Sphere-forming stem-like cell populations with drug resistance in human sarcoma cell lines

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Abstract. The presence of cancer stem cells in both solid and hematopoietic malignancies, has been recently linked to their pathogenesis. Sarcomas are rare, and diversely characterized by degrees of mesenchymal differentiation. The aim of the current study was to demonstrate whether the human sarcoma cell lines, osteosarcoma MG63, Ewing's sarcoma HTB166, fibrosarcoma HT1080, possess the stem-like properties which may contribute to the drug-resistance. All cell lines possessed an ability to form spherical, clonal expanding colonies (sarcospheres) in anchorage-independent, serum-starved conditions. Sarcospheres showed the stem-like properties with the ability of self-renewal, and increased expression of the stem cell-related genes such as Nanog, OCT3/4 SOX2 and DNA repair enzyme genes, MLH1 and MSH2. Sarcospheres showed strong resistance to doxorubicin and cisplatin, and caffeine, a DNA repair inhibitor, enhanced the efficacy of those drugs, suggesting that the drug resistance in sarcosphere cells was partly related to the efficient DNA repair ability. These results indicate that human sarcoma cell lines contain stem-like cell populations with strong drug resistance, and DNA repair inhibitor could enhance the efficacy of chemo-drugs against sarcomas.

Introduction

A small subpopulation of cancer cells that possess stem-like characteristics have been recently identified in leukemia (1,2), breast cancer (3), brain cancer (4), prostate cancer (5) and bone sarcoma (6), and these cells are referred to as cancer stem cells. These cells are considered to be associated with the ability to sustain tumor formation and growth and share many properties with somatic stem cells, including self-renewal and possibly multi-potency, in line with the idea of the tumor as an organ (7). Cancer stem cells may be involved in several tumor characteristics and progression. The bulk of a tumor consists of heterogeneous cell populations that may be formed by stem-like cells. It is also believed that these rare stem-like tumor initiators may be responsible for local and distant recurrence/metastasis in the phenomena such as tumor dormancy and seed and soil theories (8).

Previously, we have investigated the presence of stem-like cells in rat osteosarcoma and malignant fibrous histiocytoma cell lines by sphere colony forming assay (9), and demonstrated that the sphere-forming subpopulations in rat sarcoma cell lines might have the potential for self-renewal, higher tumorigenicity, and resistance to histone deacethylation inhibitor, possibly through lack of INK4a locus genes.

A recent study showed that cell surface makers such as CD133 may be marker of the cancer stem cell population in brain, prostate and pancreas cancers (10-13). Several studies have indicated that $CD133^+$ cells might contribute to the increased resistance to chemotherapeutic agents with high expression levels of specific drug transporters (14,15). Although the mechanisms of drug resistance in cancer stem cells remain to be elucidated, recent studies have shown that these may involve the ATP-binding cassette (ABC) drug transporters, overexpression of chemotherapy metabolizing enzymes such as aldehyde dehydrogenase 1, changes in cell cycle kinetics, and over-expression of anti-apoptotic proteins (14-16).

Unlike epithelial cancer, specific phenotypes of cancer stem cells in sarcoma have not yet been defined. In this study, we demonstrated the presence of a subpopulation of stem-like cells in human osteosarcoma, Ewing's sarcoma and fibrosarcoma cell lines that were formed of spherical colonies (sarcospheres) in anchorage-independent, serum-starved conditions. We showed that these sarcospheres expressed key marker genes in embryonic stem (ES) cells, including Nanog, STAT3, Oct3/4 and Sox2 with self-renewal ability. We

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Abbreviations: ES, embryonic stem; CDDP, cisplatin; DXR, doxorubicin; DMEM, Dulbecco's minimum essential medium; FBS, fetal bovine serum

Key words: cancer stem cell, osteosarcoma, Ewing's sarcoma, fibrosarcoma, drug resistance, DNA repair

also demonstrated the resistance of sarcospheres to cisplatin (CDDP) and doxorubicin hydrochloride (DXR), which are frequently used for sarcomas and elevated expression of DNA mismatch repair enzyme genes MLH1 and MSH2. The application of caffeine, a DNA repair enzyme inhibitor, in combination with CDDP and DXR against sphere forming stem-like cells was also investigated.

