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TITLE PAGE

Title

Cancer stem cell enrichment is associated with enhancement of nicotinamide N-methyltransferase expression.

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SUMMARY

The cancer stem cell theory states that a subset of tumor cells, termed cancer stem cells (CSCs), has the ability to self-renew and differentiate within the tumors. According to this theory, CSCs would be mainly responsible for tumor initiation, progression, resistance to therapy, recurrence and metastasis.

In this study, a culture system was setup to enrich CSCs from bladder cancer (T24), lung cancer (A549), colorectal cancer (CaCo-2) and osteosarcoma (MG63) cell lines, through sphere formation. Magnetic-activated cell sorting was also used to further increase CSC enrichment. Subsequently, molecular characterization of CSC-enriched cell populations and parental cells was carried out, by exploring the expression levels of stem markers and the enzyme nicotinamide N-methyltransferase (NNMT).

Results obtained showed a significant upregulation of stem cell markers in CSC-enriched populations, obtained upon sphere formation, compared with parental counterparts. Moreover, NNMT expression levels were markedly increased in samples enriched with CSCs with respect to control cells.

Considering the fundamental role played by CSCs in carcinogenesis, reported data strengthen the hypothesis that sustain a pivotal role of NNMT in cancer growth and metastasis. In addition, these findings could represent an important achievement for the development of new and effective anticancer therapies, based on CSC-associated targets. [1]

KEYWORDS

Cancer stem cells, gene expression analysis, magnetic-activated cell sorting, molecular characterization, nicotinamide N-methyltransferase, sphere-forming cells.

[1] **Abbreviations**: Cancer Stem Cells (CSCs); nicotinamide N-methyltransferase (NNMT).

INTRODUCTION

The maintenance of tissue homeostasis relies on the activity of stem cells, a relatively small population of cells endowed with self-renewing properties and the ability to differentiate into cells that are committed to a specific developmental fate. Asymmetric cell division represents a mechanism by which stem cells accomplish these two different tasks. During this process, each stem cell generates two different daughters, one with self-renewal capacity and one that undergoes differentiation [1, 2].

Recent evidences indicate that tumors are characterized by cellular heterogeneity, in which cell populations range between differentiated cancer cells and cancer cells displaying stem-like properties, these latter named cancer stem cells (CSCs). CSCs head the hierarchy within the heterogeneous cell population featuring tumor bulk and can differentiate into transient amplyfing and differentiated cancer cells [3]. According to the stem origin of tumors, or CSCs theory, a subset of tumor initating cells possess the ability to self-renew and differentiate within the tumor bulk. While the majority of well differentiated cancer cells display only limited replicative potential, CSCs can proliferate indefinitely and spread to tissues and organs distant from the site of primary tumor. According to this theory, CSCs would be responsible for tumor initiation, growth, metastasis, resistance to therapy and recurrence [4].

CSCs are likely to share many characteristics of normal stem cells, such as the capacity to remain quiescent for prolonged periods, resistance to drugs, an active DNA-repair mechanism and the ability to escape apoptosis [5]. These features contribute, at least in part, to the failure of conventional chemotherapy and radiotherapy, which mainly target proliferating cancer cells and partly affect dormant/resistant cancer cells [3]. In this light, the existance of such a population of pluripotent cells, that can survive chemotherapy and repopulate the tumor, has foundamental implications for cancer treatment and survial. Therefore, the possibility to identify and isolate CSCs from tumors, as well as to perform a comprehensive characterization of their molecular and phenotypical properties, will lead to a significant gain of knowledge for

the development of CSC-targeted therapies aimed to achieve complete eradication of tumors [6].

In the present study we focused on nicotinamide N-methyltransferase (NNMT), a cytosolic enzyme that catalyzes the N-methylation of nicotinamide, pyridines, and other structural analogues, playing a fundamental role in the biotransformation and detoxification of many xenobiotics [7, 8].

NNMT was found to be upregulated in several kind of tumors. In particular, in our previous studies, we reported enzyme overexpression in clear cell renal cell carcinoma (ccRCC) [9, 10], in oral squamous cell carcinoma (OSCC) [11, 12], in non-small cell lung cancer (NSCLC) [13], in bladder urothelial carcinoma (BUC) [14], and in cutaneous and oral malignant melanoma [15, 16].[2]

In ccRCC, OSCC and melanoma, NNMT intratumor expression levels were found to be significantly corrrelated with different clinico-pathological parameters, thus indicating the effectiveness of the enzyme as a potential prognostic marker for these neoplasms [9, 11, 12, 15, 16].

Moreover, elevated NNMT levels were also detected in salivary and urinary samples obtained from oral and bladder cancer patients, respectively, suggesting that the evaluation of enzyme expression could be used for early and non invasive diagnosis of these malignancies [17, 18].

