substrate or adhesion factors enable the selection of the most primitive cells that are able to survive, and can give rise to spherical clones.

▪**Use of specific mitogens and defined supplements**. Compounds such as epidermal, basic fibroblast and platelet-derived growth factors, leukemia inhibitory factor, progesterone, insulin, selenide, transferrin, putrescine, N2 and B27, or diverse combinations of these supplements have been used in the sphere assay. These cytokines and growth factors support the survival and expansion of responsive cells, such as stem and progenitor cells (Reynolds and Rietze, 2005; Wan *et al.*, 2010).

▪**Plating of cells at the single-cell level**. Assuring that cells are plated as single-cells increases the probability that each sphere formed is derived from a single cell and is therefore clonal. Single-cell plating also ensures that sphere-formation is due to cellular proliferation and not cellular aggregation. The presence of a semi-solid medium such as methylcellulose or collagen can help the maintenance of a single-cell culture, assuring that each clone is in fact derived from a unique suspended-growing cell. However, it should be noted that cell aggregation can still occur, being the cell seeding density and the use of a pure single-cell suspension the most determinant factors to avoid this bias.

Culturing of cells in the abovementioned conditions (or variations) has been used as the standard *in vitro* method for identifying CSCs. The sphere assay and its fundamental steps

(**Figure C6.1**) candidate competent cells to exhibit typical stem-like behavior, namely self-renewal over extended periods of time (by serial sphere dissociation and replating in sphere culture conditions) and capacity to generate differentiated progeny (Clarke *et al.*, 2006). Several studies, including our own, provide evidence that this cost- and time-effective method permits the successful isolation of CSC displaying stem-like properties and can be widely used in cancer research (Fujji *et al.*, 2009; Coulon *et al.*, 2011; Chen *et al.*, 2012).

Sphere-forming assays, like many other *in vitro* assays, are associated with some limitations. In fact, some critical considerations have to be mentioned, since over the years experimental variability has been introduced, which substantially complicates data comparison and interpretation (Pastrana *et al.*, 2011). Among the key experimental parameters essential for the accurate interpretation of sphere-forming assays are:

▪**Use of media components**. Currently there is no standardized culture media formulation that allows comparison of results between different tumor types and sometimes even within the same tumor type. Different studies have employed and optimized an enormous variety of recipes consisting of different culture media type and cocktail supplement combinations. Variability has been observed at the levels of type of supplement, concentration and cell exposure time to name a few (Wan *et al.*, 2010).

▪**Cell density**. The central tenet of the sphere assay relies on the clonal sphere origin. However, different groups have been considering different cell concentration ranges as appropriate for clonal conditions of growth. Several factors can affect clonal density, since fusion of spheres can occur, even at low density, and spontaneous and experimenter-induced locomotion can be observed using live cell imaging techniques (Coles-Takabe *et al.*, 2008). Therefore, semi-solid matrices may help avoiding intrinsic cell mobility and sphere aggregation. Nevertheless, caution should be taken when estimating stem cell frequency based on the number and size of spheres, as the most rigorous way of affirming clonality involves plating single cells in single mini-wells (clonogenic assay).

In spite of the mentioned shortcomings, the sphere assay is still widely used in CSC research; importantly, most researches are aware that this simple and easy to perform *in vitro* assay represents a phenomenon that does not occur in *in vivo* settings (Barrett *et al.*, 2012); however, the physical architecture of spheres can provide such a tridimensional environment that the hypoxic inner compartment of spheres may enhance and maintain CSC self-renewal, while the surrounding proliferating non-stem cells can provide paracrine effects, thus mimicking an *in vivo* niche (Heddleston *et al.*, 2009). In line with this, the CSC community is aware that the sphere assay mainly enriches for a population of stem and progenitor cells, together with more differentiated cells (sphere heterogeneity) and the

capacity to form spheres does necessarily mean that a certain CSC population will present tumorigenic ability (Barrett *et al.*, 2012). In fact, our own unpublished results revealed that the osteosarcoma cell line MG-63 is not tumorigenic, even after 60 days of follow-up upon cell inoculation in immunocompromised animals, and despite that it showed the highest sphere-forming efficiency among the group of cell lines explored in this thesis (**Chapter 2**).

6.2 Side-population Assay

The side-population phenotype is also used in the CSC field to identify and isolate stem-like cells, using flow cytometry (**Figure C6.2**). This technique was firstly described for use in the prospective isolation of normal hematopoietic stem cells, based on their high capacity for an active extrusion of cytotoxic compounds. In fact, the multidrug-resistance related protein BCRP has been found to be preferentially expressed in hematopoietic progenitor cells and linked to efficient efflux capacity of the nuclear-labeling dye Hoechst 33342, being the key determinant of the so-called side-population phenotype (Zhou *et al.*, 2001; Scharenberg *et al.*, 2002).

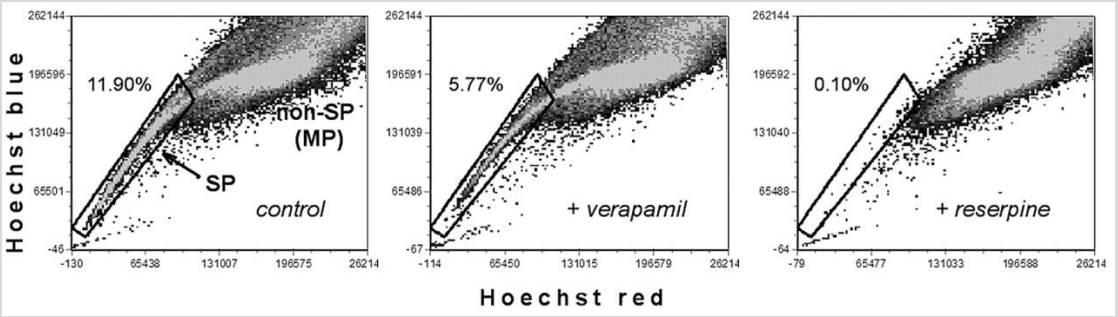

Figure C6.2. Flow cytometry of the side-population. Side-population (SP) cells can be identified using Hoechst-33342 staining. Inhibitors of ABC transporters such as verapamil or reserpine can be used to prevent Hoechst-33342 dye extrusion and therefore discriminate between SP and main population (MP) cells. Depending on the type and dosage of ABC inhibitor used, different proportions of SP cells may be found within the same sample. Adapted from (Jakubikova *et al.*, 2011).