Materials and methods

Cell culture. Human osteosarcoma cell line MG63, fibrosarcoma cell line HT1080, and Ewing's sarcoma cell line HTB166 were obtained from the American Type Culture Collection (Rockville, MD) and maintained in Dulbecco's minimum essential medium (DMEM) with 10% fetal bovine serum (FBS) for MG63 and HT1080, or RPMI-1640 with 15% FBS for HTB166 at 37˚C and a 5.0% CO_2 atmosphere.

Sphere formation assay. At ~70% confluence in DMEM/10% FBS medium or RPMI/15% FBS medium, monolayer cells were dissociated with trypsin-EDTA into single-cell suspensions. The cells were then inoculated into N2 or B27-supplemented RPMI-1640/1% methylcellulose medium without serum at a density of $1x10^5$ cells/well in ultra low attachment six-well plates (Corning Inc., Corning, NY). N2 medium consists of 2X DMEM/F12 (Invitrogen, Carlsbad, CA) supplemented with progesterone (20 nM), putrescine (100 μM), sodium selenite (30 nM), transferrin (25 μg/ml), insulin (20 μg/ml), and human recombinant epidermal growth factor (EGF; 10 ng/ml) and basic fibroblast growth factor (bFGF; 10 ng/ml) mixed with an equal volume of 2% methylcellulose (6,9). All reagents except for B27-supplement were purchased from Sigma Biochemicals (St. Louis, MO). For HTB166 Ewing's cell line spheres, RPMI-1640 supplemented with B27-supplement (Invitrogen)/1% methylcellulose medium was used. Fresh aliquots of EGF and bFGF were added every other day. After 10-14 days culture, colonies that contained >20 cells were quantitated by inverted phase contrast microscopy (Olympus CK2; Tokyo, Japan). Spheres were dissociated and re-introduced into 96-well ultra low attachment plates at least 5 times, both in normal medium and in anchorage-independent methylcellulose medium, to investigate their ability to self-renew through secondary sphere formation.

Semi-quantitative reverse transcripase-polymerase chain reaction (RT-PCR). Total RNA was extracted from frozen packed cells using the RNeasy Total RNA system (Qiagen GmbH, Hilden, Germany) and first-strand cDNAs were synthesized from 500 ng samples with the SuperscriptII RNase H Reverse Transcriptase System (Invitrogen Life Technologies, Carlsbad, CA). All PCRs were performed using 0.5 μl of a reaction mixture as templates. The primer sequences used for amplification of the STAT3, Nanog, Oct 3/4, SOX2, SOX10, hMLH1, hMSH2, $p16^{INK4a}$-$p19^{ARF}$ genes are listed in Table I. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as an internal control to adjust the amounts of template. Aliquots of 10 μl of the amplification products were separated by electrophoresis in 1.5% agarose gels and visualized by ethidium bromide staining.

CDDP and DXR treatment with/without caffeine. To assess the effect of CDDP and DXR which are drugs frequently used for chemotherapy of sarcoma, MG63, HT1080 and HTB166 cells were dissociated and inoculated into 96-well microtiter plates (Corning Inc.) at a concentration of 2000 cells/100 μl/well, and allowed to attach to the plates in DMEM/10% FBS for 8 h at 37˚C. Cells were then exposed to various concentration of CDDP or DXR. After 40-h incubation with/without drugs, cell viability was measured by MTS colorimetric assay using CellTiter 96 Aqueous One Solution Cell Proliferation Assay reagent (Promega, Madison, WI) according to the manufacturer's instructions, and compared with control cells without drugs. For the sphere conditions, MG63, HT1080 and HTB166 cells were inoculated into 96-well ultra low attachment microplates (Corning Inc.) in N2/1% methylcellulose or RPMI-1640 supplemented with B27/1% methylcellulose media at a concentration of 5000 cells/90 μl/well for 14 days to allow sphere formation. They were then treated with CDDP or DXR at a final concentration of 1 or 10 μM with/without 2 mM caffeine, and subsequently cell viability was measured by MTS assay after 48-h treatment.