Analyses carried out to investigate the functional significance of enzyme overexpression in association with neoplasms, and its role in cancer cell metabolism, showed that NNMT could represent an important molecular target for anticancer therapy. In this context, our previous studies demonstrated that enzyme exerts a great impact on tumorigenicity and proliferation of different cancer cell lines. In particular, shRNA-mediated NNMT silencing led to a significant reduction of cell viability and anchorage-independent cell growth of HeLa-derived KB cancer

[2] **Abbreviations**: clear cell renal cell carcinoma (ccRCC); oral squamous cell carcinoma (OSCC); non-small cell lung cancer (NSCLC); bladder urothelial carcinoma (BUC).

cells, OSCC cell line PE/CA PJ-15 and A549 NSCLC cells. Moreover, enzyme downregulation was significantly associated with a reduced tumor formation ability of PE/CA-PJ15 cells after subcutaneous injection into athymic mice [19-21]. On the contrary, the induction of NNMT upregulation significantly increased cell proliferation of HSC-2 OSCC cell line [22]. Moreover, NNMT mRNA levels and catalytic activity had been evaluated in cell populations enriched with CSCs, obtained from the human larynx carcinoma cell line Hep-2. Results obtained, although preliminary, showed that enzyme expression was significantly higher in CSC-enriched populations than in parental counterpart [23].

To confirm these data, a specific culture system was used to enrich CSCs from different tumor cell lines through sphere formation, by cultivating cancer cells in defined serum-free medium with specific growth factors and supplements. Subsequent analyses were performed to explore whether the obtained sphere-forming cells possessed distinct stem-like properties, by evaluating the expression levels of specific stem cell markers (CD133, CD44, Oct4, Sox2, Nanog and Nestin, ABCG2 and BMI-1) by Real-Time PCR and immunocytochemistry. Further CSC enrichment was also carried out by magnetic-activated cell sorting (MACS) and the presence of stem cell markers was evaluated by Real-Time PCR analysis. NNMT expression was also evaluated in CSC-enriched populations and parental cells by Real-Time PCR, Western blot, immunohistochemistry and an HPLC-based catalytic activity assay.[3]

[3] **Abbreviations:** Magnetic-activated cell sorting (MACS).

EXPERIMENTAL PROCEDURES

Cell culture

Different human cancer cell lines (MG63, osteosarcoma; T24, bladder carcinoma; A549, non-small cell lung cancer; CaCo-2, colon cancer) were purchased from the American type Culture Collection (ATCC, Rockville, MD, USA) and cultured in DMEM/F12 medium, supplemented with 10% fetal bovine serum and 50ug/ml of gentamicine, at 37°C in a humidified 5% CO_2 incubator.

Sphere formation

MG63, T24, A549 and CaCo-2 cells were seeded on ultra-low attachment T25 flask ($5x10^5$ cells/flask) and cultured in serum-free DMEM/F12 medium, containing 10 ng/ml human recombinant epidermal growth factor (EGF), 10 ng/ml basic fibroblast growth factor (bFGF) and N2 supplement. These latter were regularly (every 2-3 day) added to culture medium and such culturing conditions were maintained until sphere formation.[4]

RNA extraction

Upon trypsinization and counting, CSC-enriched populations and parental cells were harvested by centrifugation at 300xg for 5 minutes at 4°C. Subsequently, total RNA was isolated from pellets ($1x10^6$ cells) and reverse transcribed using the RNeasy Micro Kit (Qiagen) and M-MLV Reverse Transcriptase (Promega, Madison, WI, USA), respectively.

[4] **Abbreviations**: epidermal growth factor (EGF); basic fibroblast growth factor (bFGF).

Real-Time quantitative PCR

A Real-Time PCR assaywas setup to evaluate the expression of stem cell markers as well as NNMT. Experimets were carried out by using CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) and SsoFast EvaGreen Supermix (Bio-Rad). Amplification protocol was as previously described [23] and nucleotide sequences of primers were reported in Table 1.

Upon threshold cycle (Ct) determination, differential gene expression level (CSC-enriched populations versus parental cells) was evaluated through the formula $2^{-\Delta(\Delta Ct)}$ [24], where ΔCt = Ct (stem cell marker or NNMT) – Ct (β-actin) and Δ(ΔCt) = ΔCt (CSC-enriched populations) - ΔCt (parental cells).

Immunocytochemical analyses

Immunochitochemistry for CD133 and NNMT in CSC-enriched populations and parental cells was performed according to a previously reported protocol [23], by using a mouse monoclonal anti-CD133/1 antibody (1:50, Miltenyi, Biotec) and a rabbit polyclonal NNMT antibody (1:1000, Sigma), followed by anti-mouse or anti-rabbit biotinylated secondary antibodies (1:200, Vector Laboratories).