Although the side-population assay has been used in CSCs identification, it should be noted that, similar to what occurs in normal stem cells, dye efflux is not a common property of all stem cell populations, the side-population phenomenon is not exclusive of stem cells and also not every cancer contains a subset of side-population cells (Golebiewska *et al.*, 2011). Therefore, critical parameters of the side-population assay are the preparation of a viable single cell suspension, taking into consideration cell viability, culture density and cell dissociation; type, concentration and possible toxicity of the vital dye used; incubation

method namely temperature and duration; type and concentration of the ABC transporters' inhibitor used to establish the negative controls; and the accuracy of the discrimination of debris, dead and single cells.

6.3 ALDEFLUOR™ ASSAY

The Aldefluor™ assay has increasingly been used in the identification and isolation of CSCs in several tumor types. Enzymes belonging to the aldehyde dehydrogenase (ALDH) superfamily appear to fulfill the criteria as a marker for both normal and cancer stem cells. Two decades ago, Jones and colleagues reported for the first time that viable human hematopoietic stem cells and also leukemic stem cells could be identified by flow cytometry based on the intracellular activity of ALDH1 (Jones *et al.*, 1995). Some years later, a more efficient strategy was reported (Storms *et al.*, 1999), which placed the foundations for the currently known Aldefluor™ assay (**Figure C6.3**).

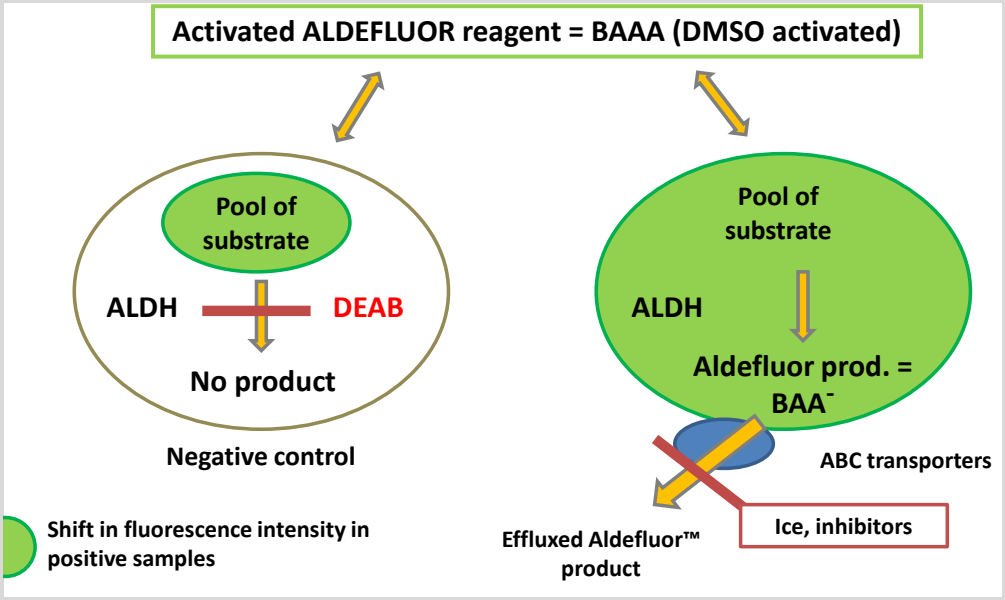

Figure C6.3. Principles of the Aldefluor™ assay. Diagram depicting the Aldefluor™ assay protocol. Activated Aldefluor™ substrate (BODIPY® -aminoacetaldehyde, BAAA) is oxidized by aldehyde dehydrogenases (ALDH) and converted into a fluorescent product (BAA^-), which accumulates at the cytoplasm in ALDH-positive cells. 4-(diethylamino)benzaldehyde (DEAB), a general inhibitor of ALDH is used to establish the control for background fluorescence.

The basic principles of the Aldefluor™ protocol are depicted in **Figure C6.3**. Essentially, cells that express ALDH activity have the capacity to uptake the uncharged activated Aldefluor™ substrate BAAA (BODIPY® -aminoacetaldehyde) by passive diffusion. The

enzymatic activity of ALDH then oxidizes BAAA into its fluorescent negatively charged form (BAA^-), which is retained inside the cell. The use of ABC transporters' inhibitors or the assay buffer provided by the manufacturer prevents the active efflux of BAA^-. Highly fluorescent cells can then be detected, gated and sorted using flow cytometric techniques. The distinction between ALDH-high or ALDH-positive from ALDH-low or ALDH-negative cell populations can be made easily and specifically via the use of the specific ALDH inhibitor DEAB (4-(diethylamino)benzaldehyde), which quenches background fluorescence intensity and is used as a negative control.