Results

Sphere formation from sarcoma cell lines. We evaluated the ability to generate spherical clones and self-renewal in anchorage-independent, serum-starved culture assays. Within several days, cells started to form spherical colonies and these were eventually formed at a frequency of ~1/400 (149.25±18.25 colonies/$6x10^4$ cells) for MG63 (Fig. 1a and b), 1/128 (466.75±41.25 colonies/$6x10^4$ cells) for HT1080 (Fig. 1c and d), and 1/180 (553.75±82 colonies/$1x10^5$ cells) for HTB166 (Fig. 1e and f) at 14 days. This frequency of sphere formation is similar to that reported by others for brain and breast tumors and sarcoma (3,6,10). To investigate cell self-renewal, cultured spheres were dissociated into single cells and allowed to grow both in monolayer cultures and in anchorage-independent, serum-starved condition with 1% methylcellulose medium repeatedly. Sarcospheres from all three kinds of sarcoma showed expansion in monolayer culture, which led to cell differentiation and self-renewal through the formation of secondary spheres (Fig. 1g and h).

Expression of STAT3, Nanog, Oct 3/4, SOX2, SOX10, hMLH1, hMSH2, $p16^{INK4a}$ and p19ARFin sphere cells. Current evidence from murine studies suggests that self-renewal and pluripotency of undifferentiated ES cells are maintained by crosstalk that involves three transcription factors: the POU family member Oct 3/4, the recently identified homeoprotein Nanog, and activated STAT3 (17,18). Expression of the genes STAT3, Nanog, Oct3/4 and Sox2, all of which are associated with the marker genes of pluripotent ES cells were investigated by semiquantitative RT-PCR analysis to determine whether these genes were expressed in both adherent and sphere conditions. All four genes were expressed in spheres from each cell line, and spheres consistently showed greater expression of Nanog gene than monolayer cells among all cell lines (Fig. 2a-c). Sphere cultures also showed similar robust levels of STAT3, Oct3/4 and Sox2 mRNA expression. Expression of SOX10,

Table I. Primer sets for RT-PCR.

	Forward (5'-3')	Reverse (5'-3')
Nanog	GCTGAGATGCCTCACACGGAG	TCTGTTTCTTGACTGGGACCTTGTC
Oct3/4	TGGAGAAGGAGAAGCTGGAGCAAAA	GGCAGATGGTCGTTTGGCTGAATA
STAT3	GGGTGGAGAAGGACATCAGCGGTAA	GCCGACAATACTTTCCGAATGC
SOX2	CCCCCCTGTGGTTACCTCTTC	TTCTCCCCCCTCCAGTTCG
SOX10	TATATACGACACTGTCCCGGC	AGTGTGGGTGCAACAGTCAAC
EWS (ex7)-FLI1 (ex9)	TCCTACAGCCAAGCTCCAAGTC	ACTCCCCGTTGGTCCCCTCC
INK4a ex2	ATGATGATGGGCAACGTTC	CAAATATCGCACGATGGTC
MLH1	GTGCTGGCAATCAAGGGACCC	CACGGTTGAGGCATTGGGTAG
MSH2	GTCGGCTTCGTGGGCTTCTTT	TCTCTGGCCATCAACTGCGGA
GAPDH	CAGCCGAGCCACATCG	TGAGGCTGTTGTCATACTTCT