The immunocytochemical expression of both proteins was evaluated under a Nikon Eclipse E600 light microscope (Nikon, Düsseldorf, Germany) (400x magnification) by two independent observers (G.L. and E.S.). Agreement between the observers was always >95%. Each experiment was carried out three times in quadruplicate and mean ± standard deviation (SD) was considered for each value. Staining intensity was assessed by using a three-tier grading system: "+" (moderate staining intensity), "++" (good staining intensity) and "+++" (strong staining intensity).[5]

[5] **Abbreviations**: threshold cycle (Ct); standard deviation (SD).

Western blot analysis

NNMT expression was evaluated by Western blot, following what reported by Sartini et al. [21]. Lysates from CSC-enriched populations and parental cells were obtained from pellets and subsequent protein quantification within extracts was carried out through Bradford's method [25]. Protein samples (50µg) were then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. Upon incubation with primary and secondary antibodies, signal ralated to NNMT was visualized through chemiluminescence. B-actin was used as internal control for data normalization.

NNMT enzyme activity

In order to evaluate NNMT activity levels in CSC-eniched populations and parental cells, an HPLC-based assay was used [20]. Cell pellets were resuspended in lysis buffer, homogenated and centrifuged. Supernatants were used as enzyme samples which were added to the assay mixture containing also NNMT substrates (nicotinamide and SAM), both kept at saturating concentration. Aliquots of assay mixture were removed at different time points and injected into a HPLC system, following previously described elution conditions [26]. Enzyme activities were determined by measuring the ammount (nmoles) of N1-methylnicotinamide produced and expressed as units (1U = 1nmol N1-methylnicotinamide per hour of incubation at 37°C) per mg of protein. The lower detection limit for NNMT catalytic assay was 0.01U/mg.[6]

Isolation of CSC-enriched population by MACS

MACS kit (Miltenyi Biotech, Bergisch Gladbach, Germany) was used to obtain CD133+ CSC-enriched population from MG63 cell line. Sphere-forming cells were treated trypsinized and

[6] **Abbreviations**: S-adenosyl-L-methionine (SAM).

counted. After centrifugation, pellets ($5x10^6$ cells) were resuspended in 300µl prechilled PBS containing 0.5% BSA and 2mM EDTA (sorting buffer). Subsequently, cell suspension was added to 200µl mixture consisting of equal volumes of FcR Blocking Reagent (Miltenyi Biotec) and MicroBeads conjugated to mouse anti-human CD133 antibody. The whole mixture was then incubated at 4°C for 30 minutes. After washing with 500µl sorting buffer, cells were centrifuged at 300xg 10 minutes at 4°C. Pellet was then resuspended in 500µl sorting buffer. Cell suspension was loaded into a MACS column within a MACS separator (Miltenyi Biotech), under the influence of a magnetic field. The labeled CD133+ cells were retained on the column while the unlabeled conterpart was eluted. Upon column removal from the separator, the magnetically retained CD133+ cells (positively selected) were collected and subjected to subsequent analysis [27].

Cells isolated using MACS technology were plated at a density of $5x10^5$ cells using ultra-low attachment T25 flask in serum-free DMEM/F12 medium supplemented with growth factors and N2 supplement for 3 weeks. Real-Time PCR was performed to analyse CD133, CD44 and NNMT expression.

Statistical analyses

Data were analyzed using GraphPad Prism software, version 6.00 for Windows (GraphPad Software Inc., San Diego, California, USA). Differences between groups were determined using the Kruskal–Wallis and Mann–Whitney U-tests. A p value < 0.05 was considered statistically significant.

RESULTS

Sphere formation from different cancer cell lines

A subpopulation of CSCs from different tumor cell lines (MG63, T24, A549 and CaCo-2) was enriched through spheres formation. Cells were cultured in DMEM/F12 serum free medium with bFGF, EGF and N2, using Ultra-low attachment T25 flasks. After being in culture for 4 weeks, cancer cells gradually started to aggregate and form sphere-like bodies, compared with the parental cells cultured in DMEM/F12 media supplemented with 10% fetal bovine serum and 50ug/ml of gentamicine. A significant volume increase of sphere-like body was observed with longer cultivation periods (6 weeks) (Fig. 1).

Expression of stem cell markers in sphere-forming cells and parental counterparts

Real-Time PCR was used to evaluate mRNA levels of stem cell-related genes in CSC-enriched populations and parental cells. Results obtained demonstrated a significant ($p<0.05$) upregulation of CD133, CD44, Oct4, Sox2, Nanog, Nestin, ABCG2 and BMI-1 in sphere-forming cell subpopulations with respect to controls (Fig. 2a-d). According to these data, CD133 immunocytochemical expression seemed to be markedly higher in CSC-enriched populations compared with that detected in parental cells (Fig. 2e).

CSC enrichment of MG63 cells through MACS technology.