6.4 Surface and CD markers

The surface markers that describe the stem cells in solid tumors are not as well-characterized as those identified for the hematopoietic cell system. Stem cells of different tissues are not all identical and the dissimilarities concerning e.g. location, self-renewal and differentiation are often reflected by specific combinations of phenotypic markers. These different combinations of markers form the basis for distinguishing a certain stem cell type from another one. In the context of cancer, it appears that CSCs express many of the markers commonly used to identify normal stem cells (either embryonic or adult somatic stem cells). In general, these cell surface markers are very advantageous to identify and isolate CSC populations using the appropriate cell sorting technologies and protocols (**Figure C6.4**). This technique has been widely used in both established cell lines and primary tumor samples.

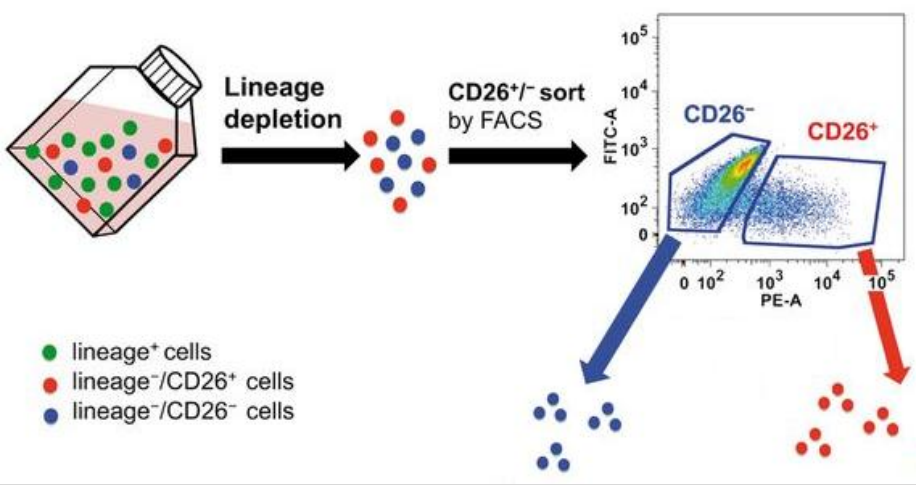

Figure C6.4. Sorting of CD-positive cells using flow cytometry. Experimental setup used to identify and isolate CD26-positive cells in chronic myeloid leukemia using fluorescence-activated cell sorting (FACS) analysis. Separated cells (CD26+ and CD26-) can be used in subsequent parallel experiments. Adapted from (Herrmann *et al.*, 2014).

The identification of CSC surface markers has been more elusive in mesenchymal tumors than in tumors originated from other tissue types. This occurs in part due to the lack of agreement on the markers that identify and unequivocally select mesenchymal progenitor cells (Chamberlain *et al.*, 2007; Kaltz *et al.*, 2010; Mohseny and Hogendoorn, 2011; Lv *et al.*, 2014). Nevertheless, for osteosarcoma CSCs several cell surface markers have been reported, including CD49 (Ying *et al.*, 2013; Penfornis *et al.*, 2014), CD133 (Li *et al.*, 2013; Ying *et al.*, 2013), CD117 (Adhikari *et al.*, 2010), CD248 (Rouleau *et al.*, 2012) and CD271 (Tian *et al.*, 2014), but these markers for osteosarcoma also require a broader validation.

Technical limitations of sorting CSC based on cell surface markers' expression include: trypsin digestion during the preparation of cell suspensions that may affect the way cells are selected, due to altered marker expression; identification of a positive phenotype is often described as *high* or simply *positive*, *middle*, *low* and *negative* to describe the properties of the sorted cells, but these terms are subjective and results depend on the protocols used for cell preparation and gating strategies, which are also commonly not described in sufficient detail to enable accurate reproduction; also, cell viability of sorted cell population can be questioned due to the normal long duration of sorting procedures (Wan *et al.*, 2010). These technical pitfalls are also applied to CSCs' identification using the Aldefluor™ and the side-population assays, especially because all these methods attempt to identify / isolate very low percentages of phenotype-positive cells within the whole tumor cell population.

6.5 References

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7

ANNEX II
COMPLEMENTARY METHODS, RESULTS AND REFERENCES

7.1 COMPLEMENTARY MATERIAL AND METHODS

7.1.1 Cytotoxicity assays - Estimation of IC_{50} values

Spheres and parental cells from MG-63, MNNG-HOS and SJSA-1 cell lines were screened for their profile of sensitivity to drugs used in the treatment of patients with osteosarcoma, which includes doxorubicin (0-100μM, hydroxydaunorubicin LUMC Pharmacy), cisplatin (0-100μM, LUMC Pharmacy) and methotrexate (0-500μM, Emthexate PF, methotrexaatnatrium, Pharmachemie Teva, Haarlem, The Netherlands).

Parental cells and spheres were dissociated, plated in 96-well plates (5,000 and 7,000 cells/well, respectively) and allowed to attach overnight. Cells at exponential growth were incubated with increasing concentrations of doxorubicin, cisplatin and methotrexate. Stock solutions of doxorubicin were stored at 4°C, and of cisplatin and methotrexate at room temperature, all protected from light. For all drugs, 10-fold working dilutions were prepared in sterile PBS, immediately before use. Forty-eight hours after drug exposure, culture media was removed and 100μL of fresh media containing WST-1 Cell Proliferation Reagent (Roche Diagnostics Netherlands B.V., 1:10 dilution) were added to each well, to estimate the number of metabolically viable cells remaining in culture after drug treatment. Quantification of the water-soluble formazan product after WST-1 mitochondrial conversion by viable cells was performed in a microplate reader operating in colorimetric mode (Perkin Elmer Victor 3 Model 1420-012 multi-label microplate reader). Cellular growth inhibition was calculated by dividing the absorbance of drug-treated cells by that of control untreated wells. These data was then used to compute dose-response curves and estimate the absolute half-maximal

inhibitory drug concentration (IC_{50}), using Origin Pro 8.0 (OriginLab Corporation) and the dose-response fitting formula:

$$y = A_1 + \frac{A_1 - A_2}{1 + 10^{[\mathrm{Log}(x_0) - x]p}}$$

In this equation, IC_{50} is represented by x_0; A_1 and A_2 are the amplitude of the baseline and maximum response, respectively; and p is the slope. In the analysis, A_2 was set equal to 1.