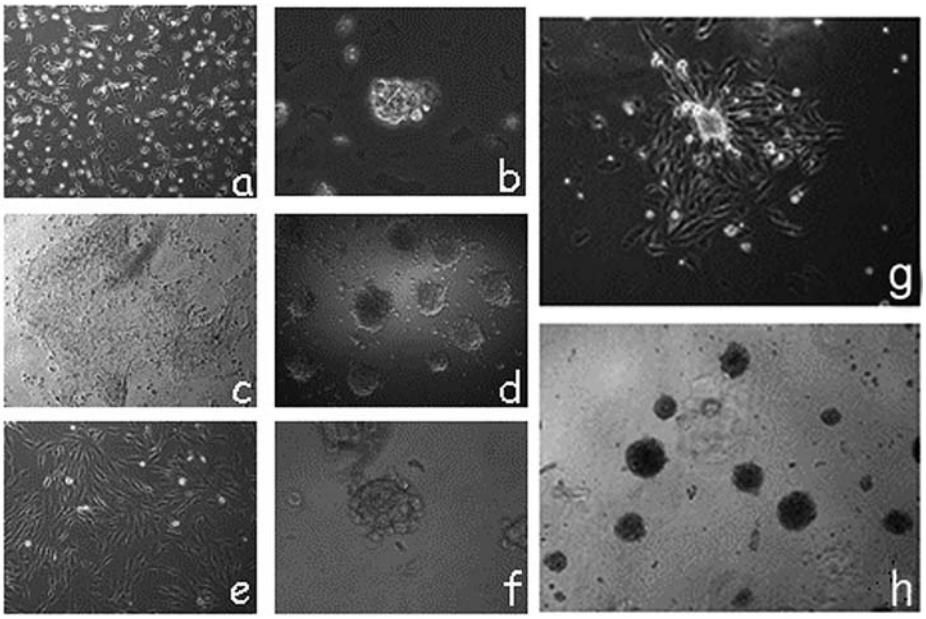

Figure 1. Microscopic appearance of human sarcoma cells in adherent, monolayer conditions (a, MG63 osteosarcoma; c, HTB166 Ewing's sarcoma; e, HT1080 fibrosarcoma) and sarcospheres in anchorage-independent, serum-starved conditions (b, MG63 spheres; d, HTB166 spheres; f, HT1080 spheres), respectively. All cell lines efficiently formed sarcospheres within 1-2 weeks. Sphere cells from MG63 showed adherent expansion by reintroduction and reculturing in monolayer, adherent conditions (g), then efficiently formed secondary spheres by repeatedly seeding into anchorage-independent, serum-starved conditions (h).

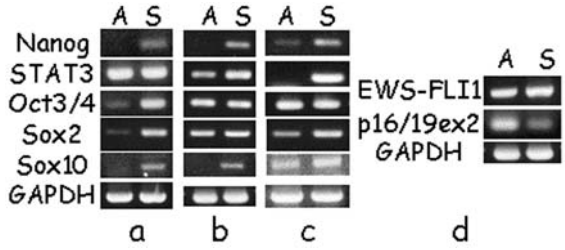

Figure 2. RT-PCR showed strong expression of Nanog, STAT3, OCT3/4, SOX2 and SOX10 in sarcospheres from all cell lines, compared with adherent cells (a, MG63 osteosarcoma; b, HTB166 Ewing's sarcoma; c, HT1080 fibrosarcoma). In the HTB166, Ewing-specific EWS-FLI1 fusion product was similarly expressed in both monolayer and sphere cultures, but INK4a locus genes expression was reduced in sphere cells (A, adherent cells; S, sarcospheres).

which encodes a member of the SOX (SRY-related HMG-box) family of transcription factors that involves the regulation of embryonic development and the determination of cell fate (19,20), was also increased under sphere conditions in all cell lines. In the HTB166 cell line, Ewing's sarcoma-specific EWS-FLI1 fusion gene was expressed in both adherent and sphere cells, but INK4a locus genes p16-p19 were decreased in sphere froming cells (Fig. 2d).

MLH1 and MSH2, which is a locus that is frequently mutated in hereditary non-polyposis colon cancer, are DNA mismatch repair proteins (21,22). DNA mismatch repair is a system for recognizing and repairing erroneous insertion, deletion and misincorporation of bases that can arise during DNA replication and recombination, as well as repairing

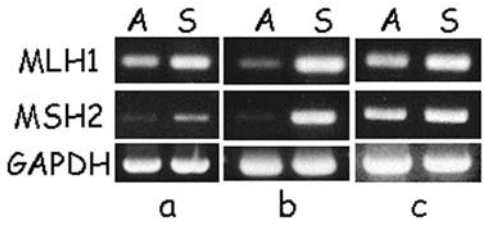

Figure 3. Expression of DNA mismatch repair enzyme genes MLH1 and MSH2 was stronger in sarcospheres compared with adherent cells (a, MG63; b, HTB166; c, HT1080, A, adherent cells; S, sarcospheres).

some forms of DNA damage (21). Expression of both MLH1 and MSH2 in sphere conditions was increased compared with monolayer conditions in all cell lines (Fig. 3).

Effect of anticancer agents on human sarcoma cells. CDDP and DXR inhibited the growth of MG63 (Fig. 4a and b) and HTB166 (Fig. 4c and d) cells in a dose-dependent manner. The survival rates of adherent and sphere cells after 48-h drug tratment are shown in Tables II and III. The difference in growth inhibition rate between adherent and sphere cells was statistically significant, i.e., 10 μM of CDDP inhibited cell growth up to 32% in MG63 and 42% in HTB166 cells in monolayer culture and 0 and 7% in sphere conditions, respectively. DRX at 10 μM concentration also inhibited cell growth up to 71% in MG63 and 49% in HTB166 cells in adherent conditions, and 16 and 0% in sphere conditions, respectively. HT1080 cells showed a simlilar tendency for cell growth inhibition (data not shown). These results suggest that the sphere cells were resistance to CDDP and DXR which are the most commonly available chemotherapeutic agents for sarcomas.