To generate MG63 CSCs-enriched population, cell line was cultured using serum-free medium supplemented with specific growth factors and N2 supplement. Further CSC enrichment was obtained by magnetic-activated cell sorting and putative cancer stem cell markers. In particular CSCs from MG63 cell line enriched through sphere formation were purified by MACS and microbeads immuno-labeled with CD133 antibody. The CD133+ cells were obtained by positive selection and directly cultured in serum free medium with growth factors and N2

supplement for 3 weeks. The presence of stem cell markers was evaluated by Real-Time PCR analysis.

Real-Time PCR assay confirmed a significant increased expression of CD133 (Fig. 3a) and CD44 (Fig. 3b) in CSC-enriched population obtained after MACS selection compared with sphere-forming cells before MACS purification, and parental cells as well. These results showed that CD133+ cells purification obtained by MACS led to further enrichment in CSCs.

NNMT expression in different tumorigenic cell lines and their CSC-enriched conterparts

Real-Time PCR analysis showed that NNMT mRNA levels were significantly ($p<0.05$) higher in sphere-forming cells compared to those detected controls, for all examined tumor cell lines (Fig. 4a).

Subsequently, enzyme expression was evaluated at protein levels by Western blot analysis and immunohistochemistry. Results obtained form Western blot showed a single immunoreactive band displaying an increased intensity in CSC-enriched populations compared to that of parental cells (Fig. 4b). Immunocytochemical analysis confirmed the increased protein expression of NNMT in CSC-enriched populations compared with parental counterparts (Fig. 4c).

According to data obtained from previous analyses, NNMT activity levels were markedly higher in CSC-enriched populations compared to those of parental cells, in which they were below the detection limit of the assay (Fig. 4d).

Regarding MG63 cell line, a significant increased enzyme expression (mRNA level) was observed in CSC-enriched population obtained after MACS with respect to CSC-enriched subpopulation before MACS purification as well as to parental cells (Fig. 4e).

DISCUSSION

The identification of tumor cells with stem-like properties (CSCs), displaying strong proliferative and differentiating capacity within cancers, revolutionized completely both molecular oncology and cancer therapy, since they are thought to play a fundamental role in cancer formation, metastasis and replapse. Experimental studies demonstrated that, among phenotypic traits highlighting CSCs, there are *in vitro* sphere-forming ability and *in vivo* tumorigenicity [28]. CSCs are also characterized by the overexpression of drug efflux pumps, like ATP-binding cassette (ABC) transporters, and specific cell-surface markers, these latter used for CSCs identification and isolation. The main surface antigens used to define CSCs from primary tumors and cancer cell lines are CD133, CD44, CD24, CD166 and the ABC transporter ABCG2 [29]. The identification of CSCs is mainly based on combined expression of several molecules including cytoplasmic proteins as well as surface markers, many of which are also expressed in adult or embryonic stem cells (ESCs). Indeed, both ESCs and CSCs share the expression of many transcription factors (Sox2, Oct4 and Nanog), surface antigens (CD133, CD44, CD177 or c-KIT, Nestin and ABCG2) and molecules involved in different signaling pathways (β-catenin, Bmi-1 and TGF-βs) [30, 31].[7] Among cell surface molecules, CD133 and CD44 have been widely used to identify and select CSCs. CD133, also known as prominin 1, is a pentaspan transmembrane glycoproteinis known to be preferentially localized in plasma membrane protrusions and microvilli, suggesting its involvement in membrane organization [32]. CD44 is a transmembrane protein that mediates cell to cell adhesion and cell to extracellular matrix interactions, being a receptor for hyaluronic acid, selectin, collagen, osteopontin, fibronectin and laminin [33].

The great contribution given by CSCs to cancerogenesis is mainly attributable to their ability to take part to several cellular processes regulating and/or promoting tumorigenesis. These

[7] **Abbreviations**: ATP-binding cassette (ABC); embryonic stem cells (ESCs).

events include oxidative stress resistance, rapid DNA damage repair, adaptation to starving conditions and enhanced inflammation, and a broad efficiency in removing chemotherapeutic drugs through efflux pumps. Taken together, all these aspects may have a significative impact in the progression of tumor microlesions that survived therapies and could be responsible for local relapse as well as metastasis [34]. Therefore, the complete eradication of tumors relies on the elimination of CSCs. To accomplish this task, the development of novel therapeutical approches targeting CSC-associated molecules will be required. In order to identify mechanisms underlying key phases of tumorigenesis, triggered by these tumor initating cells, a complete molecular characterization of CSCs, or CSC-enriched cell population, is needed.

In the present study, a culture system was setup to enrich CSCs from blader cancer (T24), lung cancer (A549), colorectal cancer (CaCo-2) and osteosarcoma (MG63) cell lines, through sphere formation. Moreover, MACS was used to further increase CSC enrichment. Subsequent molecular characterization of CSC-enriched cell populations and parental cells was performed, by exploring the expression of stem markers. Results obtained showed an upregulation of stem cell markers in sphere-forming cells compared with respective parentals cells, thus indicating CSC enrichment.

