Sara Cristina Inocêncio Neves

Master in Biomedical Engineering

Project coordinator:

PhD Célia Maria Freitas Gomes

Functional Evaluation of Cancer Stem Cells in Osteosarcoma during Differentiation and its Implications in Response to Chemotherapy

Avaliação Funcional das Células Estaminais Tumorais
Durante a Diferenciação Celular em Osteossarcoma e suas Implicações na
Resposta à Quimioterapia

University of Coimbra

2011
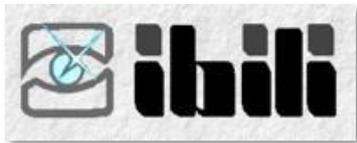
This work was developed in the following institutions:

Pharmacology and Experimental Therapeutics, Institute of Biomedical Research in Light and Image (IBILI), Faculty of Medicine, University of Coimbra, Coimbra

Institute for Nuclear Sciences Applied to Health - ICNAS, Coimbra, Portugal

Histocompatibility Center of Coimbra – University Hospital of Coimbra, Coimbra

Parts of this work are published in the following abstracts:

Sara CI Neves, Vitor EB Oliveira, Anália do Carmo, Antero J Abrunhosa, M Filomena Botelho, Célia MF Gomes. Elucidation of the drug resistance mechanisms of osteosarcoma cancer stem cells with PET tracers. *Proceedings of 1st Portuguese Meeting in Bioengineering. Bioengineering and Medical Sciences - The Challenge of the XXI century*, Lisbon, Portugal 1st-4th March 2011, pp. 53-56. (Oral presentation)

Vitor EB Oliveira, Sara CI Neves, Anália do Carmo, Antero J Abrunhosa, M Filomena Botelho, Célia MF Gomes. Cancer stem cells in osteosarcoma and drug resistance mechanisms. *Proceedings of 3rd Workshop on Biomedical Engineering,* Lisbon, Portugal, 16th April 2011, (Poster presentation)

S. I. Neves, A. J. Abrunhosa, M. F. Botelho, C. M. Gomes. Functional evaluation of osteosarcoma cancer stem cells with positron emission tomography tracers. *Proceedings of 6th International Meeting of the Portuguese Society of Stem Cells and Cell Therapies,* Cantanhede, Portugal, 28th-29th April 2011, (Poster presentation)

Aos meus Pais

Ao meu irmão

Index

Acknowledgments

Firstly, I would like to sincerely express my gratitude to Dra. Célia Gomes for all the knowledge transmitted, patience, confidence, support and above all, for always helping me becoming a better student along this year of work.

To Dr. Antero Abrunhosa, from Institute of Nuclear Sciences Applied to Health of the University of Coimbra, for all kindness, support and helpful discussions.

To Dra. Anália do Carmo from Center for Neurosciences and Cell Biology of the University of Coimbra, for support in western blot analysis and kindness.

To Professor Miguel Morgado for interest and all efforts helping Biomedical Engineering students evolving along their academic course.

To Centre of Histocompatibility of Centre of the University Hospital of Coimbra, in the person of Dr. Artur Paiva, for flow cytometry experiments for characterisation of our human osteosarcoma cells.

To Eng. Francisco Caramelo, for statistical analysis contribution and helpful suggestions.

To Dr. Carlos Alberto Fontes Ribeiro from Pharmacology and Experimental Therapeutics Service for all the facilities conceived. To all staff for the sympathy.

Às minhas colegas de casa, Sara Pipa, Patrícia Silva e Elisa Ferrão por toda a cumplicidade, amizade e apoio incondicional.

Aos meus amigos, em especial Vitor Oliveira, Sofia Sousa, Mariana Rufino, Mariana Madeira, Cátia Leitão, Isa Branco, André Sousa, Leonel Gomes, Sara Neves e André Ventura por todo o apoio, amizade e paciência em todas as circunstâncias.

Aos salsólicos Marco Bucete, Rita Videira e Lara Coelho por todo a amizade, apoio, sorrisos e hospitalidade.

À minha família, em especial aos meus Pais e irmão, Tios Manuel e Cristina, primos Miguel e Inês e Avós pelos sacrifícios, pelo apoio, união e acima de tudo pelos sorrisos em qualquer circunstância. Obrigada por acreditarem sempre em mim!

List of Figures

List of tables

List of Abreviations

[^{18}F]FDG	[^{18}F]fluoro-2-deoxyglucose
ABC	ATP-binding cassette
ALDH1	Aldehyde Dehydrogenase
AP	Alkaline Phosphatase
BCRP	Breast Cancer Resistance Protein
BMP	Bone Morphogenetic Proteins
CDK4	Cyclin-dependent Kinase 4
CE	Colony Efficiency
CIS	Cisplatin
CSC	Cancer Stem Cell
C_{ss}	Steady-state Accumulation
CTGF	Connective Tissue Growth Factor
DMEM/F12	Dulbecco's Modified Eagle Medium / Nutrient Mixture F-12 Ham
DNA	Deoxyribonucleic acid
DOX	Doxorubicin
ELISA	Enzyme-Linked Immunosorbent Assay
FBS	Fetal Bovine Serum
IC$_{50}$	Half Maximum Inhibitory Concentration
LDH	Lactate Dehydrogenase
MDM2	Murine Double Minute 2
MDR	Multidrug Resistance
MSC	Mesenchymal Stem Cell
MTT	[3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide]

MTX	Methotrexate
OCT4	Octamer-binding Transcription Factor 4
OS	Osteosarcoma
PBS	Phosphate-Buffered Saline Solution
PET	Positron Emission Tomography
Pgp	P-glycoprotein
Rb	Retinoblastoma
RNA	Ribonucleic Acid
RPM	Rotations per minute
RPMI	Roswell Park Memorial Institute (Medium)
Sox2	(Sex Determining Region Y)-box 2
TBS-T	Tris Buffered Saline − Tween20

Abstract

Background: Osteosarcoma (OS) is the most prevailing primary bone tumour among children and adolescents. This malignancy is considered a differentiation disease that arises from disruptions in the differentiation process of mesenchymal stem cells into osteoblasts. The hypothesis that cancer is driven by cells with stem-like properties (CSCs) has been widely accepted as a model for carcinogenesis. These cells are able to self-renew and to differentiate recreating the phenotypic heterogeneity of the original tumor. In this work, we aim to address the dynamic changes that occur during differentiation of CSCs regarding the expression of stemness markers and of drug efflux transporters, metabolic activity, sensitivity to DOX, clonogenic potential and pattern of motility.

Methods: CSCs were isolated from the human osteosarcoma cell line MNNG/HOS using the sphere formation assay. The isolated CSCs were characterized in terms of mesenchymal stem cells markers and multilineage differentiation. Differentiation of CSCs was induced by culturing cells in culture medium supplemented with 10% of fetal bovine serum in adherent flasks for 21 days. During this period, at different time-intervals we analyzed the expression of pluripotency markers (OCT4 and Nanog) and of drug efflux proteins (BCRP and P-glycoprotein) by Western blot. Metabolic activity was assessed based on the cellular uptake of [^{18}F]FDG. The cytotoxicity to doxorubicin was evaluated using the MTT colorimetric assay after 48h of exposure. Analysis of cells motility was assessed using the scratch assay and the clonogenic potencial was also assayed along differentiation.

Results: The isolated CSCs exhibited features of mesenchymal stem cells, and showed a higher expression of pluripotency markers OCT4 and Nanog and of drug efflux transporters BCRP and P-glycoprotein. Along differentiation of CSCs, it was observed a marked decrease in the expression levels of both stemness markers and of drug efflux transporters that was accompanied by a decrease in the metabolic activity of cells. In parallel it was observed a progressive increase in the cell's sensitivity to DOX acquiring a sensitivity profile similar to that of the parental MNNG/HOS cells. The higher clone-forming ability was observed at early stages of differentiation. Differentiated cells showed higher invasive properties.

Conclusions: Osteosarcoma contains population of cancer stem-like cells with attributes of mesenchymal stem cells, able to self-renew and to generate differentiated progeny phenotypically similar to their parental cells.

Keywords: osteosarcoma, cancer stem cells, differentiation, [^{18}F]FDG, doxorubicin

Resumo

Introdução: Osteossarcoma é o cancro primário ósseo mais comum em crianças e adolescestes. Esta patologia tem origem em perturbações que ocorrem durante o processo de diferenciação osteoblástica de células estaminais mesenquimais. A hipótese de que o cancro tem origem em células com propriedades de células estaminais (CSCs, do inglês *Cancer Stem Cells*) tem sido amplamente aceite como modelo de carcinogénese. Estas células possuem características típicas de células estaminais, nomeadamente capacidade de auto-renovação e diferenciação sendo capazes de recriar a heterogeneidade típica do tumor original. Este trabalho teve como objectivo avaliar as alterações dinâmicas que ocorrem durante o processo de diferenciação das CSCs, em termos de expressão de marcadores de células estaminais, proteínas transportadoras de fármacos, actividade metabólica, sensibilidade à doxorrubicina, potencial clonogénico e motilidade celular.

Métodos: As CSCs foram isoladas a partir da linha celular humana de osteossarcoma MNNG/HOS pelo método de formação de esferas e caracterizadas em termos de marcadores de células estaminais mesenquimais e diferencição em multiplas linhagens. A diferenciação das CSCs foi induzida em meio de cultura enriquecido com 10% de soro fetal bovino em frascos aderentes durante 21 dias. Durante este período, a diferentes intervalos de tempo, analisámos a expressão de marcadores de pluripotência OCT4 e Nanog e de proteínas transportadoras de fármacos (BCRP e glicoproteína-P) por Western blot. A actividade metabólica das células foi avaliada com [^{18}F]FDG. A citotoxicidade da doxorrubicina foi testada pelo método colorimétrico de MTT após um período de incubação de 48 h. A análise da migração celular, pelo método de Scratch, e o potencial clonogénico foram igualmente efectuados ao longo da diferenciação.

Resultados: As CSCs isoladas apresentaram características de células estaminais mesenquimais e uma elevada expressão de marcadores de pluripotência (OCT4 e Nanog) bem como de proteínas transportadoras de fármacos (BCRP e glicoproteína-P). Ao longo da diferenciação das CSCs, observou-se uma diminuição acentuada tanto dos marcadores de pluripotência OCT4 e Nanog como dos transportadores BCRP e glicoproteína-P, a qual foi acompanhada de um decréscimo da actividade metabólica das células. Em paralelo observou-se um aumento da sensibilidade das células à doxorrubicina, acabando por adquirir um perfil de sensibilidade semelhante ao das células parentais MNNG/HOS. As células apresentaram maior capacidade clonogénica numa fase mais precoce da diferenciação celular. As células mais diferenciadas apresentaram propriedades mais invasivas.

Conclusões: O osteossarcoma contém uma população celular de células estaminais tumorais com atributos de células estaminais mesenquimais capazes de se auto-renovar e de se diferenciarem gerando células fenotipicamente semelhantes às células parentais.

 Palavras-chave: osteosarcoma, células estaminais tumorais, diferenciação, [^{18}F]FDG, doxorrubicina

1.   Introduction

1.1.   Osteosarcoma

Osteosarcoma (OS) is the most common primary bone malignancy among children and adolescents. This malignancy comprises about 20% of primary bone sarcomas and represents 2.4% of all childhood cancers. In United States of America every year 400 new cases of OS are diagnosed in children and adolescents under 20 years of age. OS presents a bimodal age distribution. The first incidence peak of this disease lies between 10 and 14 years during the pubertal growth spurt. The second peak of OS incidence occurs in older adults aged over 65 years. These particular cases are frequently related Paget's disease of bone (1).

OS can occur in all bones of body although it tends to be developed in long bones in areas of rapid bone growth or turnover. OS development occurs preferentially in distal femur (40%), proximal tibia (20%) and proximal humerus (10%), although it may occur in the axial skeleton. This axial location is more frequent in older patients. Axial bone involvement in pediatric patients represents less than 10% of clinical cases. Despite of 80% of OS cases present localized disease, approximately 20% of OS patients have radiologically detectable metastases. The most common sites of metastasis are lung and other bones. Metastatic disease in OS is associated with a poor prognosis and short-time survival (2-4).

OS is a destructive process that initiates in the intramedullary region and in most of the cases grows radially towards the bone cortex resulting in cortex perforation. OS lesions can also extend to surrounding soft tissues like muscles compressing them into a pseudocapsular layer determined "reactive zone". The OS lesions are characterized by the production of malignant osteoid by the neoplasic cells which can be detected through imagiologic studies(2, 5). The imaging techniques used in osteosarcoma diagnosis are plain-film radiography, computerized tomography (CT), magnetic resonance imaging (MRI), positron emission tomography (PET) and bonescintigraphy. Despite the important information that these techniques can provide the clinicians, biopsy is the key step in osteosarcoma diagnosis (6).

The most common symptoms of OS are pain and swelling. Pain can emerge after strenuous exercise or trauma, usually 2-4 months before diagnosis, and increases progressively along time. Swelling appears later with a hard painful mass in the affected region. Despite of being uncommon, pathologic fracture can occur in OS cases (7). In addition to the physical symptoms,   OS   patients   usually   present   abnormal   high   levels   of

alkaline phosphatase (AP) and lactate dehydrogenase (LDH). These features are reported to be related to prognosis and tumor volume (8).

1.1.1. Etiology

The exact causes of OS are not completely understood, although there are some risk factors that are associated with the development of the disease. The only proven exogenous risk factor is exposure to radiation. The time period since the radiation exposure to the disease development is 10-20 years interval. Therefore radiation induced OS represents 2% of total OS cases and is more common in adult age. A feature that support the role of radiation in OS pathogenesis in the fact that 5.7% of children treated with radiotherapy develop a secondary neoplasm and 25% of these neoplasms are sarcomas (9, 10). OS is also associated with other malignancies. Patients with retinoblastoma, Ewing's sarcoma, Paget's disease of the bone, Li-Fraumeni syndrome and Rothmund-Thompson syndrome Have a higher genetic susceptibility to develop OS (11). Despite the existence of these risk factors, the majority of OS cases occur without association with predisposition factors.

The development of OS is also associated with some genetic abnormalities in both tumor suppressor genes and oncogenes. Tumor-suppressor pathways that are targets of genetic alterations in OS are retinoblastoma tumor-suppressor gene (Rb) and p53 pathways (12). Rb is a tumor suppressor gene that is entailed in development of retinoblastoma, an uncommon childhood tumor in retina. Retinoblastoma patients are reported to have a 500-fold higher risk of developing OS than healthy population. Rb gene encodes a nuclear phosphoprotein (pRb) which is responsible for the growth-suppressive effect on cell cycle regulating G1/S progression. This protein can be inactivated through phosphorilation by the protein encoded by oncogene CDK4 which is a cyclin dependent kynase. CDK4 gene is reported to be amplified or over-expressed in OS representing an important gene on OS development. p53 have been proved also to be involved in pathogenesis of OS. This gene plays an important role in tumor-suppressive function regulating genes involved in cell cycle, DNA damage response and apoptosis. Mutations in this gene have been found in human carcinomas including bone and soft tissue sarcomas. The protein encoded by p53, is inactivated by a protein encoded by MDM2 gene which is amplified or over-expressed in OS. Proto-oncogenes like C-myc and C-fos possess an important function in the regulation of cell growth and similarly to CDK4 and MDM2 are reported to be amplified or over-expressed in OS.(2, 4, 13). Despite of being known that OS have origin in the inactivation of tumor-suppressor pathways (Rb and p53) and over-expression of proto-oncongenes (C-myc and C-fos) it is still unknown the order of the events leading to OS origins (9).

Growing evidence point out that OS may be regarded as a malignant tumor of mesenchymal origin which results from genetic and epigenetic changes that interfere with the osteoblastic differentiation pathway from mesenchymal stem cells (MSC). MSC are potentially the cells of origin of OS, given their role in bone remodelling during the pubertal growth spurt and their multipotential differentiation capacity (14, 15). These cells, first discovered in the bone marrow, are non-hematopoietic and display multilineage differentiation potential particularly osteogenesis, chondrogenesis and adipogenesis (16).