The application of caffeine, a DNA repair enzyme inhibitor, in combination with either CDDP or DXR showed increased cell growth inhibition compared with non-caffeine treatment, however the growth inhibition rates were limited to only 22.2% for CDDP with caffeine and 21.7% for DXR with caffeine (Fig. 4e).

Table II. Cell survival rates (%) after 48 h drug treatments of MG63 cell line.

	CDDP		DXR	
	1 μM	10 μM	1 μM	10 μM
MG63	82.8±5.2	67.8±7.8	33.8±3.3	30.8±1.1
MG63 spheres	NA	99.9±8.1	NA	84.2±3.8

NA, not available.

Table III. Cell survival rates (%) after 48 h drug treatments of HTB166 cell line.

	CDDP		DXR	
	1 μM	10 μM	1 μM	10 μM
HTB166	76.9±3.9	58.1±3.3	60.6±3.3	51.3±2.7
HTB166 spheres	NA	93.2±4.4	NA	100.6±11.3

NA, not available.

Discussion

Recent studies have suggested that properties of normal stem cells such as self-renewal, ability to differentiate, and activation of anti-apoptotic pathways may be shared by cancer cells. According to the stem cell model, cancer is considered to be an organ, and cancer stem cells would be a origin of cellular pedigree and play a role as cancer initiator, and this concept was first developed for human myeloid leukemias in

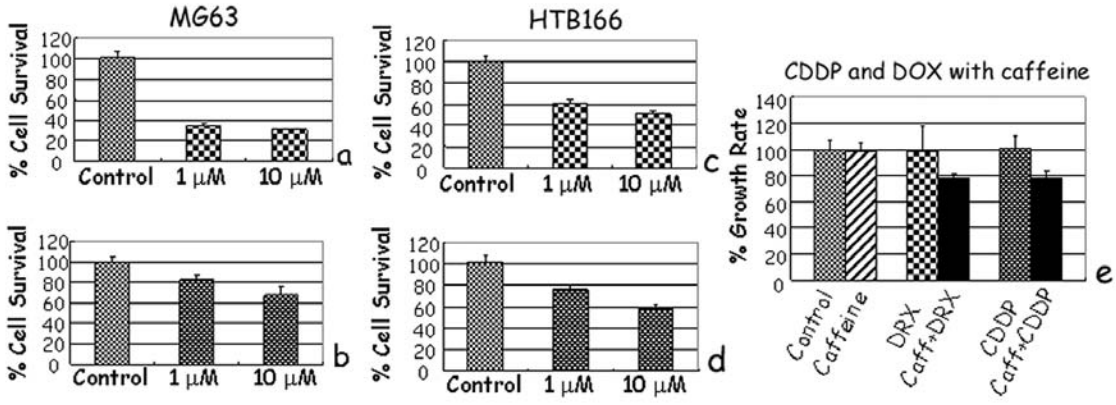

Figure 4. MG63 osteosarcoma cell growth was suppressed by DXR (a) and CDDP (b) in a dose-dependent manner. Growth inhibition rates were up to 71% by DXR and 32% by CDDP, both at 10 μM concentration. HTB166 Ewing's sarcoma cell growth was also suppressed by DXR (c) and CDDP (d) in a dose-dependent manner. Growth inhibition rates were up to 42% by DXR and 49% by CDDP, both at 10 μM concentration. Sarcospheres showed strong drug resistance. Application of caffeine, a DNA repair enzyme inhibitor, in combination with drugs increased efficacy of chemotherapeutic drugs, however the effects were limited by 23% (e) (data shown at a concentration of 2 mM caffeine in conjunction with 1 μM of DXR and CDDP. Ten μM DXR or CDDP with caffeine also showed similar results).

1970s (23,24). There are two possible ways of thinking about how the bulk of a tumor is constituted. The first is the so-called stochastic model and the second is the hierarchy model (25,26). Tumors are considered to consist of heterogeneous cell populations. In the stochastic model, every cell in the tumor can proliferate and differentiate to initiate the tumor. On the other hand, in the hierarchy model, only rare stem-like cells can differentiate and initiate the tumor, while self-renewing themselves.