In order to deeply define the molecular profile of CSC-enriched cell populations obtained from the above reported tumor cell lines, NNMT expression was further investigated in cancer cells before and after CSC enrichment. In fact, NNMT is known to be upregulated in a wide variety of neoplasms, promoting proliferative capacity and tumorigenicity of cancer cell [19-22]. Results obtained in this work showed that CSC enrichment was significantly associated with NNMT upregulation, strongly suggesting that the induction of enzyme expression is a peculiar hallmak of CSCs.

There is a lack of knowledge concerning the relation between NNMT and CSCs. In particular, few studies explored the expression levels of NNMT in CSCs or CSC-enriched populations, as well as the potential role played by the enzyme in CSC metabolism.

In 2011, D'Andrea et al. evaluated the effect of radiation treatment on survival of two different mesenchymal CSC clones (BB3 and CE8), and subsequently analyzed their respective gene expression profile. Interestingly, results obtained showed that CE8 clone displayed a significantly higher resistance to rdiation compared with that exhibited by BB3 clone. In addition, among differentially expressed genes (CE8 versus BE3), NNMT was found to be significantly upregulated [35]. As before mentioned, in our previous study, CSCs from Hep-2 laryngeal carcinoma cell line were enriched through the induction of sphere formation. Subsequently, sphere-forming cells were subjected to molecular and phenotypic characterization. NNMT expression levels were also investigated in CSC-enriched cell populations and parental counterparts. Results obtained showed that, campared with parental cells, CSC-enriched populations from Hep-2 cell line displayed an increased expression of stem cell markers and a higher *in vivo* tumorigenic potential, due to their strong capability to form tumors upon injection into athimic mice. Interestingly, NNMT levels appeared to be significantly higher in CSC-enriched populations compared with parental cells [23].

NNMT catalyses the N-methylation of nicotinamide, pyridine and structurally related compounds by using S-adenosyl-L-methionine, as methyl donor [8]. N1-methylnicotinamide so formed is mostly excreted into urine and partly oxidized to pyridones, which are also excreted into urine [36]. Therefore, nicotinamide catbolism mainly relis upon N-methylation pathway. In this light, catalysis exerted by NNMT actively takes part to homeostatic regulation of nicotinamide, and main cellular processes associated with nicotinamide involvement could be significantly affected by enzyme overexpression. Catalytic activitiy of both histone deacetylases (sirtuins) and poly(ADP-ribose) polymerases (PARPs) has been reported to be strongly inhibited by nicotinamide [37]. PARPs are described to be involved in maintaining genome integrity in response to DNA damage-associated events, such as DNA single-strand breaks induced by genotoxic agents (i.e. radiation treatment). In these light, NNMT overexpression observed upon CSC enrichment, lowering the intracellular levels of

nicotinamide, may contribute, at least in part, to a significant removal of inhibition exerted on PARPs, allowing these enzymes to promote DNA repair and genomic stability, and to counteract programmed cell death, thus confering an adaptive advantage to CSCs.[8]

During catalysis exerted by NNMT, a methyl group is transferred from SAM to nicotinamide, generating N1-methylnicotinamide and S-adenosyl-L-homocysteine (SAH).

Enhanced enzyme overexpression featuring cancer cell is able to decrease, at least in part, the cellular methylation potential (MP), defined as the ratio of SAM:SAH. This NNMT-related condition, associated with hypomethylated state of histones, was reported to be responsible for the development of aggressive phenotypes in melanoma, ovarian, kidney and lung cancer cell lines [38]. Recently, MP impairment attributable to NNMT upregulation was also decribed to alter the epigenetic status of CSCs. Interestingly, NNMT was found to be one of the few metabolic enzyme markedly overexpressed in glioblastoma stem cells (GSCs), contributing to deplete available methyl donors. In particular, NNMT-induced SAM deprivation led to a downregulation of DNA methyltransferases DNMT1 and DNMT3A. Moreover, the reduction of methionine resulted in a significant increase of cell growth *in vitro*. In the light of these evidences, it is conceivable to hypothesize a potential functional link between SAM availability and stem-like properties, such that a significant reducation of this universal methyl donor could lead to a increase of tumoricenic potential. Taken together these results strongly suggest that NNMT may play an essential role in maintenance of GSCs, whose presence is associated with poor prognosis for glioblastoma patients [39].

To the best of our knowledge, this is the first study demonstrating that NNMT upregulation is significantly associated with CSC enrichment obtained from different epithelial and mesenchymal tumor cell lines. Considering the fundamental role played by CSCs in carcinogenesis, these findings could represent an important achievement for the development

[8] **Abbreviations**: poly(ADP-ribose) polymerases (PARPs).

of new and effective therapeutic strategies, based on CSC-associated target, able to arrest cancer proliferation, relapse and metastasis.[9]

[9] **Abbreviations**: S-adenosyl-L-homocysteine (SAH); glioblastoma stem cells (GSCs).