Osteoblastic differentiation is a complex and tightly regulated process that is critical for proper bone formation and is influenced by a variety of endogenous and environmental factors (Figure 1.1 A.) If alterations in the MSC differentiation cascade block the progression to terminally differentiated osteoblasts, it is likely that tumorigenic precursors are formed and such undifferentiated OS precursors would maintain the ability to proliferate, increasing the risk for development of OS (Figure 1.1 B) (17, 18).
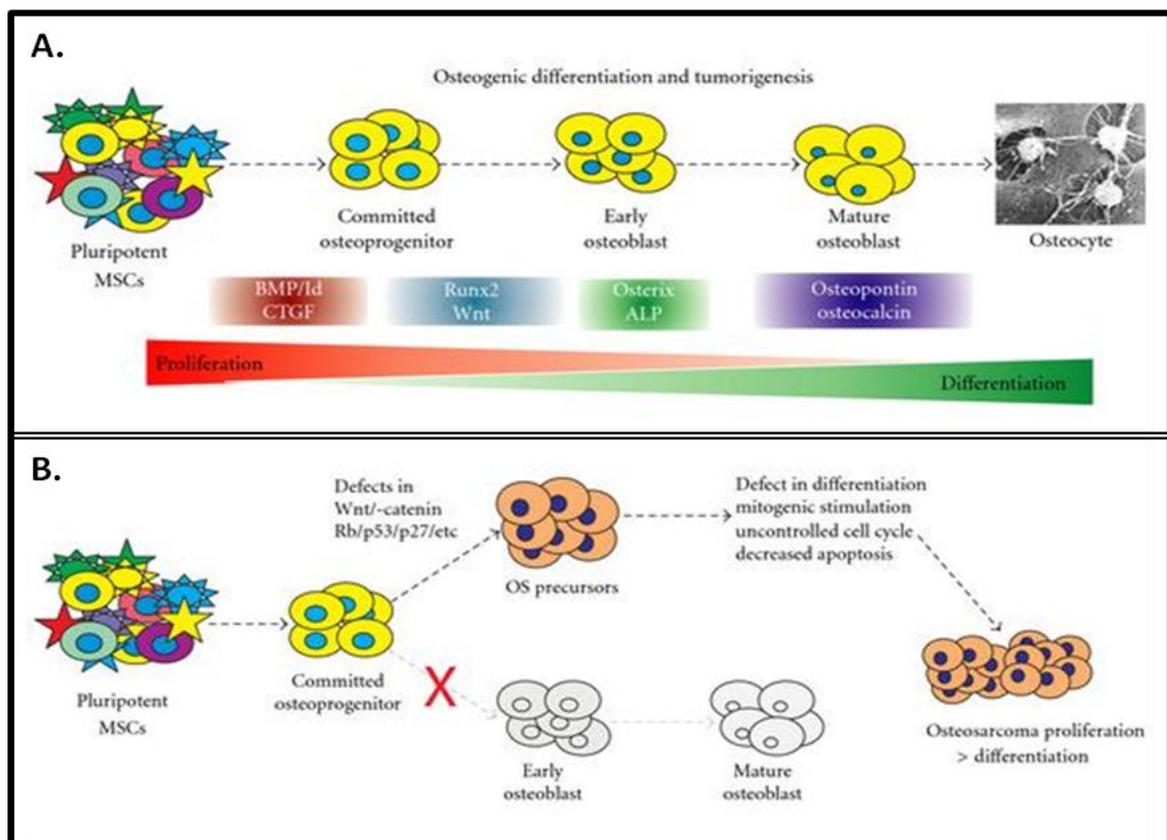
Figure 1.1. Osteogenic differentiation and osteosarcoma tumorigenesis. **A.** Mesenchymal stem cells (MSCs) progress down the osteogenic differentiation cascade by a tightly regulated process by different signalling pathways. **B.** Defects in osteogenic differentiation lead to OS development. If alterations in the MSC differentiation cascade block the progression to terminally differentiated osteoblasts, it is likely that tumorigenic precursors are formed, maintaining the ability to proliferate and increasing the risk for OS development (Adapted from (17).)

Although not completely understood, some of the potential defects may include activation of oncogenes or inactivation of p53 or Rb tumor suppressor genes and changes in Wnt signalling pathway. These defects may lead to uncontrolled cell proliferation and disrupted differentiation leading to a tumorigenic phenotype. This model is supported by the fact that OS cells share many cellular and morphologic features with undifferentiated osteoprogenitors including the high proliferative capacity, resistance to apoptosis and similar expression of many osteogenic markers such as osteopontin and osteocalcin that are highly expressed in mature differentiated osteoblasts, but minimally expressed in OS cells (19). CTGF, a multifunctional growth factor that is normally upregulated at the earliest stages of osteogenic differentiation, also shows elevated basal expression in human OS cells. Moreover osteogenic stimuli such as bone morphogenetic proteins (BMPs), failed to promote terminal differentiation of most OS cells leading to cellular proliferation and tumor growth, further supporting the existence of possible differentiation defects in OS cells (20). It is likely that defects occurring at the early stages of osteogenic differentiation may lead to a more aggressive and undifferentiated tumor and vice-versa.

1.1.2. Treatment of Osteosarcoma and prognosis

Until the 1970's, OS patients were treated surgically by amputation but, despite the good local control of the disease, the 5-year disease free survival rate was only of 12%. Seventy five percent of the patients treated surgically died after 2 years due to development of metastases mostly located in the lung. The introduction of adjuvant chemotherapy together with surgery, increased significantly the 5-year survival rate to 70% of patients with localized disease. However, for patients with metastasis, the prognosis is still poor with a 5-year survival rate of 30% (9).

Actually the treatment modalities of OS include neoadjuvant chemotherapy for elimination of micrometastasis and reduction of tumor size, followed by surgery and then additional (adjuvant) chemotherapy. Radiotherapy can be used but only for palliative care improving local symptoms and local control of the tumor (3).

The chemotherapeutic agents recommended by European and American Osteosarcoma Study Group (EURAMOS-1) include doxorubicin (DOX), cisplatin (CIS) and high-dose methotrexate (MTX) (21). These drugs target DNA of tumoral cells through different mechanisms of action. DOX intercalates at points of local uncoiling of the DNA double helix inhibiting the DNA and RNA synthesis. CIS binds directly to tumor DNA inhibiting the DNA synthesis of DNA through

formation of DNA cross-links. MTX is a folate antimetabolite that inhibits the synthesis of purine and thymidilic acid by binding dihydrofolate reductase (2).

Despite the significant improvement observed in survival of OS patients with this therapeutic approach, 25-50% without metastasis at diagnosis develop systemic disease and dye from the disease and intensifying chemotherapeutic dosages or increasing the length of treatment does not improve the outcome of osteosarcoma patients (22).

The development of resistance to chemotherapy is considered a major obstacle to the successful treatment of OS. In most of cases, despite a favourable response to chemotherapy, tumors relapse and acquire resistance to the drugs used initially and other drugs as well. Several mechanisms can account the development of multidrug resistance (MDR) such as alterations in drugs' targets like DNA topoisomerase II, increased detoxification of compounds through glutathione system, overexpression of drug efflux transporters of ATP-binding cassette (ABC) transporters, namely P-glycoprotein (Pgp), multidrug resistance related protein 1 (MRP1) and breast cancer related protein (BCRP) (23). These transporters behave as drug efflux pumps, preventing the intracellular accumulation of chemotherapeutic drugs at toxic levels, allowing the cells to survive (24). It has been proved that the over-expression of Pgp in OS is associated with a poor response to chemotherapy and is considered a poor prognostic factor (25, 26). High serum levels of alkaline phosphatase (AP) and lactate dehydrogenase (LDH) are also associated with a poor prognosis (27). Patients with elevated levels of AP have a 5-year disease free survival (54%) inferior to those cases with lower AP serum levels (64%). It is also known that higher AP levels correlate with a shorter time to recurrence (18 months) when compared with cases with normal AP levels (25 months). Abnormal elevated LDH levels, is also reported as a sign of a more biologically aggressive tumor and correlates with a worse prognosis (2).

1.2. The Cancer Stem Cell Hypothesis

Solid tumors show a considerable heterogeneity with respect to cellular morphology, proliferative index, differentiative capacity, surface antigens and genetic lesions with significant implications therapeutic response. The cancer stem cell (CSC) hypothesis is an attractive model to explain this functional heterogeneity, so commonly observed in solid tumors (28).

The CSC theory proposes a hierarchical organization of cells within tumors, in which a subpopulation of cells with stem-like properties is responsible for sustaining tumor growth. These cells share important properties with normal tissue stem cells, including their self-renewal potential, by symmetric or asymmetric division, and differentiation capacity, although aberrant (29).

The first evidence for the existence of CSCs came from studies in acute myeloid leukaemia by Lapidot *et al*.. They demonstrated that a rare population of cells comprising 0.01-1% of the total population was able to generate leukaemia when transplanted into immunodeficient mice. The cells were able to proliferate extensively *in vivo* generating a tumor with acell morphology similar to that of the observed in the original patients (30). Since then, other studies have reported the identification of CSCs in several types of cancer including including brain (31), breast (32), liver (33), kidney (34), gastric (35) and bone cancers (36).

1.2.1. Properties of CSCs

CSCs are generally defined as tumor cells with stem-like attributes that are responsible for tumor initiation and sustaining tumor growth and for the phenotypic heterogeneity observed within the tumor cell population. CSCs, similarly to normal stem cells, can divide through two division processes, symmetric and asymmetric division, although asymmetric division predominates, in which one cell give rise to an identical CSC (maintaining the pool of CSCs) and a more differentiated progenitor cell which in subsequent divisions generates the heterogeneous populations of cancer cells at various differentiation stages that constitute tumor bulk (Figure 1.2) (37, 38).
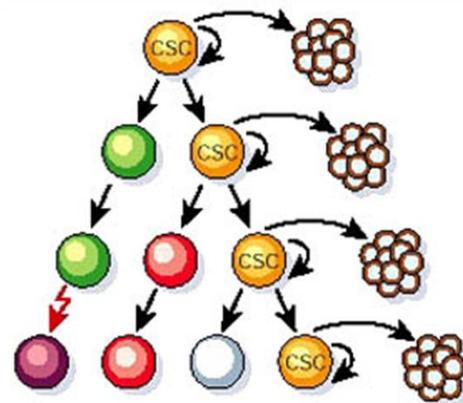
Figure 1.2. Model of tumor progression and maintenance according to cancer stem cell hypothesis. CSC – Cancer Stem Cell (Adapted from (39))

CSCs share with normal stem cells the extensive ability to self-renewal, although without a tight control of the pathways that regulate the self-renewal process, which can be

constitutively activated or improperly regulated through genetic and/or epigenetic changes leading to uncontrolled growth (40).

The exact origin of CSCs is not completely clarified. They could arise from the malignant transformation of a normal stem cell or progenitor cell that due to their long life-span accumulated oncogenic insults over time and became malignant, or in alternative can result from a more differentiated tumor cell that acquires the properties of a stem cell and developed the capacity to undergo self-renewal. In both cases, CSCs, with either inherent or acquired ability to self-renew, give rise to cells that lack the long-term self-renewal ability, but retain a finite ability to divide, similar to what happens in normal stem cells undergo differentiation, generating a phenotypically heterogeneous population of tumor cells (41).

1.2.2. Identification of Cancer Stem Cells

The isolation of the CSCs subpopulation within tumors can be performed through different techniques. One of the methods is based in the expression of a set of surface markers, usually specific for normal stem cells of the tissue of origin of the tumor, being the most common the CD133, CD44, CD24 and the epithelial adhesion molecule EpCAM that have been used to isolate CSCs from breast, brain, pancreas, prostate, lung and ovarian cancer, although the most widely used are the CD133 and CD44. Despite their usefulness in isolation of CSCs is several solid tumors it is noteworthy that none of these markers are expressed exclusively by CSCs, and there is still a need to delineate more specific markers of CSCs (42, 43).

Another technique currently used is the identification of a side-population enriched in CSC by flow cytometry. This technique is based on the extrusion of fluorescent dyes (Hoechst 33342 and Rhodamine-123), a property that is conferred by the over-expression of BCRP or Pgp in CSCs (42, 44).

CSCs can also be isolated through the under starved condition, based on the principle that cells in an undifferentiated status can grow as spherical suspended colonies when culture under starved condition in low adherent conditions. The ability of the cells to continue forming spherical colonies over more than 4-5 passages is indicative of their self-renewal potential which is a main characteristic of cells with stem-like properties (36).

The activity of the aldehyde dehydrogenase 1 (ALDH1) is also considered a marker of CSCs. ALDH1 is a detoxifying enzyme responsible for oxidizing intracellular aldehydes and converts retinol to retinoic acid, protecting cells from oxidative insults. Increased ALDH1 activity has been found in stem cell populations in human multiple myeloma, acute myeloid leukemia, brain and breast cancers, and is considered a common marker for both normal and malignant

stem cell populations. The expression of this enzyme in tumors cells is usually determined using Aldefluor assay reagent followed by fluorescence-activated cell sorting analysis (45-47).

Moreover, the transcriptor factors OCT4, Sox2 and Nanog that are required to maintain the pluripotency and self-renewal of embryonic stem cells has been found to be regularly expressed in CSCs, whereas more differentiated counterparts lack these proteins (48, 49). This feature has been observed in CSCs isolated from distinct tumors like renal carcinoma (34), gastric carcinoma (50) and OS (51), which suggest their use as promising markers of CSCs.

1.2.3. Cancer Stem Cells and response to therapy

CSCs have several characteristics that make them naturally resistant to conventional therapies. Mostly of the drugs used in cancer treatment target DNA and induce irreversible damages leading to cell death. CSCs appear to have enhanced DNA repair mechanisms, compared with more differentiated counterparts, allowing them to resist to damages induced by conventional treatments. CSCs are recognized for their ability to enter in a quiescent status, which make them refractory to conventional chemotherapeutic drugs that target preferentially cells with high proliferative activity (52). Other mechanisms that contribute for the resistant phenotype of CSC include the increased expression of specific drug-detoxifying enzymes and of specific drug efflux transporters belonging to the ABC superfamily, Pgp and BCRP. The increased expression of these transporters is considered a hallmark of CSCs and is major determinant on the side-population phenotype. These transporters recognize as transport substrates a wide variety of chemotherapeutic agents, preventing their accumulation inside the cells at toxic levels and are considered a major setback of currently applied chemotherapeutic regimens (53). There are some evidences that indicate the over-expression of these transporters in side population (SP) cells is correlated with their resistant phenotype to chemotherapeutic drugs. Hirschmann-Jax *et al.* have identified SP in neuroblastoma cell lines which were found to over-express BCRP and ABCA3. The authors have also analysed the efflux of a chemotherapeutic drug, mitoxantrone, which revealed to be increased in SP cells indicating that the resistance phenotype of these subset of cells is related with the over-expression of these transporters (54). Studies performed in human cancer cell lines have also identified successfully SP cells over-expressing ABC transporters like BCRP, Pgp among others. These cells exhibited a resistant phenotypte to a wide range of chemotherapeutic drugs including DOX and CIS (55). Similar evidences were also found in human hepatocellular carcinoma cell line. Hu *et al.* have isolated SP cells from human hepatocellular carcinoma cell lines over-expressing BCRP which were found to be responsible for the extrusion of DOX in these cells (56).

These transporters are minimally constituted by two transmembrane domains and two nucleotide-biding folds (Figure 1.3). The transmembrane domains are responsible for substrate recognition and subsequently extrusion while the nucleotide-biding folds bind and hydrolyze ATP. This process is energy-dependent once the efflux of the drugs mediated by these transporters occurs against their concentration gradient (24, 57).
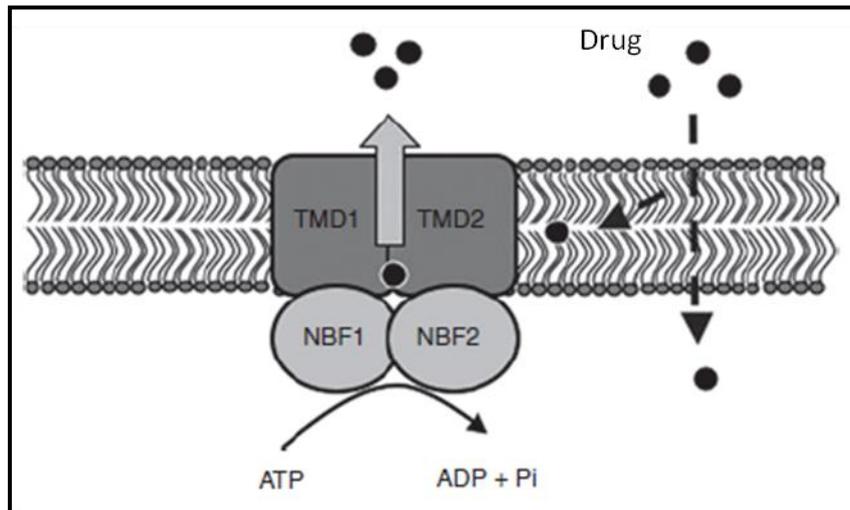
Figure 1.3. Generic structure of an ABC transporter. TMD – Transmembrane Domain; NBF – Nucleotide-binding fold; ATP – Adenosine Triphosphate; ADP – Adenosine Diphosphate; Pi – Inorganic Phosphate (Adapted from (57))

These transporters are not exclusive of cancer cells. They are expressed in a broad variety of normal tissues with excretory function (small intestine, liver and kidney) and at blood-tissue barriers (e.g. blood–brain barrier, blood–cerebral spinal fluid barrier, blood–testis barrier and placenta). These transporters are known for their ability to modulate the absorption, distribution, metabolism, and elimination of xenobiotics in these tissues playing an important role in tissue defence (58).