7.1.2 Multilineage differentiation studies

Cells were tested for multilineage differentiation, as previously described with some modifications regarding cell density and compound-treatment schedule (Mohseny *et al.*, 2011). Adipogenic and osteogenic differentiation were induced by culturing 6,000 cells/mL and 5,000 cells/mL, in 24-well and 12-well plates for 2.5 and 3 weeks, respectively, using the basal culture medium α-MEM (Lonza, Cat. No. BE12-169F) supplemented with 10% v/v FBS, 1% v/v P/S and 1% v/v GlutaMAX™. For chondrogenic differentiation, 1×10^6 cells/mL were pelleted and cultured in suspension in U-shaped 96-well plates (Greiner Bio-One) for 5 weeks. Basal medium contained DMEM high-glucose (Invitrogen) supplemented with 1% v/v P/S, 1% v/v GlutaMAX™, proline (40 µg/mL, Sigma), sodium pyruvate (100 µg/mL, Sigma) and insulin, human transferrin and selenous acid (ITS premix, 10µg/mL, BD™ 354350). Human mesenchymal stem cells (MSCs) were used as a positive control in all assays. For all experiments, fresh culture media and specific supplements were added to cells twice a week (**Table** C7.1 **C7.1**). Cells were stained as previously described (Mohseny *et al.*, 2011). Stained sections were viewed using a Leitz DMRD microscope on brightfield mode, in order to analyze extracellular matrix deposition and the presence of cells with chondrocyte-like morphology. Extent of differentiation was scored semi-quantitatively: 0 (no staining), 1 (moderate) and 2 (strong).

Table C7.1. Specific supplements used in adipogenic, osteogenic and chondrogenic differentiation studies.

Compound	Concentration	Notes
Adipogenic differentiation		
Ascorbic acid 2-phosphate	50 µg/mL	From day 0
Dexamethasone	0.25 µM	From day 0
Insulin	100 µg/mL	From day 0
3-Isobutyl-1-methylxanthine (IBMX)	0.5 mM	From day 0
Indomethacin	50 µM	From day 4
Osteogenic differentiation		
Ascorbic acid 2-phosphate	50 µg/mL	From day 4
Dexamethasone	10^{-7} M	From day 0
β-glycerolphosphate	5 mM	From day 11
Chondrogenic differentiation		
Ascorbic acid 2-phosphate	50 µg/mL	From day 0
Dexamethasone	10^{-7} M	From day 0
Transforming growth factor beta 3 (TGF-β3)	10 ng/mL	From day 0
Bone morphogenetic protein-6 (BMP-6)	500 ng/mL	From day 0

***Note**:* All compounds are from Sigma, except TGF-β3 and BMP-6 (R&D Systems, Cat. No. 243-B3 and 507-BP, respectively).

7.2 Complementary Results

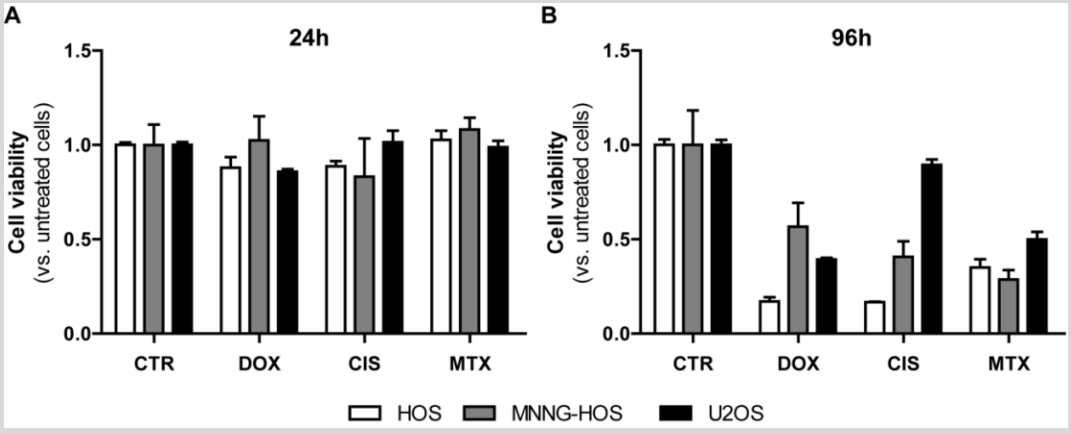

Figure C7.1. Viability of HOS, MNNG-HOS and U2OS cells after treatment with doxorubicin 0.5µM, cisplatin 5µM or methotrexate 0.05µM for 24h (A) or 96h (B). Cell viability was determined using the MTT assay, according to the manufacturer instructions.