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CONFLICT OF INTEREST

None of the authors have a conflict of interest to declare.

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FIGURE LEGENDS

Fig. 1 Sphere formation in tumor cell lines. Phase contrast microscopy observation of parental cells (100x original magnification) and CSC-enriched populations (200x original magnification) from MG63, T24, A549 and CaCo-2 cell lines. CSCs, but not contols cultivated under standard conditions, were able to form spheres in suspension.

Fig. 2 Stem cell marker expression in CSC-enriched populations and parental cells. Real-time PCR (a-d) and immunocytochemical analysis (e) were performed to evaluate mRNA and protein expression levels of putative stem cell markers in CSC-enriched population compared with parental cells obtained from MG63, T24, A549 and CaCo-2 cell lines (*$p < 0.05$) ("+" = moderate staining intensity, "++" = good staining intensity; "+++" = strong staining intensity).

Fig. 3 Expression of CD133 and CD44 in MG63 cells subjected to MACS. Real-Time PCR assay was carried out to analyze mRNA levels of CD133 (a) and CD44 (b) in CSC-enriched population obtained after MACS sorting compared with CSC-enriched subpopulation before MACS purification and parental cells (*$p < 0.05$).

Fig. 4 NNMT expression upon CSC enrichment. Real-time PCR analyses (a), western blot (b), immunoistochemistry (c) and catalytic activity assay (d) were performed to evaluate NNMT expression in parental cells and CSC-enriched populations obtained from MG63, T24, A549 and CaCo-2 cell lines. NNMT mRNA levels were also determined upon MACS purification (e) (*$p < 0.05$) ("+" = moderate staining intensity, "++" = good staining intensity; "+++" = strong staining intensity) (n.d. = not detectable or < 0.01 U/mg).

Table 1. Primers used for quantitative Real-Time PCR.

Target gene	Sequence
CD133	forward 5'-TGCTGCTTGTGGAATAGACAGAATG-3' reverse 5'-AGGAAGGACTCGTTGCTGGTGAA-3'
CD44	forward 5'-CGGACACCATGGACAAGTTT-3' reverse 5'-GAAAGCCTTGCAGAGGTCAG-3'
NESTIN	forward 5'-CAGCTGGCGCACCTCAAGATG-3' reverse 5'-AGGGAAGTTGGGCTCAGGACTGG-3'
OCT4	forward 5'-CTTGCTGCAGAAGTGGGTGGAGGAA-3' reverse 5'-CTGCAGTGTGGGTTTCGGGCA-3'
NANOG	forward 5'-GATTTGTGGGCCTGAAGAAA-3' reverse 5'-AAGTGGGTTGTTTGCCTTTG-3'
SOX2	forward 5'-ACTTTTGTCGGAGACGGAGA-3' reverse 5'-CATGAGCGTCTTGGTTTTCC-3'
ABCG2	forward 5'-CACCTTATTGGCCTCAGGAA-3' reverse 5'-TGCCACAGCAGTGGAATCT -3'
BMI-1	forward 5'-ATGATAAAAGATACTTACGATGCCCAG-3' reverse 5'-GAACTCTGTATTTCAATGGAAGTGGAC-3'
NNMT	forward 5'-GAATCAGGCTTCACCTCCAA-3' reverse 5'-TCACACCGTCTAGGCAGAAT-3'
β-ACTIN	forward 5'-TCCTTCCCTGGGCATGGAGT-3' reverse 5'-AGCACTGTGTTGGCGTACAG-3'