Considering that CSCs have tumorigenic potential and a selective advantage over their differentiated counterparts, it is plausible that these cells resist chemotherapy and support tumor regrowth increasing the risk of tumor relapse (Figure 1.4). In order to avoid this situation, it is crucial to develop CSC-targeted therapies to achieve a more effective treatment of cancer (53).
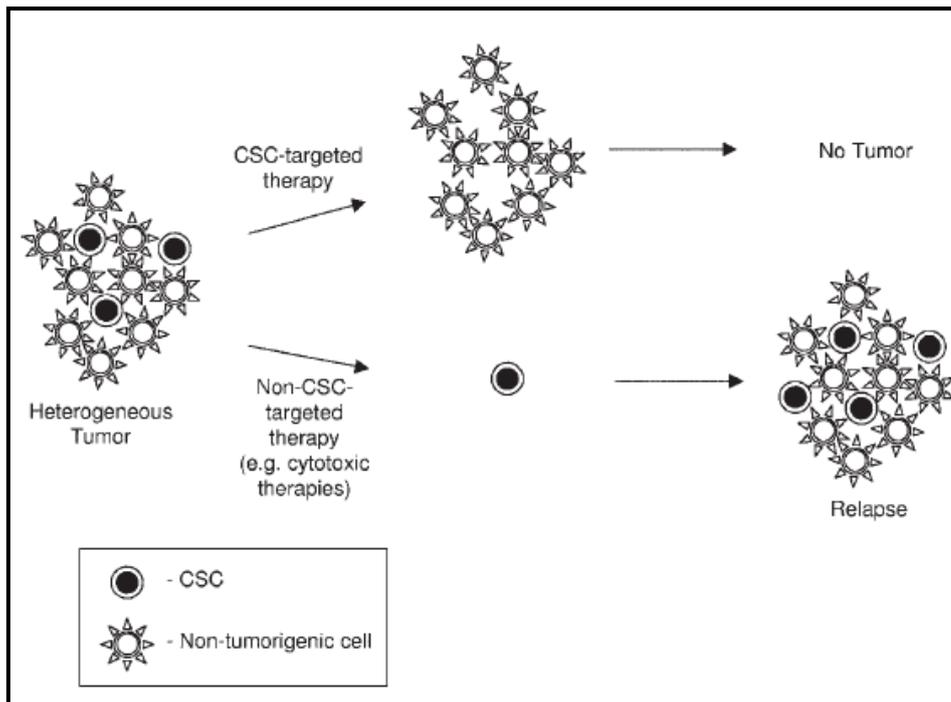
Figure 1.4. Implications of the CSC hypothesis for cancer treatment. Convetional cytotoxic therapies can eradicate more differentiated cells in the tumor but spare CSC that can lead to a relapse of the tumor. CSC – Cancer Stem Cell. (Extracted from (53).)

1.2.4. Cancer stem cells in osteosarcoma

Recent studies have successfully identified a subset of cells with stem-like features among OS cell populations. Gibbs *et al*. have successfully isolated the CSCs subpopulation from nine established cultures from untreated OS biopsies and a OS cell line (MG 63) through sphere formation assay. Sarcospheres-derived cells expressed the MSC surface markers Stro-1, CD105 and CD44 and over-expressed embryonic stem cells pluripotency markers (OCT4 and Nanog) (51). Wang *et al*. observed similar results in four more human OS cell lines (36). Murase et al. also reported the existence of a subset of CSCs in human OS cell lines identified through the extrusion of Hoechst 33324. These cells revealed higher tumorigenicity *in vivo* and *in vitro* (59). Studies performed in our laboratory also demonstrated that CSCs isolated from a human OS cell line have higher resistance to chemotherapeutic drugs (DOX, CIS and MTX) and to ionizing irradiation when compared with parental cells (60, 61). Altogether, these findings strongly suggest that OS are enriched in cells with stem-like properties and that these cells are responsible for drug resistance.

1.3. Objectives

Being OS regarded as a differentiation disease that arises from mesenchymal stem cells due to a disruption in the osteoblastic differentiation pathway, and the compelling evidences that osteosarcoma possess CSCs, it is likely that mesenchymal stem cells are the cells of origin of CSCs in OS.

The main goal of this study was to isolate and characterize CSCs population from a human osteosarcoma cell line and to evaluate the dynamic changes that occurred during their differentiation, regarding changes in expression levels of stemness markers and of MDR-related proteins, changes in metabolic activity and sensitivity to DOX, changes in clonogenic potential and on pattern of motility.

2. Materials and Methods

2.1. Cell Culture

The adherent human osteosarcoma cell line MNNG/HOS was purchased from the American Type Culture Collection (ATCC, Rockville, MD). Cells were cultured in adherent flasks (Orange Scientific, Belgium), with RPMI-1640 (R4130, Sigma-Aldrich®) medium supplemented with 10% (v/v) of heat-inactivated fetal bovine serum (FBS, Gibco® Invitrogen Life Technologies), 1% (v/v) of penincilin/streptomicin mixture (15070-063, Gibco® Invitrogen Life Technologies) and 0.1% (v/v) of amphotericin B (5290-018, Gibco ® Invitrogen Life Technologies). Cells were kept at 37°C in a humidified atmosphere with 5% of CO_2 and 95% of air.

2.1.2. Cell viability

Cell viability was assessed before all experiments through the trypan blue exclusion method. This method is based in the principle that viable cells with intact cytoplasmic membrane extrude the dye and thereupon come out with a clear cytoplasm, whereas dead cells take up the dye and appear blue under the microscope. For this assay equal volumes (20 µl) of cell suspension and of 0.4% (w/v) Trypan Blue (Sigma Chemicals, St. Louis, USA) solution were mixed and transferred into a Neubauer hematocytometer (F009-111020, Optik Labor®). Cells were observed and counted under an inverted microscope (Nikon, Eclipse TS 100). Cellular viability was calculated as percentage of viable cells relative to the total number of cells. Only cellular suspensions with viability higher than 90% were used in all experiments.

2.2. Sphere formation assay

Cells with stem-like properties were isolated from the MNNG/HOS cell line through the sphere formation assay under anchorage-independent conditions in serum-free medium. The adherent MNNG/HOS cells at a confluence of 70-80% were detached from the culture flask (Orange Scientific, Belgium) with trypsin-EDTA (Sigma-Aldrich®) and seeded in 6-well plates (Orange Scientific, Belgium) coated with poly-HEMA 0.8mg/cm^2 at a density of 6×10^4 cells/well in serum free N2-methylcellulose based medium. The N2 medium is composed of Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 Ham (DMEM/F12, D2906, Sigma-Aldrich®) supplemented with sodium bicarbonate 1.2 g/L (S6297, Sigma-Aldrich®), progesterone 20 nM (P7556, Sigma-Aldrich®), putrescine 100 µM (P5780, Sigma-Aldrich®), insulin-transferrin-

selenium-A supplement 1% (v/v) (Gibco® Invitrogen Life Technologies) and penincilin/streptomicin mixture 1% (v/v) and amphotericin B 0,1% (v/v) mixed with equal volume of sterile 2% methylcellulose solution (M0387, Sigma-Aldrich®). Fresh aliquots of epidermal growth factor (EGF, E9644, Sigma-Aldrich®) and of human basic fibroblast growth factor (bFGF, Peprotech EC, London, UK) were added twice a week at a final concentration of 10 ng/mL. Cells were maintained in a humidified atmosphere of 5% CO_2 and 95% air at $37^{o}C$.

After 7-10 days of culture, the formed spherical colonies, termed as sarcospheres, were collected and transferred to culture flasks where were maintained in RPMI medium containing 10% FBS and allowed to grow in monolayer. After reaching a confluence of 70-80%, cells were detached and then re-seeded again in serum-free medium in non-adherent conditions as previously described for the formation of secondary spheres. This procedure was repeated three to four times to evaluate the self-renewing capacity of cells growing in serum-free medium in non-adherent conditions. Spherical colonies of 3rd and 4th generation were termed as cancer stem cells (CSCs) and were used in subsequent studies.

2.3. Characterization of CSCs

2.3.1. Multilineage differentiation potential into mesenchymal lineages

The differentiation assays of the isolated CSCs into mesenchymal lineages (adipogenic, chondrogenic and osteoblastic) were performed using the STEMPRO® differentiation kits (Gibco® Invitrogen Life Technologies) according to the guidelines of the manufacturer.

Before inducing differentiation, the sarcospheres (3rd or 4rd generation) were transferred to a culture flask and expanded in Mesenchymal Stem Cell Growth medium consisting of DMEM low glucose medium supplemented with 10% (v/v) of MSC-qualified serum, 2mM glutamine (59202C, Sigma-Aldrich®), 1% (v/v) of penincilin/streptomicin and 0.1% of amphotericin B until reaching 60-80% of confluence.

2.3.1.1. Adipogenic differentiation

To induce adipocyte differentiation, cells were detached and plated in a 12 well plate (Orange Scientific, Belgium) at a density of 3×10^4 cells/well in MSC growth medium. After 24 hours the culture medium was replaced by Complete Adipogenesis Differentiation Medium consisting of STEMPRO® Adipocyte Differentiation Basal Medium supplemented with 10% (v/v) of STEMPRO® Adipogenesis Supplement, 1% (v/v) of penincilin/streptomicin and 0.1% of

14

amphotericin B. Cells were kept at 37°C in a 5% CO_2 incubator for 14 days. The culture medium was replaced every 3 to 4 days. After the incubation period, the culture medium was removed and the cells were rinsed with phosphate-buffered saline solution (PBS). Cells were fixed with 4% formaldehyde solution (143091.1214, Panreac Quimica SAU) for 30 minutes at room temperature. Thereafter, cells were rinsed with distilled water, incubated for 5 minutes in isopropanol 60% solution and stained with 0.3% Oil Red O solution (w/v) (O065, Sigma-Aldrich®, USA) in 60% isopropanol for 5 minutes. Cells were washed several times with distilled water until the water became colourless, and then incubated with hematoxilin for 1 minute for nuclei staining. After removal hematoxilin, cells were rinsed with distilled water and observed using a contrast phase inverted microscope for visualization of lipid droplets and images were captured.

2.3.1.2. Chondrogenic differentiation

To induce chondrogenic differentiation, a high density cell suspension containing 1.6×10^7 cells/mL in MSC growth medium was prepared to generate micromass cultures. Five µL droplets of the cell suspension were seeded onto the bottom of 6-well plate. Cultures were kept at 37°C in a 5% CO_2 incubator along 2 hours, and then incubated with 2 mL of Chondrogenesis Differentiation Medium consisting of STEMPRO® Chondrocyte Differentiation Basal Medium supplemented with 10% (v/v) of STEMPRO® Chondrogenesis Supplement, 1% (v/v) of penincilin/streptomicin and 0.1% (v/v) of amphotericin B. The cultures were reefed every 2-3 days for a culture period up to 14 days. After the incubation period, the medium was removed and the cultures rinsed with PBS and fixed with a 4% formaldehyde solution during 30 minutes and washed gently once with PBS. After fixation cells were stained with Alcian Blue 1% solution (A5268, Sigma-Aldrich®, India), prepared in 0.1M hydroxide chloride (HCl) during 30 minutes and then gently washed with 0.1M HCl 3 times. Stained differentiated cells were observed and captured in a contrast phase inverted microscope.

2.3.1.3. Osteogenic differentiation

To induce osteogenic differentiation, cells were plated in a 12-well plate at a density of 2×10^4 cells/well in MSC growth medium. After 24 hours the culture medium was replaced by Complete Osteogenesis Differentiation Medium consisting of STEMPRO® Osteocyte Differentiation Basal Medium supplemented with 10% (v/v) of STEMPRO® Osteogenesis supplement, 1% (v/v) of penincilin/streptomicin and 0.1% (v/v) of amphotericin B in order to induce cells' differentiation into osteocytes. Cells were maintained at 37°C in a 5% CO_2 incubator

during 21 days. The cultures were reefed every 3 to 4 days with the osteogenesis differentiation medium. Following the incubation period, the culture medium was removed and cells were rinsed with PBS and then fixed with 4% formaldehyde solution for 30 minutes at room temperature. After fixation cells were washed twice with distilled and stained with 2% of Alizarin Red S (A5533, Sigma-Aldrich®, China) solution (pH=4.2) during 2-3 minutes, followed by washing with distilled water until the water becomes colorless. Stained differentiated cells were observed and captured in a contrast phase inverted microscope.

2.3.2. Expression of mesenchymal stem cell surface markers

Both isolated CSCs and parental MNNG/HOS cells were analyzed by flow cytometry for the expression of cell surface antigens associated with mesenchymal stem cells (MSC). According to the recommendations of the International Society for Cellular Therapy (62) MSC should be positive for CD73, CD90 and CD105 surface markers and negative for CD11b, CD19, CD34, CD45 and HLA-DR. Adherent MNNG/HOS at 70-80% of confluence were detached with trypsin-EDTA, counted and suspended in PBS at a concentration of 1.5×10^6 cells/mL. Third generation sarcospheres were collected, dissociated with accutase (Gibco® Invitrogen Life Technologies) and resuspended in PBS at a concentration of 1.1×10^6 cells/mL. Two hundred microliters of each single-cell suspension were incubated for 10 minutes in the dark with fluorescent-labelled monoclonal antibodies. After the incubation period, cells were washed twice with PBS for removal of unlabelled antibodies and were re-suspended in 100 µl of PBS. Cells were analyzed in a cytometery BD FACS Canto™ II Flow Cytometer (Becton Dickinson, S.A., USA) and analysed using the CellQuest software (BD Biosciences). The fluorescent-labelled monoclonal antibodies analyzed were:

- Phycoerythrin (PE)-conjugated CD73 (BD Pharmingen™)
- Phycoerythrin (PE)-conjugated CD105 (Immunostep)
- Allophycocyanin (APC)-conjugated CD90 (BD Pharmingen™)
- Pacific blue (PB)-conjugated CD11b (BD Pharmingen™)
- Phycoerythrin (PE)-Cy7-conjugated CD19 (eBioscience)
- Peridinin-cholophyll-protein complex (PerCP)-Cy5.5-conjugated CD34 (BD Pharmingen™)
- Pacific orange (PO)-conjugated CD45 (Gibco® Invitrogen Life Technologies)
- Fluorescein isothiocyanate (FITC)-conjugated HLA-DR (eBioscience)

2.3.3. Expression of pluripotency markers and drug efflux transporters by Western Blot Analysis

The expression of the transcriptional factors OCT4 and Nanog that are required for maintaining the pluripotency and self-renewal of embryonic stem cells and of the drug efflux transporters P-glycoprotein and BCRP that have been proposed as markers of CSCs was analyzed in the isolated CSCs and were compared to those determined in parental MNNG/HOS cells.

- **Cell lysates**

Adherent cells with a confluence of 70-80% % were rinsed with PBS and scrapped in 3 mL of ice-cold PBS and transferred to microcentrifuge tubes. Cells were centrifuged at 5000 rpm during 5 minutes at 4°C. The supernatants were discarded and pellets were homogenized at 4°C in 150 µl of a radioimmune precipitation lysis buffer (RIPA) supplemented with a mixture of proteases and phosphatase inhibitors (Roche®) and 1mM dithiothreitol (DTT). The spherical colonies were collected and washed twice with PBS before incubation with RIPA buffer at 4°C. RIPA buffer contains 150 mM sodium chloride (NaCl, Merck), 1% (v/v)Triton X-100 (X114, Sigma-Aldrich®), 0.5% (w/v) sodium deoxycholate (D6750, Sigma-Aldrich®), 0.1% (w/v) sodium dodecyl sulphate (SDS, L3771, Sigma-Aldrich®), 50 mM trizma base (T1378, Sigma-Aldrich®,pH 8.0) and 2 mM EDTA. Thirty minutes after incubation in RIPA buffer, cell lysates were sonicated at 40 MHz (3-5 times) during 5 seconds.