7.2.1 Elevated expression of ABC transporters and aldehyde dehydrogenases may mediate osteosarcoma spheres chemoresistance

Osteosarcoma CSCs isolated using the sphere assay displayed increased resistance to conventional chemotherapies. To further characterize possible signaling pathways mediating this resistance profile, we analyzed the expression of ABC drug efflux transporters know to play a key role in actively extruding cytotoxic compounds from the cells, and also the expression of isozymes involved in the aldehyde dehyodrogenase signaling, well-known to mediate active detoxifying functions within the cell cytoplasm. We selected these two classes of biological pathways based on our previous results, where we showed that *ABCG2* expression correlated with the side-population phenotype (**Chapter 2**). Additionally, increased Aldefluor™ activity was also found in both osteosarcoma parental cells and spheres (**Chapter 2**). Furthermore, doxorubicin, cisplatin and methotrexate induced an increase in the expression of *ABCG2*, *ABCB1*, Aldefluor™ enzymatic activity and the expression of related isozymes *ALDH1A1*, *ALDH2* and *ALDH7A1* (**Chapter 4**).

In this part of the work, we then characterized the expression of the aforementioned transcripts in osteosarcoma CSCs (spheres) and compared with the expression in parental cells. Results revealed that the expression of drug efflux transporters *ABCG2* and *ABCB1* was at least 2-fold and 1.5-fold, respectively, significantly higher in MG-63, MNNG-HOS and SJSA-1 spheres than in corresponding parental cells (**Figure C7.2A**). Also, expression of *ALDH1A1* and *ALDH2* isozymes was at least 2-fold higher in osteosarcoma spheres of the three cell lines. However, *ALDH7A1* expression was significantly increased only in MG-63 spheres, compared to parental cells, with no relevant differences being observed for MNNG-HOS and SJSA-1 cells (**Figure C7.2B**).

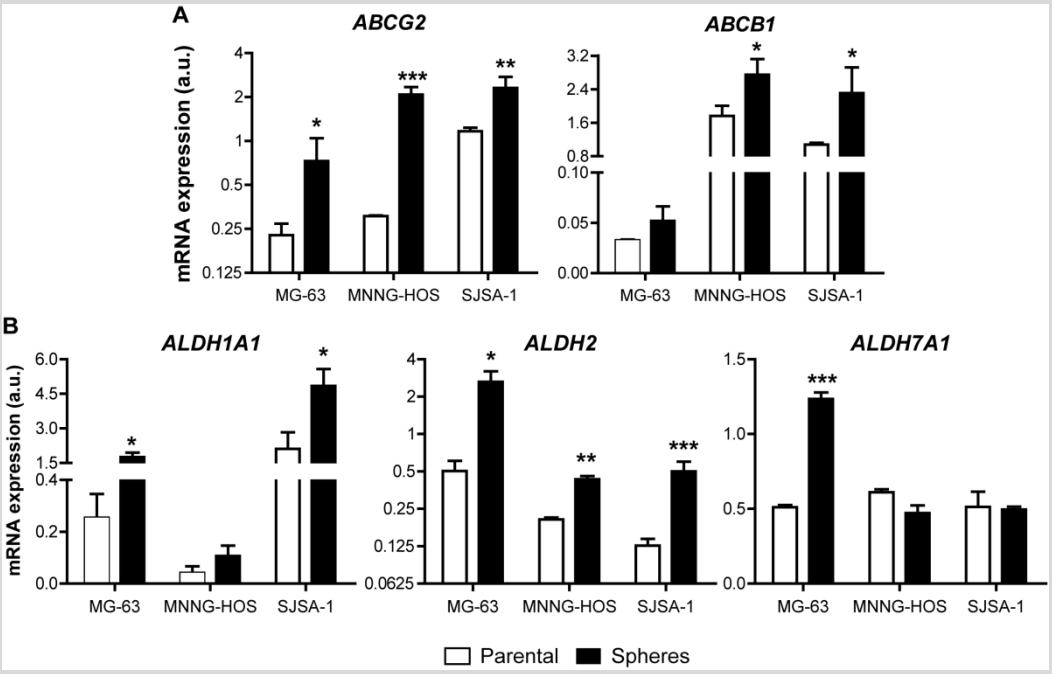

Figure C7.2. Increased expression of drug efflux-related transporters and aldehyde dehydrogenase isozymes may mediate spheres chemoresistance. A. Constitutive mRNA expression of drug efflux transporters *ABCG2* and *ABCB1* is higher in spheres than in parental cells. **B.** mRNA expression of aldehyde dehydrogenase isozymes *ALDH1A1* and *ALDH2* is higher in spheres than in parental cells. *ALDH7A1* is increased in MG-63 spheres compared to parental cells, but not in MNNG-HOS and SJSA-1 cell lines. Expression of these genes was analyzed by quantitative RT-PCR, as described in **section 2.3.9**. Bar graphs represent mRNA expression ± SEM in parental cells and spheres, after normalization of Ct values to two housekeeping genes. *P<0.05, **P<0.01, ***P<0.001, compared to parental cells, Mann-Whitney test.

7.2.2 Osteosarcoma spheres display increased resistance to conventional chemotherapeutics

We tested whether CSCs (spheres), isolated from diverse human osteosarcoma cell lines using the sphere-formation assay, were more resistance to doxorubicin, cisplatin and methotrexate than the main parental population. Cells were exposed to increasing concentrations of the three drugs during 48h and afterwards assayed with a cell viability test (**Figure C7.3**). In fact, spheres from MG-63, MNNG-HOS and SJSA-1 cell lines showed reduced sensitivity to all drugs in test, with IC_{50} values being significantly higher than those of corresponding monolayer-growing parental cells (**Table C7.2**). Overall, MG-63 spheres displayed the highest resistance profile of the three cell lines, and methotrexate was the least harmful drug to the sphere cell populations.