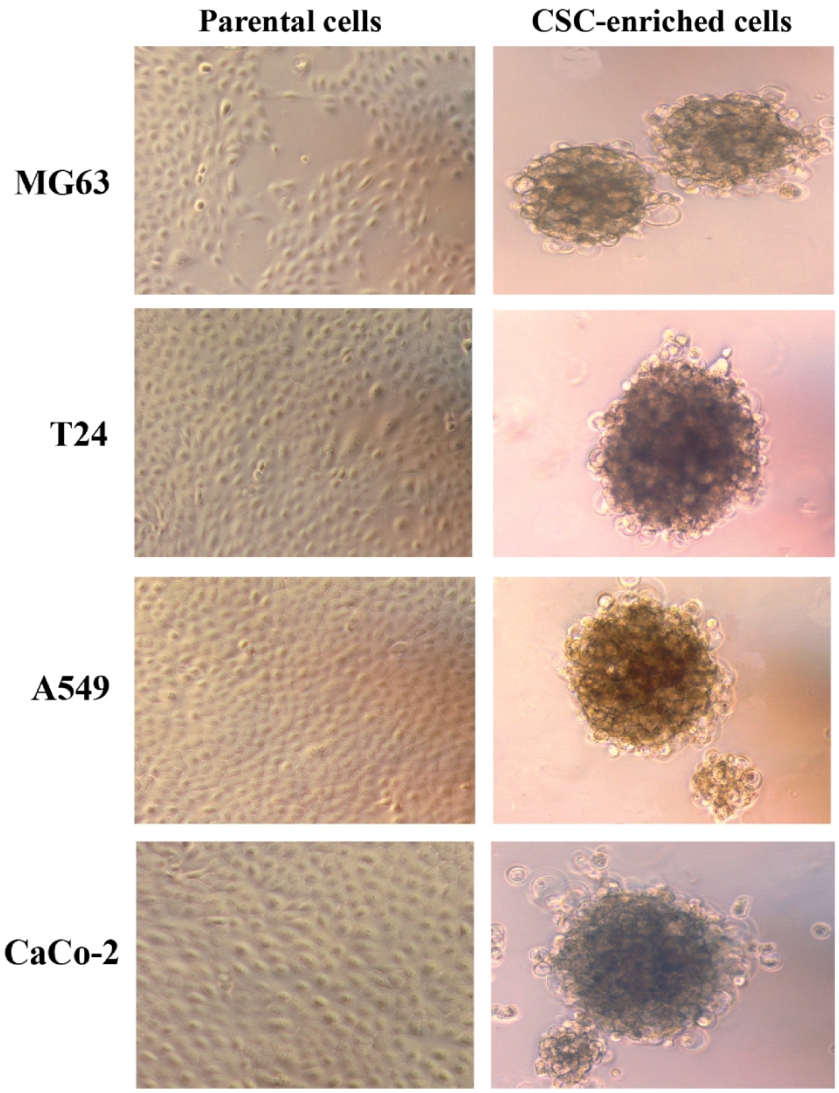


Figure 1

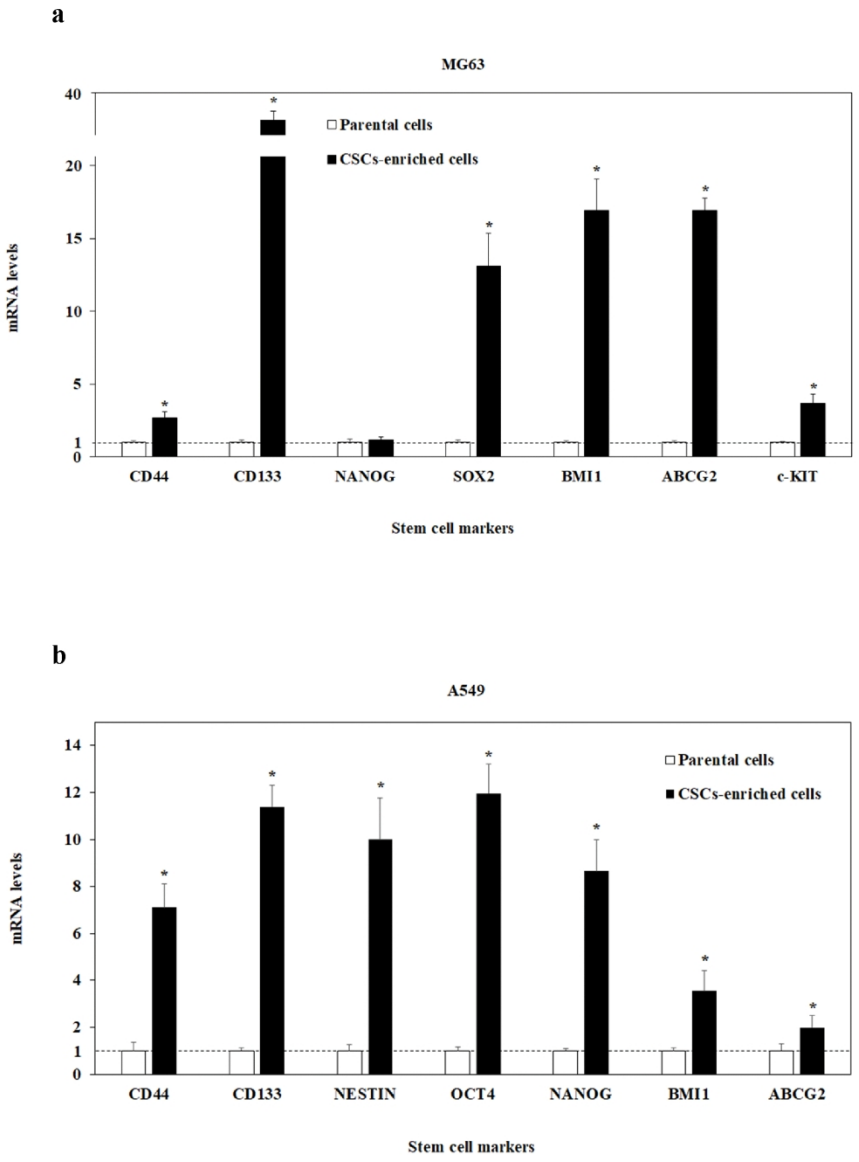
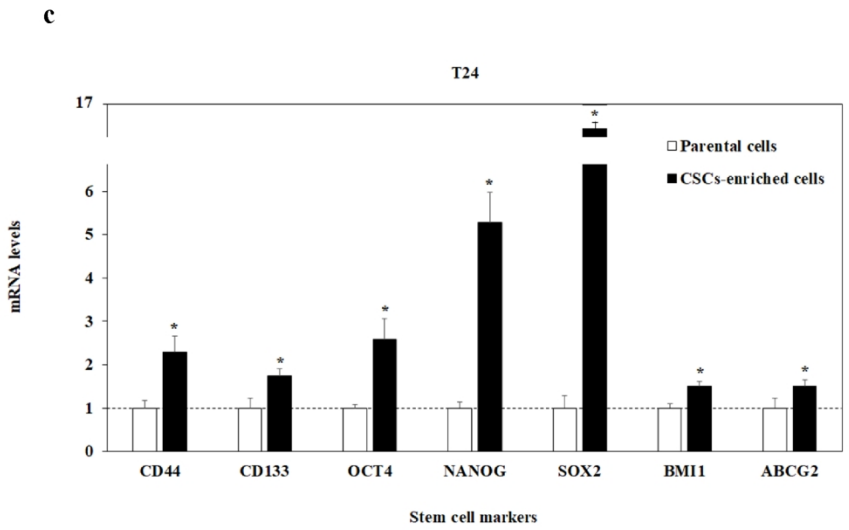