- **Protein Quantification**

The quantification of protein in cell lysates was measured through the bicinchoninic acid (BCA) assay which combines the reduction of Cu^{2+} to Cu^{1+} by protein in an alkaline medium (biuret reaction) with the highly sensitive colorimetric detection of the product formed by the chelation of two molecules of BCA with one cuprous cation (Cu^{1+}). In alkaline conditions, the chelation of BCA with Cu^{1+} develops a blue/purple complex that can be read in an automatic ELISA microplate reader at a wavelength of 562 nm.

Eight diluted bovine serum albumin (BSA, A2153, Sigma-Aldrich®) standards with concentrations between 25-2,000 µg/mL were prepared to generate a standard curve. A 25 µL of each standard and an aliquot of 5 µL of each cell lysate diluted with 20 µL of water, were added to each well of a 96 well-plate (Orange Scientific, Belgium) previously filled with 200 µL/well of the working reagent containing BCA (B9643, Sigma®) and Copper (II) sulphate 4% solution in a

proportion of 50:1. The microplate was kept at 37°C for 30 minutes. The absorbance was read at 562 nm in an automatic ELISA reader (SLT Spectra-II™, Austria). The *protein concentration was determined* from *the calibration curve.*

The protein samples were mixed with an equal volume of 2× denaturing solution [trizma base 0.25 M (pH 6.8), DTT 200 mM, glycerol 20% (w/v) (G5516, Sigma-Aldrich®), SDS 4% (w/v) and bromophenol blue 0.05% (w/v)] and were heated at 95°C for 5 minutes for protein denaturation. Samples were stored frozen at -20°C until their use.

- **Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransference**

The separation of proteins was performed based on their molecular weight by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Polyacrylamide gels are neutral, hydrophilic three-dimensional networks of long hydrocarbons cross-linked by methylene groups that are formed by the polymerization of acrylamide and N,N-methylene-bis-acrylamide (Bis) which is the cross-linking agent. The separation of the proteins within a gel is determined by the relative size of the pore formed, which is dependent on the total amount of acrylamide present and the amount of the cross-linker. Greater the percentage of acrylamide, smaller is the pore size. Lower percentage gels are better for resolving high molecular weight, whereas small proteins are separated better in a gel with large pores*.*

Fourty µg of the of the total extract protein were carefully loaded in SDS-polyacrylamide gels 8 or 12% of acrylamide (161-0146, Bio-Rad Laboratories, Inc.), depending on the size of the protein (Table 2.1). Proteins were separated by electrophoresis at 110 V in buffer solution 50 mM trizma base-HCl containing 50 mM bicine (B3876, Sigma-Aldrich®) and 0.1% (w/v) SDS. A molecular weight marker (161-0373, Bio-Rad™) was included to determine the protein size and to monitor the progress of the electrophoretic run.

Table 2.1. Gel percentage of running buffers

Protein	Protein Size (kDa)	Gel Percentage (%)
OCT4	45	12
Nanog	42	12
BCRP	70	12
PgP	170	8

Following electrophoresis, the proteins were electrotransferred from the electrophoresis gel onto activated polyvinylidenedifluoride membranes (PVDF, Boehringer Mannheim,

Alemanha) in transfer buffer (12.5 mM trizma base containing 96 mM glycine (G8898, Sigma-Aldrich®) and 20% (v/v) methanol (20837.320, VWR®, Carnaxide, Portugal). The tranfer was performed at 110 V for 90 minutes at 4°C.

- **Immunoblot and quantification**

After transfer, the PVDF membranes were blocked for 1 hour in 5% non-fat milk solution in TBS-T [20 mM Tris, 137 mM NaCl and 0.1% (v/v) Tween20 (Merk)] to prevent any nonspecific binding of antibodies to the surface of the membrane and then were rinsed once in TBS-T to remove the excess of blocking solution. Afterwards, the membranes were incubated overnight at 4°C with primary antibodies at appropriated dilutions (see Table 2.2) in TBS-T containing a carrier protein (1% non-fat dry milk or 5% BSA(w/v). After incubation, the membranes were washed 5× for 5 minutes with TBS-T, followed incubation with alkaline phosphatase-conjugated secondary antibodies (anti-mouse or anti-rabbit) for 1 hour at room temperature with gentle agitation. Finally, the membranes were washed 5× for 5 minutes with TBS-T and then revealed using the chemofluorescence substrate ECF (ECF, Western blotting Reagent Pack, GE Lifesciences, Pittsburg, PA).

Table 2.2. Composition of the primary antibodies' solutions used and respective secondary antibodies.

Protein	Primary antibody (dilution)	Preparation	Secondary antibody (dilution)
OCT4	1:500 (Cell signalling®)	5% BSA	Anti-Rabbit (1:20000)
Nanog	1:500 (Cell signalling®)	5% BSA	Anti-Rabbit (1:20000)
BCRP	1:250 (Milipore™)	1% non-fat milk	Anti-Mouse (1:20000)
PgP	1:150 (Calbiochem®)	1% non-fat milk	Anti-Mouse (1:20000)

The expression levels of all analyzed proteins were quantified relative to a structural protein, β-actin. The membranes were stripped using 0.2 M NaOH and then reprobed with the anti-β-actin (Milipore™) diluted 1:10000 in TBS 1% non-fat milk. The incubation was performed for one hour at room temperature followed by 5 consecutive washes of 5 min with TBS-T. Ultimately, the membranes were incubated with an anti-mouse secondary antibody in a dilution of 1:2000 prepared in a 1% non-fat milk solution containing 0.02% sodium azide and detected using chemiluminescence.

The immunoreactive bands were visualized on a Typhoon FLA 9000 (GE Healthcare Bioscience AB, Uppsala, Sweden) and were quantified using the ImageJ software (Resarch Service Branch).

The levels of the proteins of interest were normalized to β-actin and were expressed as a ratio of the levels found in parental MNNG/HOS cells that was set as 1.

2.4. Differentiation of CSCs

Sarcospheres of 3rd or 4th generation were transferred to adherent flasks and cultured in RPMI medium supplemented with 10% FBS without growth factors to induce differentiation. Cells were maintained under these differentiating culture conditions along 21 days and were sub-cultured twice a week after reaching 70-80% confluence.

Several biological parameters namely, the metabolic activity, drug sensitivity, expression of stemness markers and of MDR-related proteins, clonogenicity and migration ability were evaluated along the 21 days differentiation period of cells at various time intervals.

2.4.1. Analysis of dynamic alterations of protein expression profiles during differentiation of CSCs

The dynamic alterations in the expression of proteins related with pluripotency (OCT4 and Nanog) and of MDR-related transporters (P-glycoprotein and BCRP) were analyzed along the differentiation of CSCs by Western blot analysis as previously described in section 2.3.2 This analysis was performed in CSCs at 2, 7, 14 and 21 days under differentiation culture conditions.

2.4.2. Sensitivity to Doxorubicin – MTT colorimetric assay

The sensitivity of CSCs along differentiation to DOX was analyzed using the [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide] (MTT) colorimetric assay, which enables the quantification of viable cells. The mitochondrial enzyme succinate dehydrogenase of metabolic active cells reduces the yellow tetrazolium salt to purple formazan crystals. The colored product can be quantified, after dissolution with acidified isopropanol, by spectrophotometry in an ELISA microplate reader.

The cytotoxicity of DOX was analyzed in MNNG/HOS cells, in isolated CSCs (3rd or 4th generation) and in CSCs at 2, 7, 14 and 21 days under differentiation conditions. Adherent cells were were detached from the culture flask with trypsin-EDTA and plated in 24 well plates (Orange Scientific, Belgium) at a density of 3×10^4 cells/well in RPMI culture medium containing 10% FBS. Sarcosheres were collected, dissociated with accutase and plated at a density of

3×10^4 cells/well in MSC Growth Medium. After 24 hours cells were incubated with increasing concentrations of doxorubicin in the range of 0.001 to 100 µM during 48 hours. Following this period, the culture medium was removed and 200 µL of a 5 mg/mL MTT solution (M2128, Sigma®) was added to each well. The plate was incubated during 4 hours at 37°C. The formazan crystals produced in viable cells were dissolved by adding 200 µL of acid isopropanol (HCl 0.04). After dissolution, 300 µL of the content of each well were transferred to a 96-well culture plate, and the absorvance was read in an automatic ELISA reader (SLT Spectra-II™, Austria) at 570 nm with a 620 nm reference filter. The percentage of cell viability for each concentration of DOX was normalized to that of the controls (untreated samples) through the following equation:

$$\% \ Cell \ viability \ = \frac{Absorvance \ of \ treated \ samples}{Absorvance \ of \ control's \ (untreated \ samples)} \times 100$$

Dose-response curves were plotted in a semi-logarithmic scale and were fitted to a sigmoidal function according to the equation:

$$y \ = A1 + \frac{A2 - A1}{1 + 10^{(\log x0 - x)p}}$$

where y is the response, $A1$ is the minimum value of y, $A2$ is the maximum value of y, $x0$ is the half maximal inhibitory concentration (IC$_{50}$) and p is the slope of the curve. The curves' fit was performed in Origin software (Version 8, OriginLab Corporation).

2.4.3. Metabolic activity [^{18}F]FDG Uptake assay

The metabolic activity of cells along differentiation was assessed based on the cellular uptake of the radiopharmaceutical [^{18}F]FDG, which is an analogue of glucose that accumulates preferentially in cells with high metabolic rates.

This study was performed in MNNG/HOS cells, in CSCs (3rd or 4th generation) and in CSCs with 2, 7, 14, and 21 under differentiating conditions.

For this assay single-cell suspensions of each cell type were prepared by enzymatic dissociation at a density of 2×10^6 cells/mL in respective culture media. Cells were kept in the incubator for 1 hour before the onset of the experiments for cells recovery.

After the recovery period, cells were incubated with 0.75 MBq/mL of [^{18}F]FDG at 37°C in a heating plate. At 15, 30, 45 and 60 minutes, samples of 200 µL were collected to microcentrifuge tubes filled with 500 µL ice-cold PBS and centrifuged during one minute at 10^4 rpm. Supernatants were collected to plastic tubes and pellets were washed with 500 µL of ice-

cold PBS. Radioactivity of both cell pellets and supernatants was measured in a radioisotope calibrator well counter (SR3, Nuclear Enterprises, Reading, UK) within the ^{18}F sensitivity energy window. The cellular uptake was calculated as percentage of total activity normalized to one million of cells. The collected data points of each experiment were fitted to an exponential growth function in order to calculate the steady-state accumulation of [18F]FDG according to the following formula:

$$C_t = C_{ss} \times \left(1 - e^{-kt}\right)$$

Where C_t is the cell content of [18F]FDG at time t (%/10^6 cells), C_{ss} is the steady-state [18F]FDG concentration (%/10^6 cells), k is the uptake rate (min^{-1}) and t is the time (minutes).

2.4.4. Evaluation of cells' migration capacity – Scratch assay

The migratory capacity of tumor cells was analyzed using the Scratch assay or wound healing assay, in which we measure the propensity of cells to close an open wound in a cellular confluent monolayer. The more quickly cells populate the wound higher is their migratory ability.

This assay was carried out in four groups of cells: parental MNNG/HOS cells, isolated CSCs and CSCs with 2 and 5 days of differentiation.

Cells were seeded in a 12-well plate at a density of 2.5×10^5 cells/well in specific culture medium and were allowed to reach a confluent monolayer. The wound was created with a plastic pipette tip. The cells were washed to remove debris and were replenished with fresh medium. The wound area was photographed at time 0 and every two hours until the wound is completely closed. Photographs were taken in a phase contrast microscope. Images were further analyzed qualitatively.

2.4.5. Clonogenic assay

For the analysis of clonogenic activity of cells along differentiation, cultured CSCs with 2 and 14 days under differentiation conditions and MNNH/HOS cells were tripsinized to obtain single-cell suspensions and were plated in a 6-well plate at a density of 150 cells/well in RPMI culture medium containing 10% FBS. Spherical colonies were dissociated and plated at the same density in MSC Growth Medium. Cells were maintained in culture for 12 days at 37°C in a CO_2 incubator and the medium was refreshed once a week. The cell growth was monitored microscopically to ensure that each colony originates from a single cell. At the end of the

incubation period (12 days), culture media were removed and wells were washed twice with PBS. The formed colonies were fixed with formaldehyde 1% (v/v) during 15 minutes at room temperature and then stained with a crystal violet solution 0.1% during 15 minutes. Crystal violet solution was aspirated and the wells were washed with distilled water until the water became clear. The plate was photographed with a digital photographic camera (D40, Nikon®, Japan). Clusters of cells were considered colonies when they were visible macroscopically. The colony efficiency (CE) was calculated from the formula:

$$CE\ (\%) = \frac{number\ of\ colonies}{number\ of\ cells\ seeded} \times 100$$

2.5. Statistical analysis

Non-parametric Kruskal-Wallis test was used to perform multiple comparisons between multiple cell types under different or same conditions. The Mann-Whitney non-parametric test was used to perform comparisons between two different cell types under the same condition. The p-value < 0.05 was considered statistically significant. All statistical analysis was performed by using the Statistical Package for the Social Sciences (SPSS) software (version 19; SPSS, Inc., Chicago, IL).

3. Results

3.1. Isolation of a cancer stem cell (CSC) population from the human osteosarcoma cells line MNNG/HOS

The existence of a subpopulation of cancer stem cells in the MNNG/HOS cell line was demonstrated by the formation of spherical colonies after culturing the adherent MNNG/HOS cells (Figure 3.1 A) in methylcellulose-based serum-free medium under ultra-low adherence conditions.
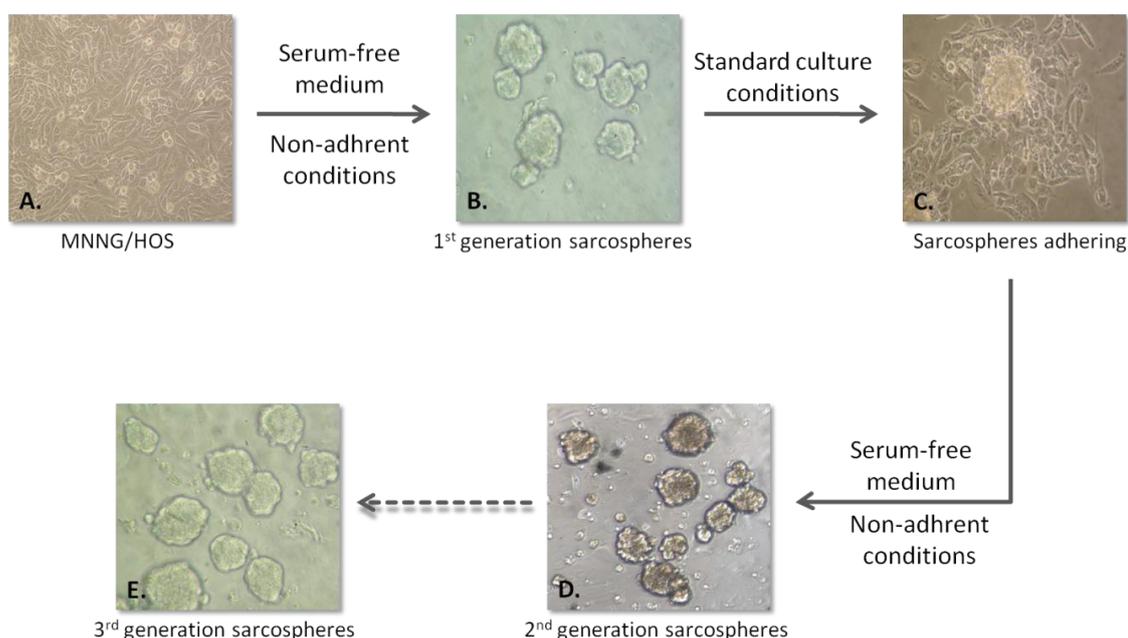
Figure 3.1. Isolation of cancer stem-like cells from the human MNNG/HOS OS cell line through the sphere formation assay. **A.** Adherent MNNG/HOS cells. **B.** Sarcospheres derived from MNNG/HOS cell line after 7 days of culturing in serum-free medium and ultra-low adherence conditions. **C.** The collected sarcospheres when cultured in serum-containing medium and in adherence conditions were able to expand and to attach to the surface of the flask. **D.** Formation of secondary sarcospheres from attached cells. **E.** Tertiary sphere formation by repeating the protocol performed for secondary sphere formation. (Original magnification: 200×)

After 7 days of culture, cells formed floating spherical colonies named as sarcospheres (Figure 3.1 B). When collected and cultured in adherent flasks in RPMI medium supplemented with 10% FBS, cells derived from colonies began to migrate and to adhere to the surface of the flask acquiring a morphology similar to that of the adherent MMNG/HOS cells (Figure 3.1 C). At near-confluence, when the cells were re-seeded as single-cells in non-adherent conditions and in serum-free methylcellulose-based medium, a secondary generation of spheres was formed with the same efficiency as the previous one. (Figure 3.1 D). The ability of forming spherical colonies

was observed after serial *in vitro* passages from adherent to non-adherent conditions, which demonstrates the self-renewal potential of the isolated cells (Figure 3.1 E). Sarcospheres of third and fourth generation were termed as cancer stem-like cells (CSCs) and were used in subsequent studies.