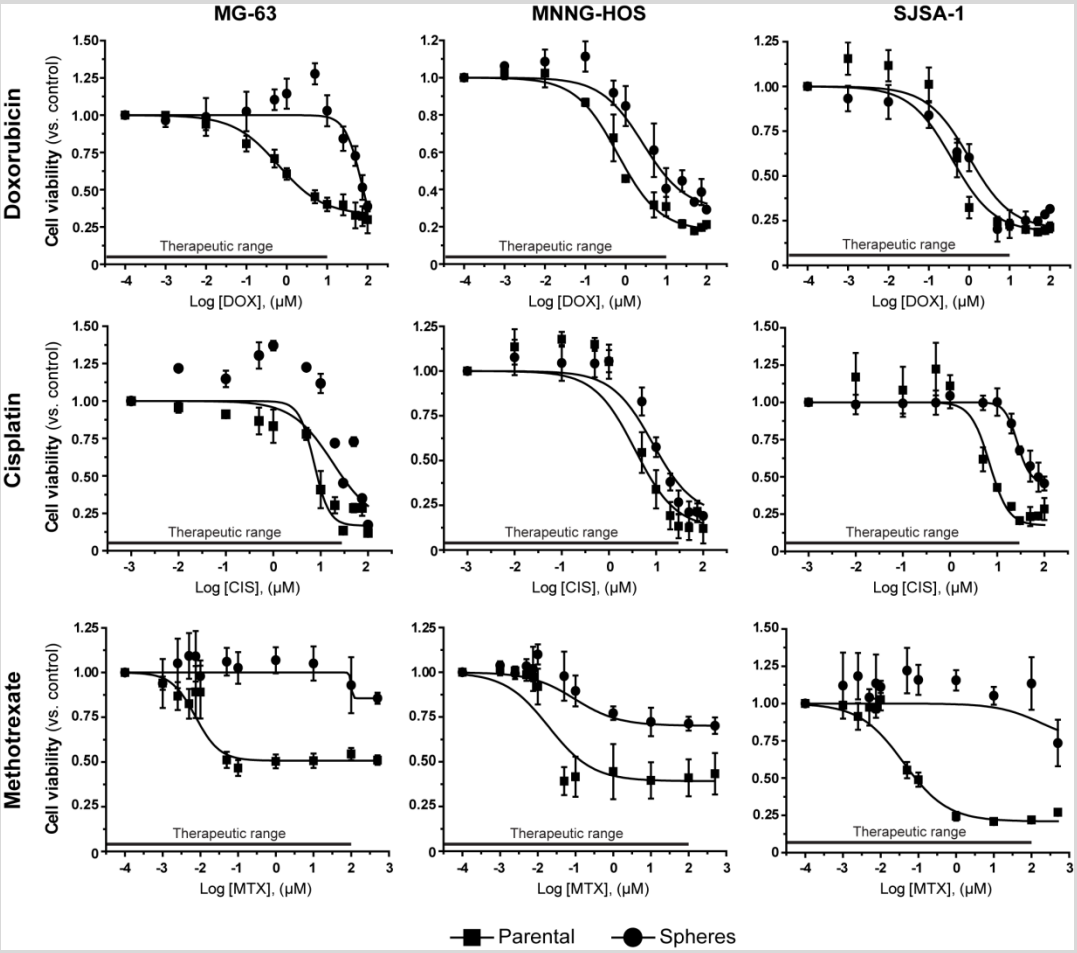

Figure C7.3. Osteosarcoma stem cells are highly resistant to conventional drugs. Concentration-viability curves of MG-63, MNNG-HOS and SJSA-1 parental cells and spheres to doxorubicin (DOX, 0-100μM), cisplatin (CIS, 0-100μM) and methotrexate (MTX, 0-500μM). Cells were incubated with increasing doses of chemotherapeutic compounds during 48h. Cell survival was tested using the WST-1 assay, and show a significantly higher resistance of spheres to chemotherapy than parental cells. Data represent mean values ± SEM performed on triplicate experiments. The lines correspond to the fitting using a sigmoidal model.

Table C7.2. IC_{50} values of doxorubicin, cisplatin and methotrexate in osteosarcoma parental cell lines and corresponding spheres.

IC_{50}	MG-63		MNNG-HOS		SJSA-1	
	Parental	**Spheres**	**Parental**	**Spheres**	**Parental**	**Spheres**
DOX	0.40 ± 0.15	48.74 ± 12.44**	0.79 ± 0.25	2.46 ± 0.70*	0.67 ± 0.21	1.03 ± 0.16*
Fold diff.	**>100**		**3.11**		**1.54**	
CIS	7.46 ± 3.17	17.55 ± 2.59*	7.13 ± 0.65	11.25 ± 1.71*	6.74 ± 1.59	29.35 ± 5.55**
Fold diff.	**2.35**		**1.58**		**4.35**	

MTX	0.011 ± 0.006 100[&]**	0.017 ± 0.006 0.069 ± 0.017**	0.058 ± 0.025 187[&]*
Fold diff.	**>100**	**4.06**	**>100**

Note: Results are expressed as mean ± standard deviation of three independent experiments performed in duplicate. *P<0.05 and **P<0.01 compared to parental cells (non-parametric Kruskal-Wallis test for multiple comparisons between independent samples). [&]Values are approximations as IC_{50} values were not achieved in experiments. *Abbreviations*: IC_{50} – half-maximal inhibitory concentration, DOX, doxorubicin; CIS, cisplatin; MTX, methotrexate.