The presence of cancer stem cells in the bulk of the tumor was first described in the hematopoietic system, with the identification of acute myeloid leukemia stem cells in 1994 (27). It is considered that tumor-initiating cells are characterized by stem-like properties, such as the ability to undergo self-renewal and high tumorigenicity. Now cancer stem cell populations have been prospectively identified from various tumors of epithelial origin, including the breast, colon and prostate cancer, as well as bone sarcoma (3-6,10-15,28). We demonstrated that osteosarcoma, Ewing's sarcoma and fibrosarcoma cell lines possess the ability to form sarcospheres and self-renew in anchorage-independent, serum-starved culture conditions. In some epithelial tumors, isolation of stem-like cells is based on phenotypic positive combination for epithelial-specific antigens such as CD34, CD44, CD105 or CD133, with efficient tumorigenesis by a small number of cells (11-15,29-31). However, to date, we have not found any such surface marker antigens for identification of stem-like populations in sarcoma. Thereupon, we have shown that sarcospheres express key marker genes of ES cells, Nanog, STAT3, Oct3/4 and Sox2, more strongly than adherent cultures. ES cells are derived from the inner cell mass of blastocysts and proliferate indefinitely while maintaining pluripotency (32). Sox family transcription factors play an essential role in cell differentiation, development and sex determination. Sox2 was previously thought to be the sole Sox protein expressed in ES cells (33). Oct3/4 (also known as OTF3, POU5F1) is a POU-domain, octamer-binding transcription factor expressed in ES cells (34). Sox2 associates with Oct3/4 to maintain self-renewal of ES cells. Nanog is a recently identified divergent homeoprotein that can maintain self-renewal in ES cells (17,18). STAT3 plays an important roles in regulating cell growth, differentiation, apoptosis, angiogenesis, and immune responses (35). STAT3 and over-expression of Nanog is associated with increased self-renewal ability and maintenance of pluripotency in ES cells (18,33). Although we did not demonstrate a relationship between these proteins and the genesis of sarcoma cells, these date provide compelling evidence of the presence of side-populations with stem-like properties in sarcomas. We propose that sphere-forming stem-like cells have self-renewal potency, with increased expression of proteins that are related to maintaining stem-like features in ES calls, and these spheres are possibly more primitive populations than adherent cells. Dysregulation and constitutive activation of Oct3/4, Nanog or STAT3 have been found in numerous primary cancers such as embryonal carcinoma, lymphoma, leukemia, prostate, breast and lung cancer and sarcoma (6,31,36-38). Expression of some of these genes was demonstrated in cells under adherent, monolayer conditions; therefore, some key marker genes in ES cells might play a role in sarcoma oncogenesis.

We demonstrated that sphere cells have the potential for resistance to CDDP and DXR. In sphere cells, expression of DNA repair enzyme genes MLH1 and MSH2 is increased, especially in MG63 and HTB166 cell lines. This indicates that drug resistance in cancer stem cells in sarcoma may be related to increased levels of DNA repair enzyme. Under this result from RT-PCR, we treated MG63 sphere cells with caffeine, a DNA repair enzyme inhibitor, in combination with CDDP or DXR. Both CDDP and DXR with caffeine treatment had a suppressive effect of cell growth, but this was limited to only 20-25%. In the present study, CDDP and DXR with caffeine were not satisfactorily effective against sarcospheres, possibly because of various drug resistance mechanisms other than DNA repair enzyme inhibitors. Goodell *et al* have demonstrated that multidrug resistance transporter 1 (MDR1), a member of the ATP-binding cassette (ABC) transporter transmembrane proteins, may be involved in the efflux capacity of cancer stem cells (39). These transporters have been shown to contribute to Hoechst dye efflux and produce a cancer stem cells phenotype in a wide variety of tissues, and are associated with drug resistance in tumor cell lines. The expression of these transporters has been analyzed in various malignancies in relation to the drug resistance of cancer stem cells (31).

The origin of cancer stem cells has not yet been defined. Recent studies have suggested that hereditary and sporadic breast tumors may originate through the deregulation of self-renewal pathways in normal mammary stem and/or progenitor cells (40). The heterogeneity of the tumor cells may reflect the different cells of origin and/or different mutation profiles in these cells. These heterogeneous cancer stem cell populations, in turn, may determine the overall molecular phenotype of the tumors. The development of suitable *in vitro* and animal models that aim to identify the diverse cancer stem cell populations may facilitate the discovery of possible targets for novel cancer therapies.

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