3.2. Characterization of Cancer Stem Cells

3.2.1. Expression of Mesenchymal Stem Cell surface markers

The expression of MSC surface markers were assessed in both CSCs and MNNG/HOS cells by flow cytometry according to the criteria of the International Society for Cellular Therapy (62).

The obtained results revealed that both MNNG/HOS and CSC cells were positive for the MSC markers CD13, CD73, CD90 and CD105 and negative for CD45 which is a leukocyte common antigen (Figure 3.2). Both cell types were also negative for the expression of HLA-DR, CD34, CD11b and CD19 (data not shown) which exclude the existence of hematopoietic and endothelial cells that are commonly found in a mesenchymal stem cell culture.
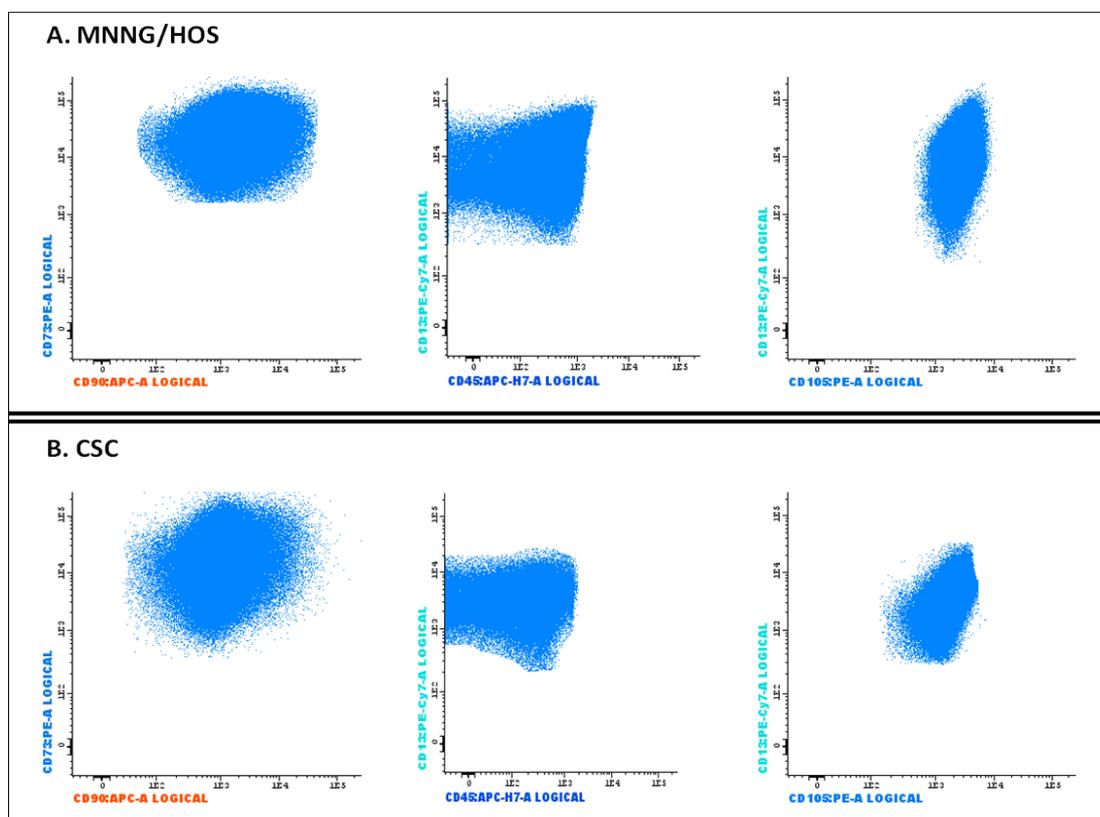
Figure 3.2. Dotplots for CD13, CD45, CD73, CD90 and CD105 expression in MNNG/HOS (**A.**) and CSC (**B.**).

3.2.2. Differentiation into mesenchymal lineages

The multipotency of the isolated CSCs was assessed by inducing their *in vitro* differentiation into adipocytes, chondroblasts and osteoblasts upon culturing under specific differentiation conditions. The obtained results showed that the isolated CSCs were able to differentiate into the three lineages that MSC can differentiate as illustrated in Figure 3.3.
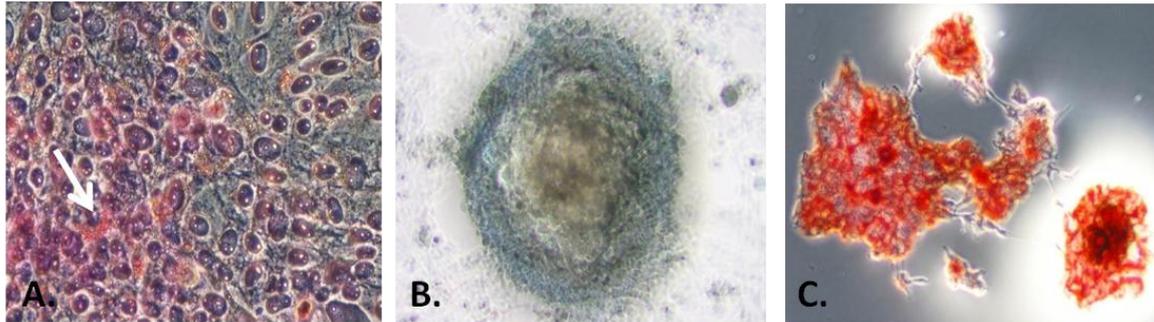
Figure 3.3. Mesenchymal lineages obtained through CSCs' differentiation. **A.** Oil Red O staining of adipocytes 14 days after CSCs culturing in Adipogenesis Differentiation Medium. (Original magnification: 400×) **B.** Alcian Blue staining of the obtained chondroblasts with 14 days of culturing in Chondrogenesis Differentiation Medium. (Original magnification: 200×) **C.** Alizarin Red staining of the osteocytes with 21 days in culture with Osteogenesis Differentiation Medium. (Original magnification: 200×)

CSCs cultured under Adipogenesis Differentiation Medium for 14 days showed adipogenic differentiation as indicated by the presence of lipid vacuoles stained with Oil Red O (white arrow in Figure 3.3 A).

The differentiation of CSCs in the chondroblastic lineage is evidenced by the intense Alcian Blue staining of proteoglycans (Figure 3.3 B) that were produced after 14 days under chondrogenic conditions.

The ability of CSCs to differentiate into the osteoblastic lineage was demonstrated by the positive red staining with Alizarin Red S that reveals the deposition of a calcified matrix upon osteogenic induction (Figure 3.3 C).

Taken together, these data suggest that the isolated CSC are multipotent and can differentiate along the three main mesenchymal lineages. These results suggest that CSCs are derived from mesenchymal stem cells and preserve their multipotency capacity.

3.2.3. Expression of pluripotency markers

The expression of the pluripotency markers OCT4 and Nanog was analyzed in CSCs and compared with that in parental MNNG/HOS cells. OCT4 and Nanog are transcription factors that are required for maintaining the pluripotency and self-renewal capacity of embryonic stem cells and have been found to be regularly expressed in CSCs. Western blot analysis showed a significant ($p < 0.05$) increased expression of both proteins in CSCs as compared with that in MNNG/HOS cells (Figure 3.4).
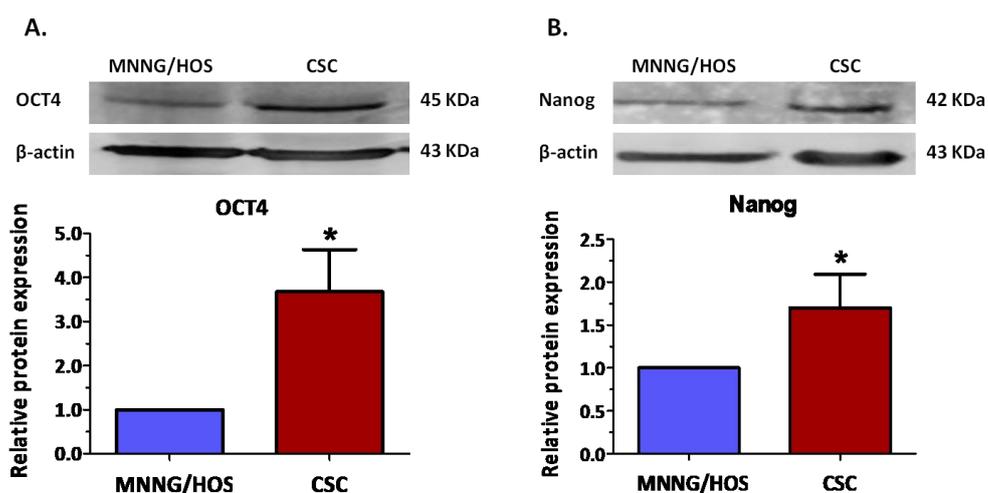

Figure 3.4. Representative Western Blot analysis of OCT4 (**A.**) and Nanog (**B.**) in both MNNG/HOS and CSC. β-actin was blotted as loading control. The lower panel represent the quantitative analysis of proteins (normalized to β-actin) expressed as a ratio of the levels found in MNNG/HOS cells that was set as 1 for both proteins. Data represent the mean ± SEM of three (n=3) independent experiments. *$p < 0.05$ as compared with MNNG/HOS

The quantitative analysis of protein expression levels indicated a 3.7-fold increase of OCT4 (Figure 3.4 A) and of 1.7-fold of Nanog (Figure 3.4 B) in CSCs in relation to MNNG/HOS cells. The higher expression of these transcription factors in CSC support the hypothesis they possess attributes of stem cells.

3.2.4. Expression of ABC transporters

The over-expression of the ABC transporters BCRP and Pgp that are associated with the multidrug resistance phenotype in tumors cells have been found to be upregulated in stem cells as a mechanism of self-defence. Our Western blot analysis revealed that both BCRP and Pgp are significantly ($p < 0.05$) over-expressed in CSCs comparatively to parental MNNG/HOS cells (Figure 3.5).
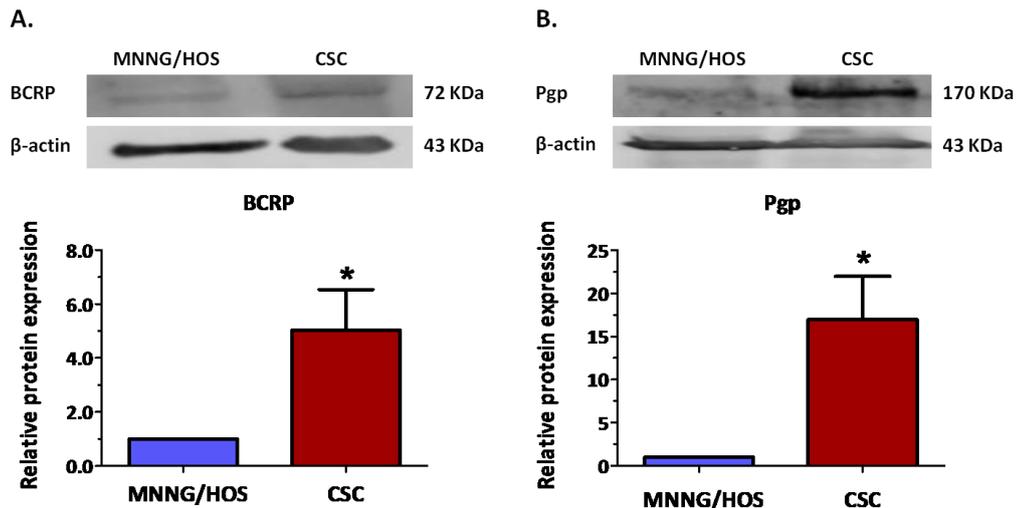
Figure 3.5. Representative Western Blot analysis of BCRP (**A.**) and Pgp (**B.**) in both MNNG/HOS and CSC. β-actin was blotted as loading control. The lower panel represent the quantitative analysis of proteins (normalized to β-actin) expressed as a ratio of the levels found in MNNG/HOS cells that was set as 1 for both proteins. Data represent the mean $\pm$ SEM of three (n=3) independent experiments.
*$p<0.05$ as compared with MNNG/HOS

The expression levels of BCRP in CSCs were 5-fold higher superior to those levels found in MNNG/HOS (Figure 3.5 A). Regarding Pgp, we found a difference even more pronounced, with 17-fold increase in CSCs in relation to MNNG/HOS cells (Figure 3.5 B). These findings are consistent with the hypothesis that these ABC transporters serves as phenotypic markers for stem cell populations.

3.3. Dynamic alterations occurring along the differentiation process of CSCs

Cells from the 3rd or 4th generation of sarcospheres (Figure 3.6 A) were induced to differentiate by culturing the colonies in RPMI medium supplemented with 10% FBS in adherent flasks. Cells were expanded under these culture conditions for 21 days. Immediately after transfer to adherent flasks, cells start to migrate from the colonies and to adhere to the surface, acquiring a spindle-shaped morphology (Figure 3.6 B). After 7 days the cells acquired the typical morphological features of the parental MNNG/HOS cells (Figure 3.6 C) that was maintained during the entire period of culture under differentiation conditions (Figure 3.6 D).
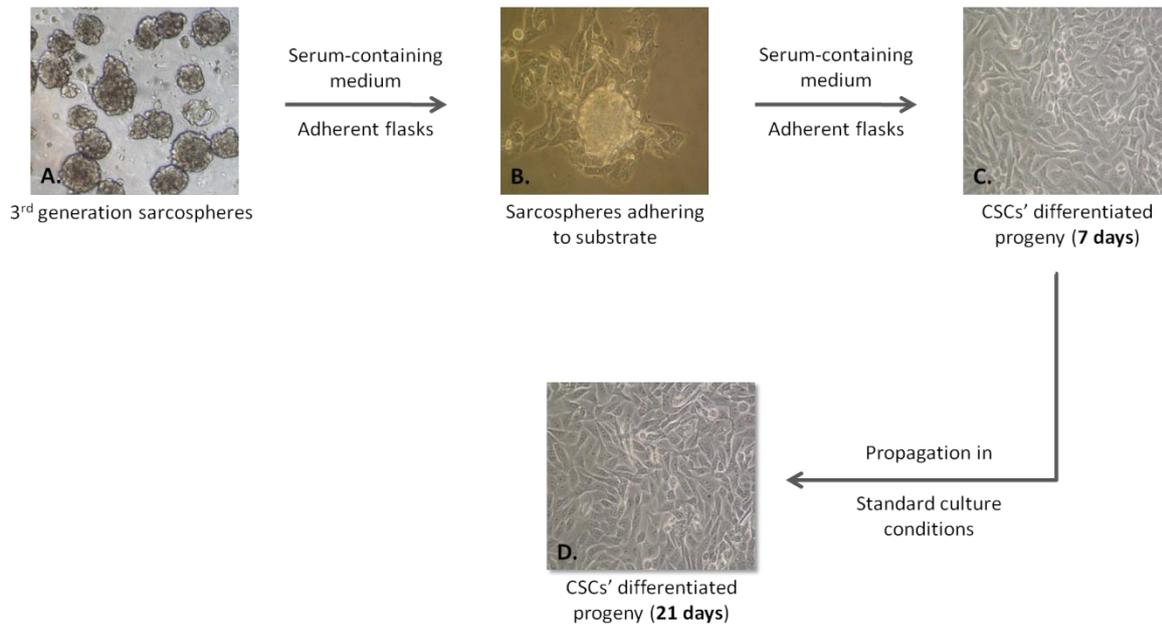
Figure 3.6. Differentiation of CSCs induced by standard culture conditions. **A.** 3rd generation spherical colonies. **B.** Adhesion of a colony to the bottom of the culture flask. **C.** CSC's differentiated progeny after 7 days of culturing in standard culture conditions. **D.** Cell population obtained 21 days after culturing spherical colonies under standard culture conditions (Original magnification: 200×)

These results demonstrate the ability of CSCs to generate differentiated progeny which is considered a fundamental property of CSCs.