7.2.3 Osteosarcoma spheres proliferate less than parental cells and are less susceptible to doxorubicin-induced cell cycle arresting

To further explore whether osteosarcoma spheres had a differential cell cycle response to doxorubicin, the most commonly used chemotherapeutic for osteosarcoma, we treated parental cells and spheres with 0.75μM doxorubicin for 48h and then subjected all the cells to flow cytometric analysis using propidium iodide staining (**Figure C7.4**). Comparing firstly the cell cycle profile of parental cells with spheres it is possible to observe that parental cells of the three osteosarcoma cell lines have a higher proportion of actively proliferating cells, either in the S-phase or G2/M phase, than that found in corresponding spheres (S+G2/M percentage range: parental cells – 21.71% - 56.9%; spheres – 7.16% - 31.99%). These results are in agreement with our previous results showing that osteosarcoma CSCs display a behavior consistent with that of quiescent cell populations, based on either glucose uptake (Martins-Neves *et al.*, 2012) or immunohistochemical staining for the proliferation-related marker Ki-67 (**Chapter 2**).

Concerning the cell cycle response to drug exposure, we found that doxorubicin caused an expressive accumulation of cells in S and G2/M phases in parental cells, consistent with a cell cycle arrest that was accompanied by a proportional decrease in the percentage of cells in the G1 phase (**Figure C7.4**). However, despite that a similar effect was observed in osteosarcoma spheres, the proportion of arrested cells was lower than that observed in parental cells (S-phase percentage range in doxorubicin-treated cells: parental cells – 5.45%–25.19%, spheres – 7.8%-9.07%; G2/M phase – parental cells 46.07% - 86.62%; spheres – 11.94% - 49.01%).

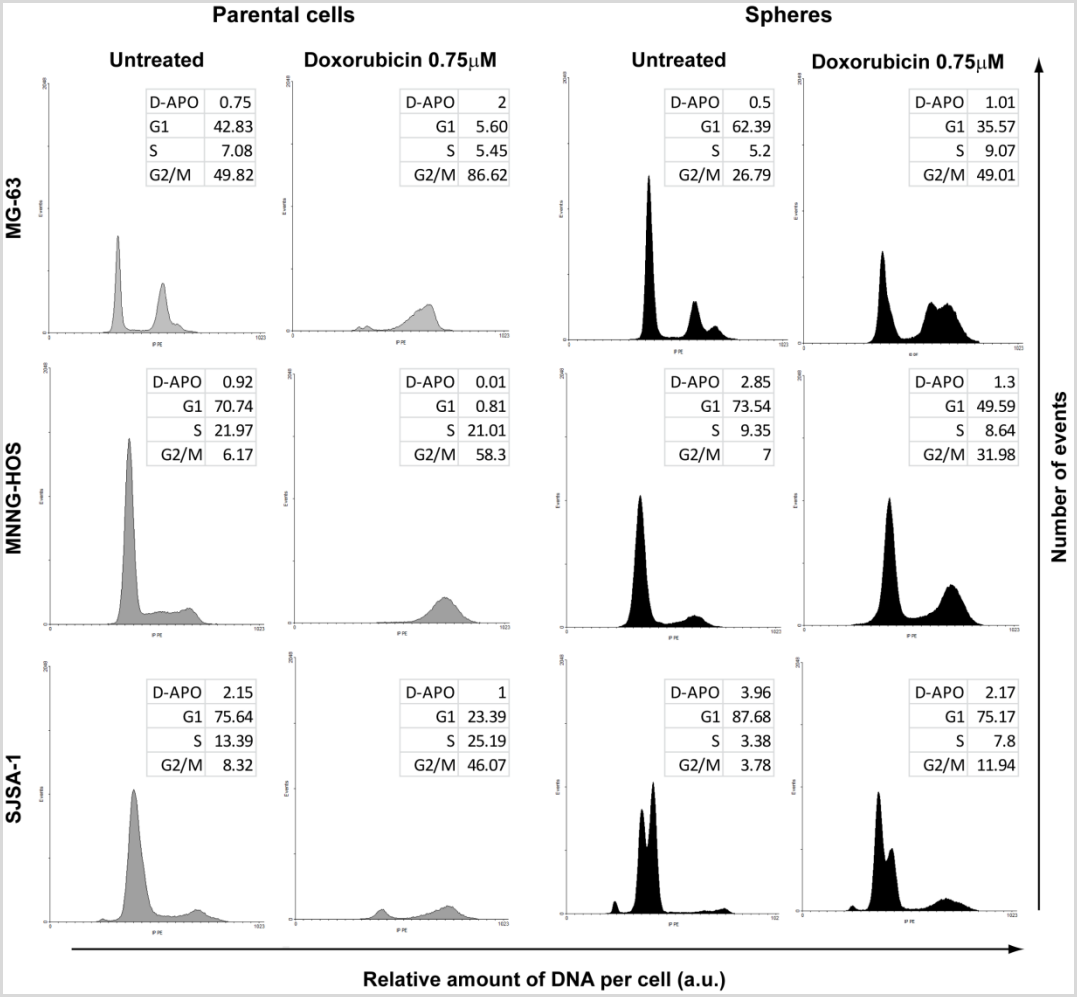

Figure C7.4 Flow cytometric cell cycle analysis of osteosarcoma parental cells and spheres upon doxorubicin treatment. Cell cycle phase distribution of parental MG-63, MNNG-HOS and SJSA-1 and corresponding spheres after treatment with doxorubicin for 48h. Cells were fixed and stained with propidium iodide for cell cycle analysis by flow cytometry. Doxorubicin induced a cell cycle arrest in parental cells, but this effect was less prominent in spheres. *Abbreviations*: D-APO, debris+apoptotic cells.

7.2.4 Osteosarcoma spheres have mesenchymal multilineage differentiation capacity

We further characterized osteosarcoma stem cells populations by testing their capacity to differentiate into several mesenchymal lineages, given the fact that osteosarcoma is a mesenchymal-like tumor and MSCs are considered as its cells-of-origin. Moreover, adult multipotent stem cells are also characterized by their ability to

differentiate into multiple lineages (Pittenger *et al.*, 1999). We observed fields of Oil Red O-positive cells indicative of adipogenic differentiation, discrete foci of Alizarin Red S positivity indicative of mineralization (calcium deposits) in osteogenic medium, and matrix deposition or chondrocyte-like structures on chondrogenic differentiation (**Figure C7.5**). For all parental cell lines other than MG-63, which differentiated towards two lineages, specific differentiation was achieved towards only one lineage (**Table C7.3**). In contrast, for all cell lines, spheres differentiated towards at least two lineages, either partially or in larger fields of the monolayer culture, resembling therefore a more plastic and stem-like phenotype. Noteworthy, we observed heterogeneous patterns of positively stained cells on differentiated spheres, which can be attributed to progressive loss of differentiation capability due to culture on serum-supplemented media.

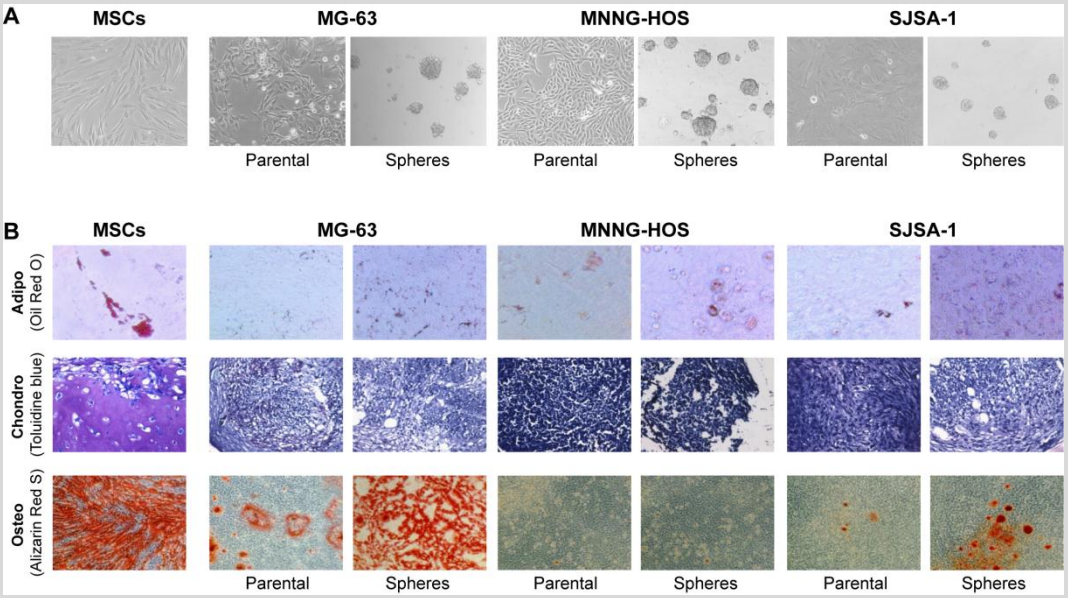

Figure C7.5. Osteosarcoma spheres display multilineage differentiation capacity. A. Morphology of osteosarcoma parental cells and spheres cultured in their regular media and adherent (parental) or suspended (spheres) conditions. Magnification: 100×. **B.** Representative images of mesenchymal stem cells (MSC), osteosarcoma parental cells and spheres, after culturing in specific conditions leading to mesenchymal-related differentiation. Adipocytes (Adipo) were stained with Oil Red O, chondrocytes (Chondro) with toluidine blue and osteocytes (Osteo) with Alizarin Red S, after 2.5, 5 and 3 weeks, respectively. Magnification: 400× – adipocytes; 100× – chondrocytes; 100× – osteocytes. See also **Table C7.3**.

Table C7.3. *In vitro* capacity of human MSCs, parental cells and spheres to differentiate into multiple mesenchymal lineages.

		MG-63		MNNG-HOS		SJSA-1	
	MSCs	**Parental**	**Spheres**	**Parental**	**Spheres**	**Parental**	**Spheres**
Adipocytes	2	0	**2**	2	**2**	0	**2**
Chondrocytes	2	2	**1**	0	**1**	0	**2**
Osteocytes	2	1	**2**	0	**0**	1	**2**
	3/3	**2/3**	**3/3**	**1/3**	**2/3**	**1/3**	**3/3**

Note: After incubation with compounds inducing specific differentiation as described in the methods sections, cells displaying adipogenic differentiation were stained with Oil Red O, paraffin-embedded cells showing chondrogenic differentiation with Toluidine Blue and cells displaying osteogenic differentiation with Alizarin Red S. This table summarizes whether indicated cells could differentiate and into which specific lineage(s) of differentiation, based on respective dye staining. Human mesenchymal stem cells (MSCs) were used as a positive control. Extent of differentiation was scored semiquantitatively as 0 (no staining), 1 (moderate) and 2 (strong).

7.3 Complementary References

- Martins-Neves S, Lopes A, do Carmo A, Paiva A, Simoes P, Abrunhosa A, Gomes C. 2012. Therapeutic implications of an enriched cancer stem-like cell population in a human osteosarcoma cell line. BMC Cancer 12:139. http://dx.doi.org/10.1186/1471-2407-12-139

- Mohseny AB, Machado I, Cai Y, Schaefer KL, Serra M, Hogendoorn PCW, Llombart-Bosch A, Cleton-Jansen AM. 2011. Functional characterization of osteosarcoma cell lines provides representative models to study the human disease. Lab Invest 91:1195-1205. http://dx.doi.org/10.1038/labinvest.2011.72

- Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR. 1999. Multilineage Potential of Adult Human Mesenchymal Stem Cells. Science 284:143-147. http://dx.doi.org/10.1126/science.284.5411.143