3.3.1. Expression of stemness markers OCT4 and Nanog along differentiation

To investigate whether transcription factors OCT4 and Nanog that regulates the maintenance of the stem cell phenotype change along differentiation of CSCs, we analyze the expression levels of OCT4 and Nanog by Western blot along the differentiation of CSCs at specific times. This analysis was performed in the isolated CSCs and after 2, 7, 14 and 21 days of culture under differentiation conditions. The expression levels, after normalizing to β-actin, were presented in relation to the levels found in parental MNNG/HOS cells.

The expression levels of both transcription factors OCT4 and Nanog that were found to be significantly higher in CSCs than in parental MNNG/HOS cells, decreased substantially after culturing under differentiation conditions, and reached the levels found in parental cells (Figure 3.7).
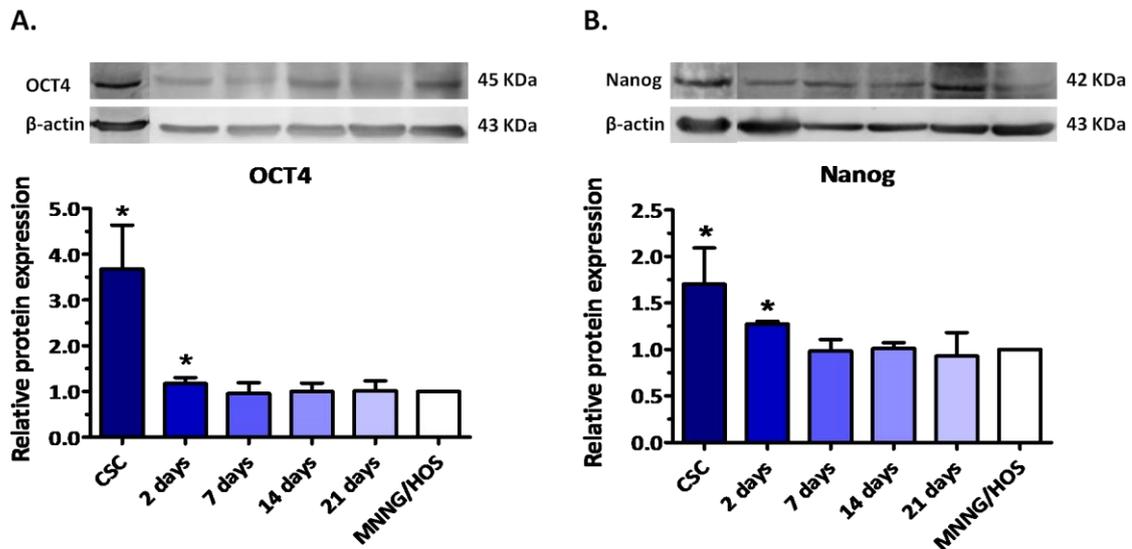
Figure 3.7. Representative Western Blot analysis of OCT4 (**A.**) and Nanog (**B.**) in CSC, CSC with 2, 7, 14 and 21 days of differentiation and MNNG/HOS. β-actin was blotted as loading control. The lower panel represent the quantitative analysis of proteins (normalized to β-actin) expressed as a ratio of the levels found in MNNG/HOS cells that was set as 1 for both proteins. Data represent the mean $\pm$ SEM of three (n=3) independent experiments.
*p<0.05 as compared with MNNG/HOS

The expression levels of OCT4 that was highly expressed in CSCs (3.7-fold higher compared with MNNG/HOS cells) decreased substantially reaching the levels found in MNNG/HOS cells after 7 days of culturing under differentiation conditions and remained almost constant throughout the differentiation period (Figure 3.7 A.). Despite this abrupt decrease in the OCT4 levels that was observed after just 2 days of differentiation, the differences in relation to the levels in CSCs were not statistically significant (p=0.05), a result that can be related with the small number of tested samples (n=3).

Similar results were observed with Nanog. The expression levels of this protein also decreased after 2 days after culture under differentiation conditions and after 7 days, the expression levels were in the same range as parental MNNG/HOS cells. In the same way as with OCT4, the decrease observed in the expression levels of Nanog in cells under differentiation were not significant (p=0.05) in relation to the levels in CSCs (Figure 3.7 B).

Although, we have not observed statistically significant differences (probably due to the small number of tested samples), our data indicates clearly that there was a tendency to a decrease in the expression levels of these stemness markers following differentiation, indicating that they lose their stemness phenotype.

3.3.2. Expression of ABC transporters BCRP and Pgp

To further investigate whether differentiation could induce changes on the expression levels of the ABC transporters (Pgp and BCRP), that we found to be up-regulated in CSC; we evaluated their expression along differentiation of CSCs. This analysis was performed at 2, 7, 14 and 21 days under differentiation culture conditions. The expression levels, after normalizing to β-actin, were presented in relation to the levels found in parental MNNG/HOS cells.

The results showed a substantial decrease in the expression levels of both transporters BCRP and Pgp in CSCs after culturing under differentiation conditions, similar to what was observed with stemness markers (Figure 3.8).

In comparison with MNNG/HOS cells, CSCs express 5-fold high levels of BCRP. After culturing under differentiation conditions, the expression levels of this protein decrease progressively reaching the values found in MNNG/HOS, after 14 days (Figure 3.8 A). However when compared with CSCs, the differences on the expression levels of BCRP were not significant for any time of differentiation, a result that is probably related with the small number of tested samples (n=3), since the p value was of 0.05 which is considered in the border line of significance.

The results presented here regarding Pgp expression along differentiation corresponds to only one experiment, and no significant conclusions can be drawn. However, these preliminary results give us an indication that Pgp is highly expressed in CSCs and decreased drastically as these cells begin to differentiate (Figure 3.8 B).
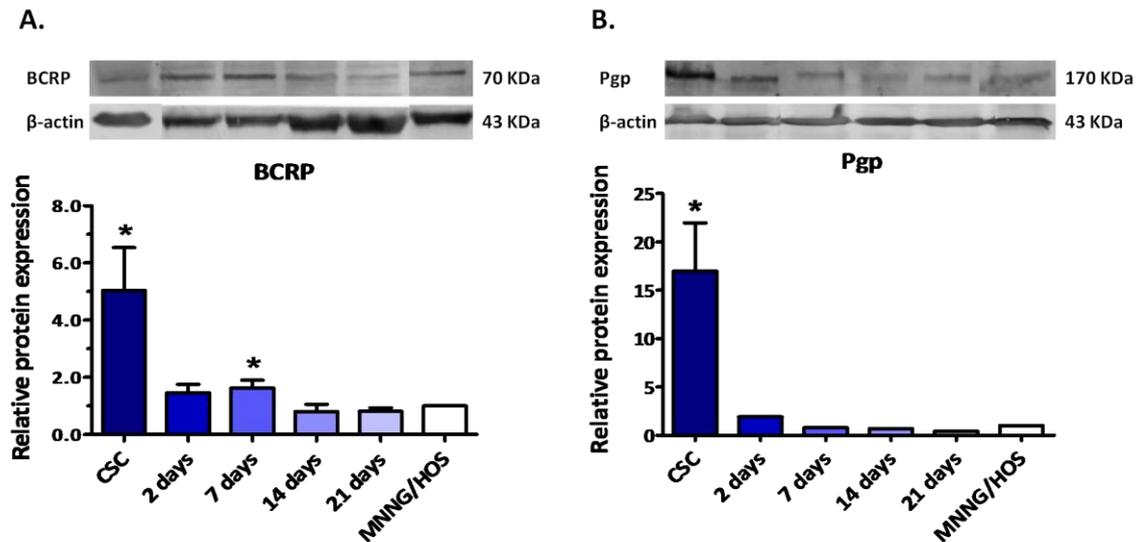
Figure 3.8. Representative Western Blot analysis of BCRP (**A.**) and Pgp (**B.**) in CSC, CSC with 2, 7, 14 and 21 days of differentiation and MNNG/HOS. β-actin was blotted as loading control. The lower panel represent the quantitative analysis of proteins (normalized to β-actin) expressed as a ratio of the levels found in MNNG/HOS cells that was set as 1 for both proteins. Data represent the mean $\pm$ SEM of three (n=3) independent experiments with the exception of the data corresponding to Pgp expression in CSCs under differentiation which correspond to a single experiment (n=1).
*$p<0.05$ as compared with MNNG/HOS

3.3.3. Sensitivity to DOX

To further investigate whether the sensitivity of cells to DOX may change along differentiation, we analyzed the cytotoxicity of DOX in CSCs with 2, 7, 14 and 21 days after culture under differentiation conditions. This study was also performed in CSCs (before inducing differentiation) and in parental MNNG/HOS cells.

The cytotoxicity of DOX was assessed using the MTT colorimetric assay after incubating cells with different concentrations of DOX up to 100 μM during 48h.

The obtained dose-response curves were fitted with a sigmoidal function for calculation the IC_{50} values, which is indicative of cells' sensitivity to DOX.

The dose-response curves to DOX of MNNG/HOS and of CSCs at different days of differentiation and their corresponding IC_{50} values are presented in Figure 3.9 and Table 3.1, respectively. Treatment with DOX induced a decrease in cell viability in a dose-dependent manner, in all cells tested (Figure 3.9). Nevertheless, there is a progressive displacement of the curves to the left as cells move towards differentiation which indicates that cells in an undifferentiated status are less sensitive to DOX than more differentiated cells. The mean IC_{50} value of CSCs was of 0.89 ± 0.01μM, and decreased as cells are more committed with differentiation, reaching the values in MNNG/HOS cells (0.54 ± 0.03μM) after 7 days under

differentiation conditions. On subsequent weeks under differentiation, the IC_{50} values remained almost constant and closer to the MNNG/HOS cells (Table 3.1). At 2 days under differentiation, cells exhibited the same resistant phenotype to DOX of the CSCs, as indicated by the overlapped dose-response curves and of the IC_{50} values (Table 3.1).
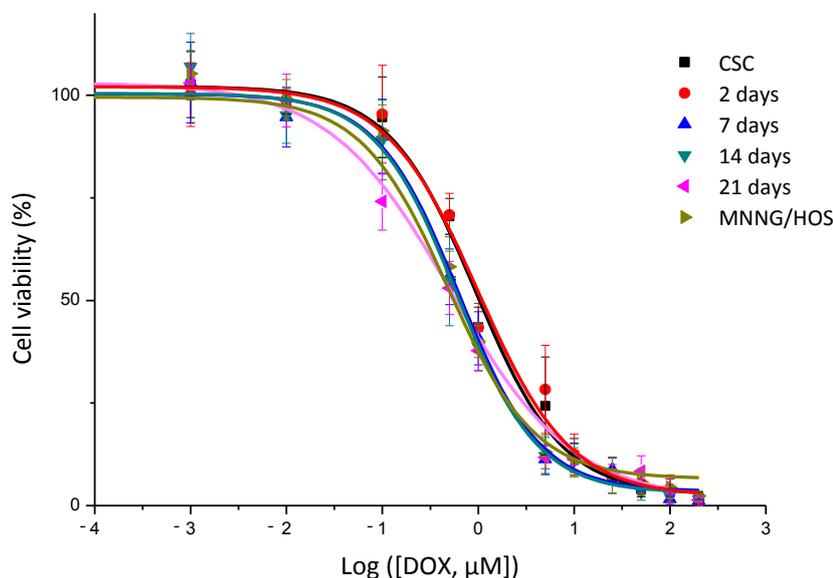
Figure 3.9. Dose-response curves to DOX for CSC, CSC with 2, 7, 14 and 21 days of differentiation and MNNG/HOS. Data-points correspond to the mean ± SD of the 5 (n=5; CSC and MNNG/HOS), 4 (n=4; 7 days) and 3 (n=3; 2 days, 14 days and 21 days) independent assays performed in duplicate.

Table 3.1. IC_{50} values of DOX in CSC under differentiation.

	IC_{50} (µM)
CSC	0.89 ± 0.01* (n=5)
2 days	0.97 ± 0.14* (n=3)
7 days	0.62 ± 0.05# (n=4)
14 days	0.60 ± 0.09# (n=3)
21 days	0.44 ± 0.04# (n=3)
MNNG/HOS	0.54 ± 0.03# (n=5)

*$p < 0.05$ when compared with MNNG/HOS
#$p < 0.05$ when compared with CSC
Abbreviations: IC_{50}, half maximal inhibitory concentration; DOX, doxorubicin. Cells were incubated with increasing concentrations of DOX (0-100 µM) for 48 hours. Cytotoxicity was evaluated using the MTT colorimetric assay. The IC_{50} values were obtained from a sigmoidal fitting of the dose-response curves. Results are expressed as mean ± SEM of 5 (n=5; CSC and MNNG/HOS), 4 (n=4; 7 days) and 3 (n=3; 2 days, 14 days and 21 days) independent assays performed in duplicate.

3.3.4. Metabolic activity

The metabolic activity of cells along differentiation was assessed based on the cellular uptake of the glucose analogue [^{18}F]FDG. This study was performed in parental MNNG/HOS cells, isolated CSCs and in cells at 2, 7, 14 and 21 days after culturing under differentiation conditions.

In all cell tested groups it was observed a progressive increase in the cellular uptake [^{18}F]FDG along the 60 min period study (Figure 3.10 and Table 3.2). However, the percentage of [^{18}F]FDG uptake in CSCs was significantly ($p < 0.05$) lower, when compared with parental MNNG/HOS cells. At 60 min the mean values for CSCs was of 3.90 ± 0.31 %, about 3-fold lower than that in MNNG/HOS cells (10.78 ± 0.59 %). After culturing under differentiation conditions it was observed a substantial increase in the cellular metabolic activity, as indicated by the significant increments in [^{18}F]FDG uptake that were observed within the first 2 days of inducing differentiation. As soon as cells start to differentiate, the cellular uptake of [^{18}F]FDG increased significantly reaching the values of the parental MNNG/HOS cells (Figure 3.10 and Table 3.2).
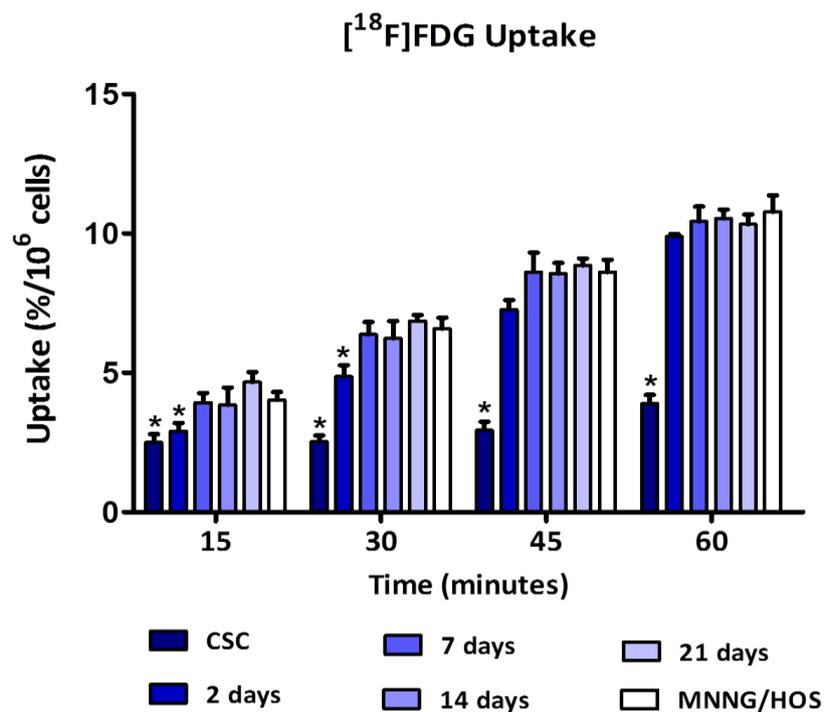
Figure 3.10. Cellular uptake of [^{18}F]FDG along differentiation of CSCs. accumulation for CSC, CSC with 2, 7, 14 and 21 days of differentiation and MNNG/HOS. Data is represented as mean ± SEM of 3 (n=3; CSC and CSC with 2, 7, 14, and 21 days of differentiation) and 4 (n=4; MNNG/HOS) independent assays performed in triplicate.
*$p < 0.05$ when compared with MNNG/HOS

Table 3.2. [^{18}F]FDG accumulation and Steady-state concentration values for CSC, CSC with 2, 7, 14 and 21 days of differentiation and MNNG/HOS.

	[^{18}F]FDG uptake (%/10^6 cells)				
	15 minutes	30 minutes	45 minutes	60 minutes	Css
CSC (n=3)	2.50±0.03*	2.53±0.22*	2.94±0.31*	3.90±0.31*	3.54±0.28*
2 days (n=3)	2.91±0.29*	4.86±0.41*	7.26±0.35	9.89±0.09	11.87±1.23
7 days (n=3)	3.93±0.34	6.38±0.44	8.61±0.70	10.43±0.53	16.64±1.56
14 days (n=3)	3.85±0.62	6.24±0.62	8.56±0.38	10.54±0.32	15.02±1.09
21 days (n=3)	4.67±0.35	6.86±0.21	8.85±0.25	10.32±0.36	12.7±0.85
MNNG/HOS (n=4)	4.03±0.28	6.58±0.40	8.61±0.45	10.78±0.59	15.78±1.35

*p<0.05 when compared with MNNG/HOS
Abbreviations: C$_{ss}$, Steady state [^{18}F]FDG concentration; [^{18}F]FDG uptake and Css are reported as the percentage of cell radioactivity associated with the total radioactivity added, normalized per million cells. Results are expressed as mean ± SEM of 3 (n=3; CSC and CSC with 2, 7, 14, and 21 days of differentiation) and 4 (n=4; MNNG/HOS) independent assays performed in triplicate.

Accordingly, the steady-state accumulation of [^{18}F]FDG in CSCs, calculated after fitting data points to an exponential growth function, was of 3.54 ± 0.28%, significantly lower than that in parental MNNG/HOS cells (15.78 ± 1.35%). After 2 days under differentiation conditions, the steady-state accumulation increases significantly (11.87 ± 1.23%, p<0.05) reaching the values of parental cells (Table 3.2) and remained constant throughout the differentiation period.

These results suggest that CSCs have lower energy requirements than their parental MNNG/HOS, which is probably related with the ability of CSCs to enter in quiescence. The higher uptake of [^{18}F]FDG that was observed in cells under differentiation, suggest that cells in a more differentiated status have high metabolic rates and therefore high energy demands.

3.3.5. Migratory capacity

A comparative analysis of the cells motility along differentiation was performed using the scratch assay. This study was carried out in parental MNNG/HOS cells, in the isolated CSCs and in CSCs with 2 and 5 days of differentiation. The change in the width of the scratched region was monitored during 24h with intervals of 4 hours after scratching. The change in the width of the scratched region is displayed in Figure 3.11.

We observed a significant reduction in the width of the scratch region of the MNNG/HOS cells as compared to CSCs. The MNNG/HOS cells moved to the wound area at a higher migration rate, being the wound completely closed after 12h. For CSCs, the reduction in the width of the

scratch occurred more slowly, taken more than 25 hours to close the wound. After inducing differentiation, it was observed an increase in the number of cells in the wounded as compared with CSCs at the same time-points. Cells repopulated the wound area, at approximately, the same migration rate as of MNNG/HOS cells. The wound area of cells with 2 or 5 days of differentiation was almost closed after 12h (Figure 3.11).

Gathering together, these results suggest that undifferentiated CSCs have lower mobility than MNNG/HOS but, as soon as they start to differentiate they acquire a mobility similar to that of the parental MNNG/HOS.
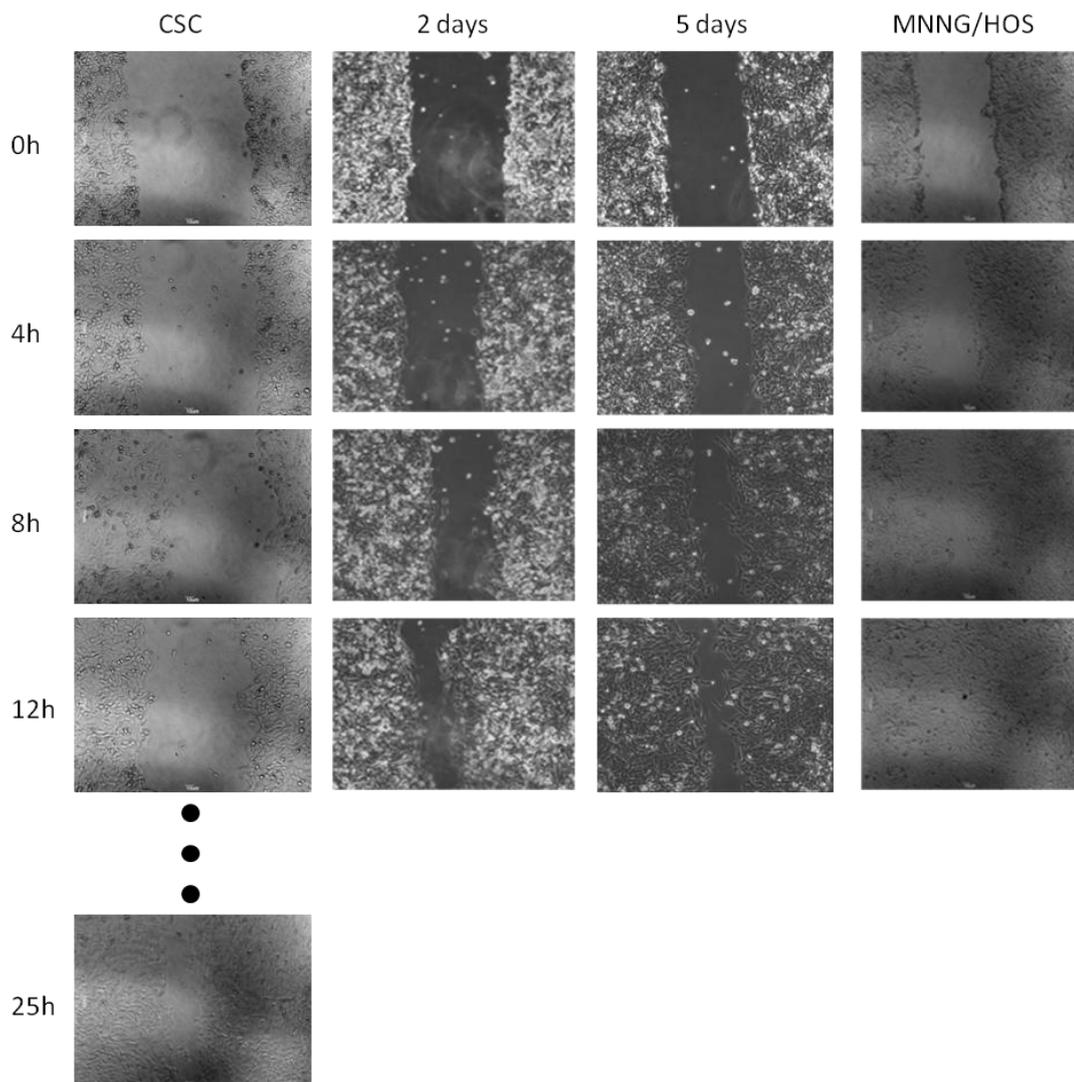
Figure 3.11. Representative phase-contrast images of cells migrating into the wounded area in an *in vitro* scratch wound healing assay. Photographs taken every 4 hours after performing the scratch. (Original magnification: 200×)

3.3.6. Clonogenic potential

The ability of cells to form colonies when seeded at low density provides an indication of their tumorigenic potential, which is proportional to the number of adherent colonies formed. Clonogenic assay was performed in CSCs, MNNG/HOS and in CSCs with 2 days and 14 days of differentiation.

As shown in Figure 3.12, the lowest clonogenic efficiency was observed in CSCs with a colony efficiency of 3.3% (Figure 3.12 A). After 2 days of culturing under differentiation conditions, the colony efficiency increased substantially to 34% (Figure 3.12 B) and then decreased to 21.3% following 14 days (Figure 3.12 C). For parental MNNG/HOS cells the colony-forming efficiency was of 18.7% (Figure 3.12 D).
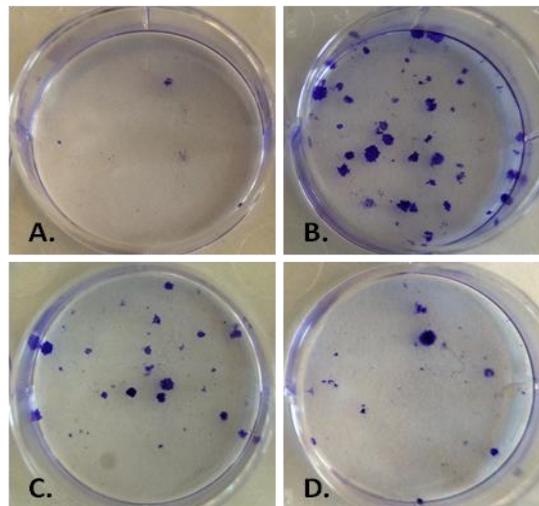
Figure 3.12. Clonogenic efficiency analysis. Fotographs of colonies formed by CSCs (**A.**) cells with 2 days of differentiation (**B.**), cells with 14 days of differentiation (**C.**) and MNNG/HOS cells (**D.**).

4.   Discussion

In this work we planned to assess the dynamic changes that occur during the differentiation of CSCs previously isolated from a human osteosarcoma cell line MNNG/HOS. We addressed changes in expression levels of stemness markers and of MDR-related proteins, changes in metabolic activity and sensitivity to DOX, changes in clonogenic potential and on pattern of motility. This analysis was performed at different time-intervals during a period of 21 days.

The population of CSCs was isolated from the MNNG/HOS cell line using the sphere formation assay. This technique allows the selection of cells with stem-like properties when cultured under serum-starved and anchorage-independent conditions. Under these stressful conditions, only cells with a stem-like phenotype can survive whereas more differentiated cells are unable to survive and die (63). After 7 days of culturing MNNG/HOS cells in serum-free medium and non-adherent conditions was observed the formation of suspended spherical colonies (sarcospheres). After 3-4 consecutive passages under these stressful conditions the cells maintained the ability to form sarcospheres providing evidences for their self-renewal potential which is a fundamental characteristic of cancer stem cells. These results are in accordance with those of Gibbs *et al.*, Wang *et al.* and Fuji *et al.* that also were able of isolate the population of CSCs from human OS cell lines and from untreated OS biopsies through this assay (36, 51, 64).

Assuming that MSCs are the cells of origin of CSCs in OS, the isolated CSCs were characterised for the expression of surface markers associated with MSCs and multilineage potential according to the recommendation of the International Society for Cellular Therapy that propose the minimal criteria to define human MSC (62).

Flow cytometry analysis of CSCs revealed a positive expression of CD73, CD13, CD90 and CD105 and negative expression of CD45, CD34, CD11b, CD19 and HLA-DR, which meet the immunophenotypic criteria to define MSCs. Additionally, we verified that CSCs differentiated towards the three mesenchymal lineages (osteogenic, adipogenic and chondrogenic) when cultured under specific induction medium, fulfilling the criteria for defining multipotent MSCs. When cultured in MSC growth medium in adherent culture flasks, cells adhere to the bottom and propagated as adherent monolayer culture, which is a well-described property of MSCs. Taken together, these results suggest that CSCs in OS arises from MSCs and preserve some degree of their plasticity.

The analysis of the expression levels of the transcription factors OCT4 and Nanog commonly associated with pluripotency, revealed that these factors were over-expressed in

sarcospheres comparatively to the levels found in parental MNNG/HOS cells. These transcription factors are involved in the self-renewal, maintenance of undifferentiated state and pluripotency of embryonic stem cells and have been proposed as surrogate markers for CSCs (65).The increased expression of both OCT4 and Nanog in sarcospheres is consistent with the results obtained by other groups (36, 51, 64) and provide compelling evidences for the undifferentiated status of these cells.

We also observed a higher expression of the transmembrane drug efflux proteins, Pgp and BCRP in sarcospheres, in comparison with parental MNNG/HOS cells. Although not related with maintenance of pluripotency, these transporters are regularly over-expressed in CSCs and are considered additional markers for CSCs (66). These proteins are proposed to protect long-lived cells like normal stem cells from naturally occurring xenobiotic toxins. The presence of these efflux transporters is CSCs is associated with the side-phenotype and resistance to chemotherapy, due their ability to extrude cytotoxic agents from the cells.

Upon induction the differentiation of CSCs by culturing under standard culture conditions (RPMI medium supplemented with 10% FBS in adherent flasks), we observed that CSCs expanded in monolayer and acquired the typical morphological features of the parental MNNG/HOS cells with decreased expression of the stemness markers OCT4 and Nanog, in parallel with a diminution in the expression levels of the drug efflux transporters Pgp and BCRP approaching to levels of the MNNG/HOS parental cells. The results from Western analysis showed a dramatic decrease in the expression levels of the stemness transcription factors, as well as a marked reduction in the levels of the drug efflux transporters. This decrease was observed just after 2-7 days upon culturing under differentiation conditions, which indicates that CSCs rapidly re-acquire the differentiated phenotype of the parental cells. These findings demonstrated that CSCs are able to differentiate and generate progeny phenotypically similar to the original parental cells. This ability is conferred by the stem-cell-like behaviour of asymmetrical division, in which CSC are able to replicate themselves and generate progeny that can differentiate into the bulk of proliferating cancer cells within the tumor.

Our results from chemosensitivity assays to DOX showed that CSCs were relatively more resistant to DOX than their parental MNNG/HOS cells, a result that can be explained by the higher expression of the drug efflux transporters BCRP and Pgp in CSCs, that recognize DOX as a transport substrate (57).

After culturing under differentiation conditions, we observed that cells become progressively more sensitive to DOX acquiring a sensitivity profile similar to that of the parental MNNG/HOS cells. This finding appears to be related with the down-regulation of the Pgp and

BCRP that was observed in more differentiated cells. Other attribute of CSCs that may contribute for their higher resistance to DOX is the ability of cells to enter in quiescence or slow dividing state, which is considered an intrinsic defense mechanism against cytotoxic drugs that target rapidly dividing cells, while sparing quiescent cells.

The metabolic activity of cells along differentiation was assessed based on the uptake of [^{18}F]FDG, positron emitter radiotracer analogue of glucose, currently used in the clinical setting for detecting and staging malignant tumors (67). [^{18}F]FDG accumulates preferentially in tumor cells due to the higher metabolic activity and energy requirements as compared with non-malignant tissues. The molecules of [^{18}F]FDG are transported into the cell by glucose transporters (GLUT 1 and GLUT 3) that are up-regulated in tumor cells. Once in the cytosol, [^{18}F]FDG is phosphorilated by the enzyme hexokinase into [^{18}F]FDG-6-phophate that is no further metabolized and accumulates into the cytosol. The degree of [^{18}F]FDG uptake, based on enhanced glycolysis, is considered a good indicator of the metabolic status of the cells (68, 69).

Our data demonstrated that CSCs accumulates significantly lower amounts of [^{18}F]FDG as compared with parental MNNG/HOS cells, suggesting that CSCs have low metabolic activity and lower energy requirements, a trait that can related with the ability of these cells to enter in quiescence. As in this state cells are not dividing they do not have high energy demands to synthesize proteins, lipids or DNA. After being placed in differentiation culture conditions, it was observed a marked increase in the uptake of [^{18}F]FDG that rapidly reached the values of the parental MNNG/HOS cells. This increase suggests there are energy-consuming processes occurring in the early transition to a differentiated state and that cells in a more differentiated status have high energy demands as compared with undifferentiated cells. These observations suggest that the quiescence of the CSC might contribute to the higher resistance to DOX, since this drug target the DNA of proliferating cells.

The *in vitro* scratch wound-healing assay mimics to some extent the migration of cells *in vivo*, which provides an estimation of their invasiveness (70). We found that the MNNG/HOS cells moved to the wound area at a higher migration rate, being the wound completely closed after 12h, whereas CSCs took more than 25 hours to close the wound area. After inducing differentiation, cells acquired motility similar to that observed in parental MNNG/HOS cells. These results suggest that cells at an undifferentiated status like CSCs have impaired motility and invasive properties, compared with more differentiated counterparts.

The ability of cells to form colonies when seeded at low density provides an indication of their tumorigenic potential, which is proportional to the number of adherent colonies formed (71). The clonogenic efficiency of CSCs was very low of about 3.3%, but increased significantly

upon induction of differentiation. The higher clone-forming ability was observed after 2 days (34%) at the early stages of differentiation, and then began to decline acquiring a clonogenic potential similar to that of the parental MNNG/HOS cells (18.7%). The limited clonogenic capacity of CSCs is likely to be related with the slow turnover of the quiescence cells, as indicated by the low uptake of [^{18}F]FDG. However the high clonogenicity that was observed at 2 days under differentiation, indicates that cells at early stages of differentiation have higher tumorigenic potential then their terminally differentiated counterparts, which is in agreement with the CSC theory. It is noteworthy that this assessment was performed by an in vitro experiment which can be a simplistic model to assess the tumorigenic potential of cells. In order to confirm these findings, these cells should be injected in athymic mice to assess their ability to generate tumors

5. Conclusions

In this study we aimed to evaluate the dynamic changes that occur along the differentiation of CSCs regarding expression of stemness markers and of MDR-related proteins, metabolic activity, sensitivity to DOX, clonogenic potential and pattern of motility.

Our findings demonstrated the existence of a population of CSCs within the MNNG/HOS OS cell line, with attributes of MSCs and ability to able to self-renew and to generate differentiated progeny phenotypically similar to their parental cells.

The culture of CSCs under differentiation conditions induced the down regulation of the transcription factors OCT4 and Nanog, which are involved in the self-renewal and in the maintenance of an undifferentiated state.

The drug efflux transporters Pgp and BCRP that were found to be upregulated in CSCs and are associated with drug resistance, decreased markedly after inducing differentiation, which explains the increased sensitivity to DOX that was observed in more differentiated cells.

The lower $[^{18}F]$FDG uptake found in CSCs suggests they are in quiescent or slow-dividing state and thereby are more resistant to DOX that target the highly proliferative cells. The increase in metabolic activity that was observed after being placed in differentiation culture conditions suggest they start to proliferate at high rate with high energy demands, becoming more susceptible to cytotoxic effects of DOX.

Taken together these results suggest that CSCs undergo processes that are analogous to the self-renewal and differentiation of normal stem cells, and that the administration of drugs inducing differentiation of CSCs could be an interesting approach to sensitize CSCs to conventional therapies and therefore to improve the outcome of osteosarcoma patients.

6.   Reference List

(1) Giullia Ottaviani, Norman Jaffe. The Epidemiology of Osteosarcoma. In: Norman Jaffe, Stefan Bielack, Øyvind S.Bruland, editors. Pediatric and Adolescent Osteosarcoma. Springer; 2009.

(2) Ta HT, Dass CR, Choong PF, Dunstan DE. Osteosarcoma treatment: state of the art. Cancer Metastasis Rev 2009; 28: 247-63.

(3) Gorlick R, Khanna C. Osteosarcoma. J Bone Miner Res 2010; 25: 683-91.

(4) Marina N, Gebhardt M, Teot L, Gorlick R. Biology and Therapeutic Advances for Pediatric Osteosarcoma. The Oncologist 2004; 9: 422-41.

(5) Sandberg AA, Bridge JA. Updates on the cytogenetics and molecular genetics of bone and soft tissue tumors: osteosarcoma and related tumors. Cancer genetics and cytogenetics 2003; 145[1]: 1-30.

(6) Kellar-graney K, Shmookler B, Malawer MM. Osteosarcoma:A Multidisciplinary Approach to Diagnosis and Treatment. American Family Physician 2002; 65[6]: 1123-1132.

(7) Beckingsale TB, Gerrand CH. (i) Osteosarcoma. Orthopaedics and Trauma 2010; 24[5]: 321-331.

(8) Klemens T, Rainer K. Proteins expressed in osteosarcoma and serum levels as prognostic factors. The International Journal of Biochemistry & Cell Biology 2001; 33: 11-7.

(9) Longhi A, Errani C, De Paolis M, Mercuri M, Bacci G. Primary bone osteosarcoma in the pediatric age: state of the art. Cancer treatment reviews 2006; 32[6], 423-436.

(10) Broadhead ML, Clark JC, Myers DE, Dass CR, Choong PF. The molecular pathogenesis of osteosarcoma: a review. Sarcoma 2011; 2011: 1-12.

(11) Hamre MR, Severson RK, Chuba P, Lucas DR, Thomas RL, Mott MP. Osteosarcoma as a second malignant neoplasm. Radiotherapy and oncology : Journal of the European Society for Therapeutic Radiology and Oncology 2002; 65[3]: 153-157.

(12) Cleton-Jansen AM, Buerger H, Hogendoorn PCW. Central high-grade osteosarcoma of bone: Diagnostic and genetic considerations. Current Diagnostic Pathology 2005; 11[6]: 390-399.

(13) Tang N, Song WX, Luo J, Haydon RC, He TC. Osteosarcoma development and stem cell differentiation. Clin Orthop Relat Res 2008; 466: 2114-30.

(14) Thomas D, Kansara M. Epigenetic modifications in osteogenic differentiation and transformation. J Cell Biochem 2006; 98: 757-69.

(15) Haydon RC, Luu HH, He TC. Osteosarcoma and Osteoblastic Differentiation: A New Perspective on Oncogenesis. Clin Orthop Relat Res 2007; 454: 237-246.

(16) Xu W, Bian Z, Fan Q, Li G, Tang Tt. Human mesenchymal stem cells (hMSCs) target osteosarcoma and promote its growth and pulmonary metastasis. Cancer letters 2009; 281[1]: 32-41.

(17) Li N, Yang R, Zhang W, Dorfman H, Rao P, Gorlick R. Genetically transforming human mesenchymal stem cells to sarcomas. Cancer 2009; 115: 4795-806.

(18) Wagner ER, Luther G, Zhu G, Luo Q, Shi Q, Kim SH, et al. Defective osteogenic differentiation in the development of osteosarcoma. Sarcoma 2011, 1-12. 2011.

(19) Zhong-Liang D, Katie AS, Ni T, Wen-Xin S, Jinyong L, Xiaoji L, et al. Regulation of osteogenic differentiation during skeletal development. Frontiers in Bioscience 2008; 13: 2001-2021.

(20) Luo X, Chen J, Song WX, Tang N, Luo J, Deng ZL, et al. Osteogenic BMPs promote tumor growth of human osteosarcomas that harbor differentiation defects. Laboratory investigation; a journal of technical methods and pathology 2008; 88: 1264-77.

(21) Zalupski MM, Rankin C, Ryan JR, Lucas DR, Muler J, Lanier KS, et al. Adjuvant therapy of osteosarcoma—A Phase II trial. Cancer 2004; 100: 818-25.

(22) Grimer RJ, Sommerville S, Warnock D, Carter S, Tillman R, Abudu A, et al. Management and outcome after local recurrence of osteosarcoma. European journal of cancer 2005; 41[4]: 578-583.

(23) Lehnert M. Clinical multidrug resistance in cancer: a multifactorial problem. European journal of cancer 1996; 32A[6]: 912-920.

(24) Chang G. Multidrug resistance ABC transporters. FEBS letters 2003; 555[1], 102-105.

(25) Chan HSL, Grogan TM, Haddad G, Deboer G, Ling V. P-glycoprotein Expression : Critical Determinant in the Response to Osteosarcoma Chemotherapy. Journal of the National Cancer Institute 1997; 89[22]: 1706-1715.

(26) Hornicek FJ, Gebhardt MC, Wolfe MW, Kharrazi FD, Takeshita H, Parekh SG, et al. P-Glycoprotein Levels Predict Poor Outcome in Patients With Osteosarcoma. Clin Orthop Relat Res 2000; 373.

(27) Whelan JS. Paediatric Update Osteosarcoma. Science 1997; 33[10]: 1611-1618.

(28) Ward RJ, Dirks PB. Cancer Stem Cells: At the Headwaters of Tumor Development. Annu Rev Pathol Mech Dis 2007; 2: 175-89.

(29) Bjerkvig R, Johansson M, Miletic H, Niclou S. Cancer stem cells and angiogenesis. Seminars in Cancer Biology 2009; 19: 279-84.

(30) Lapidot T, Sirard C, Vormoor J, Murdoch B, Hoang T, Caceres-Cortes J, et al. A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. Nature 1994; 367: 645-8.

46

(31) Lei Q, Yong Y, Yan-Jun M, Fei-Hong C, Ling-Bo Z, Wei L, et al. Isolation and characterization of cancer stem like cells in human glioblastoma cell lines. Cancer Lett 2009; 279: 13-21.

(32) Muhammad Al-Hajj, Max S.Wicha, Adalberto Benito-Hernandez, Sean J.Morrison, Michael F.Clarke. Prospective identification of tumorigenic breast cancer cells. Proc Natl Acad Sci U S A 2003; 100: 3983-8.

(33) Yi S, Nan K, Yuan A, Lu C. Isolation and characterization of cancer stem-like cells from MHCC97H Cell Lines. Journal of Nanjing Medical University 2009; 23: 194-8.

(34) Zhong Y, Guan K, Guo S, Zhou C, Wang D, Ma W, et al. Spheres derived from the human SK-RC-42 renal cell carcinoma cell line are enriched in cancer stem cells. Cancer letters 2010; 299[2]: 150-160.

(35) Cao H, Xu W, Qian H, Zhu W, Yan Y, Zhou H, et al. Mesenchymal stem cell-like cells derived from human gastric cancer tissues. Cancer letters 2009; 274[1]: 61-71.

(36) Wang L, Park P, Lin CY. Characterization of stem cell attributes in human osteosarcoma cell lines. Cancer Biology & Therapy 2009; 8: 543-52.

(37) Lobo NA, Shimono Y, Qian D, Clarke MF. The Biology of Cancer Stem Cells. Annu Rev Cell Dev Biol 2007; 23: 675-99.

(38) Zimmerman AL, Wu S. MicroRNAs, cancer and cancer stem cells. Cancer Lett 2011;300:10-9.

(39) Reya T, Morrison SJ, Clarke MF, Weissman IL. Stem cells, cancer, and cancer stem cells. Nature 2001; 414: 105-11.

(40) Patrick C.Hermann, Sonu Bhaskar, Michele Cioff, Christopher Heeschen. Cancer stem cells in solid tumors. Seminars in Cancer Biology 2010; 20: 77-84.

(41) Craig T.Jordan, Monica L.Guzman, Mark Noble. Cancer Stem Cells. The New England Journal of Medicine 2006; 355: 1253-61.

(42) Visvader JE, Lindeman GJ. Cancer stem cells in solid tumours: accumulating evidence and unresolved questions. Nat Rev Cancer 2008; 8: 755-68.

(43) Lauren L Campbell Marotta, Kornelia Polyak. Cancer stem cells: a model in the making. Current Opinion in Genetics & Development 2009; 19: 44-50.

(44) Wu C, Wei Q, Utomo V, Nadesan P, Whetstone H, Kandel R, et al. Side population cells isolated from mesenchymal neoplasms have tumor initiating potential. Cancer Research 2007; 67: 8216-22.

(45) Kitamura H, Okudela K, Yazawa T, Sato H, Shimoyamada H. Cancer stem cell: Implications in cancer biology and therapy with special reference to lung cancer. Lung cancer 2009; 66[3]: 275-281.

(46) Todaro M, Francipane MG, Medema JP, Stassi G. Colon Cancer Stem Cells: Promise of Targeted Therapy. Gastroenterology 2010; 138[6]: 2151-2162.

(47) Deng S, Yang X, Lassus H, Liang S, Kaur S, Ye Q, et al. Distinct Expression Levels and Patterns of Stem Cell Marker, Aldehyde Dehydrogenase Isoform 1 (ALDH1), in Human Epithelial Cancers. PLoS ONE 2010; 5[4]: e10277.

(48) Shin-Ya H, Masako T, Hironobu K, Shinpei Y, Tomohiro K, Toru N, et al. Pluripotential competence of cells associated with Nanog activity. Mechanisms of Development 2005; 122: 67-79.

(49) Pardo M, Lang B, Yu L, Prosser H, Bradley A, Babu MM, et al. An Expanded Oct4 Interaction Network: Implications for Stem Cell Biology, Development, and Disease. Cell stem cell 2010; 6[4]: 382-395.

(50) Matsuoka J, Yashiro M, Sakurai K, Kubo N, Tanaka H, Muguruma K et al. Role of the Stemness Factors Sox2, Oct3/4, and Nanog in Gastric Carcinoma . The Journal of surgical research 2011.

(51) C.Parker G, Valery GK, John DR, Olga T, Oleg NS, Edward WS, et al. Stem-Like Cells in Bone Sarcomas: Implications for Tumorigenesis. Neoplasia 2005; 7: 967-76.

(52) Miller SJ, Lavker RM, Sun TT. Interpreting epithelial cancer biology in the context of Stem Cells. Biochimica et Biophysica acta 2005; 1756: 25-52.

(53) Maximilian D, Robert WC, Michael FC. Therapeutic implications of the cancer stem cell hypothesis. Seminars in Radiation Oncology 2009; 19: 78-86.

(54) Hirschmann-Jax C, Foster AE, Wulf GG, Nuchtern JG, Jax TW, Gobel U, et al. A distinct "side population" of cells with high drug efflux capacity in human tumor cells. Proceedings of the National Academy of Sciences of the United States of America 2004; 101: 14228-33.

(55) Maria MH, Alvin VN, Stephen L, Jaclyn YH. Side Population in Human Lung Cancer Cell Lines and Tumors Is Enriched with Stem-like Cancer Cells. Cancer Research 2007; 67: 4827-33.

(56) Chen Hu, Hong Li, Jinjun Li, Zheng Zhu, Shengyong Yin, Xiangfang Hao, et al. Analysis of ABCG2 expression and side population identifies intrinsic drug efflux in the HCC cell line MHCC-97L and its modulation by Akt signaling. Carcinogenesis 2011; 29: 2289-97.

(57) Moitra K, Lou H, Dean M. Multidrug Efflux Pumps and Cancer Stem Cells: Insights Into Multidrug Resistance and Therapeutic Development. Clin Pharmacol Ther 2011; 89: 491-502.

(58) Elaine ML, Roger GD, Susan CC. Multidrug resistance proteins: role of P-glycoprotein, MRP1, MRP2, and BCRP (ABCG2) in tissue defense. Toxicology and Applied Pharmacology 2005; 204: 216-37.

(59) Murase M, Kano M, Tsukahara T, Takahashi A, Torigoe T, Kawaguchi S, et al. Side population cells have the characteristics of cancer stem-like cells/cancer-initiating cells in bone sarcomas. Br J Cancer 2009; 101: 1425-32.

(60) Áurio OL, Sara RN, Anália C, Artur AP, Maria B, Célia MG. Indentification of cancer stem-like cells in osteosarcoma and their implications in response to chemotherapy. BMC Proceedings 2010; 4: P41.

(61) Sara RM Neves, Aurio OG Lopes, Anália do Carmo, Antero J Abrunhosa, Paulo CPS Simões, Artur A Paiva, et al. Osteosarcoma contains a subpopulation of cancer stem-like cells that are highly resistant to radiotherapy. BMC Proceedings 2010; 4: P40.

(62) Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 2006; 8: 315-7.

(63) Yu SC, Ping YF, Yi L, Zhou ZH, Chen JH, Yao XH, et al. Isolation and characterization of cancer stem cells from a human glioblastoma cell line U87. Cancer Lett 2008; 265: 124-34.

(64) Fujii H, Honoki K, Tsujiuchi T, Kido A, Yoshitani K, Takakura Y. Sphere-forming stem-like cell populations with drug resistance in human sarcoma cell lines. International Journal of Oncology 2009; 34: 1381-6.

(65) Zhang L, Rayner S, Katoku-Kikyo N, Romanova L, Kikyo N. Successful co-immunoprecipitation of Oct4 and Nanog using cross-linking. Biochemical and Biophysical Research Communications 2007; 361: 611-4.

(66) Felix Roman Moltzahn, Jens-Peter Volkmer, Daniel Rottke, Rolf Ackermann. "Cancer stem cells"—Lessons from Hercules to fight the Hydra. Urologic Oncology 2008; 26: 581-9.

(67) Pantaleo MA, Nannini M, Maleddu A, Fanti S, Ambrosini V, Nanni C, et al. Conventional and novel PET tracers for imaging in oncology in the era of molecular therapy. Cancer treatment reviews 2008; 34[2]: 103-121.

(68) Pauwels EK, Ribeiro MJ, Stoot JH, McCready VR, Bourguignon M, Mazière B. FDG accumulation and tumor biology. Nuclear Medicine and Biology 1998; 25: 317-22.

(69) Hani AN, José MZ. Clinical Applications of 18F-FDG in Oncology. Journal of Nuclear Medicine Technology 2002; 30: 3-9.

(70) Chun-Chi L, Ann YP, Jun-Lin G. In vitro scratch assay: a convenient and inexpensive method for analysis of cell migration in vitro. Nature Protocols 2007; 2: 329-33.

(71) Nicolaas A P Franken, Hans M Rodermond, Jan Stap, Jaap Haveman, Chris van Bree. Clonogenic assay of cells in vitro. Nature Protocols 2006; 1: 2315-9.