a
MG63
Parental cells
CSCs-enriched cells
mRNA levels
40
20
15
10
5
1
0
CD44
CD133
NANOG
SOX2
BMI1
ABCG2
c-KIT
Stem cell markers
b
A549
Parental cells
CSCs-enriched cells
mRNA levels
14
12
10
8
6
4
2
1
0
CD44
CD133
NESTIN
OCT4
NANOG
BMI1
ABCG2
Stem cell markers

Figure 2ab


c
T24
17
6
5
4
3
2
1
0
mRNA levels
Parental cells
CSCs-enriched cells
CD44
CD133
OCT4
NANOG
SOX2
BMI1
ABCG2
Stem cell markers

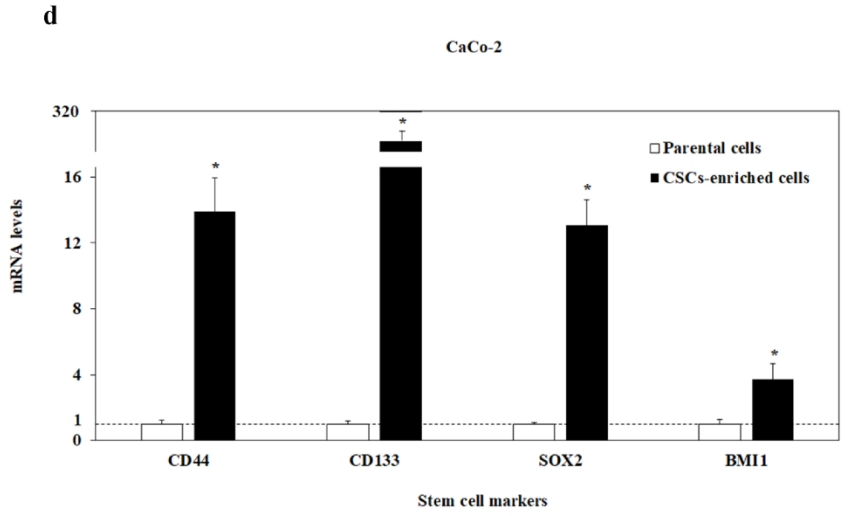
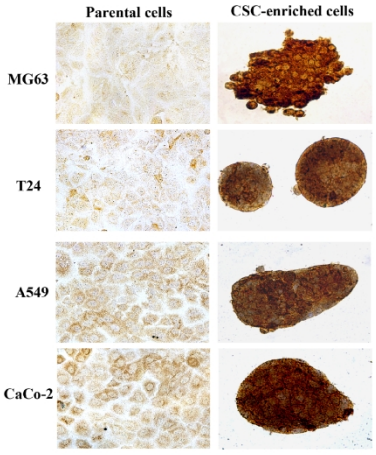

d
CaCo-2
320
16
12
8
4
1
0
mRNA levels
Parental cells
CSCs-enriched cells
CD44
CD133
SOX2
BMI1
Stem cell markers

Figure 2cd

e



Cell Lines	CD133 expression	
	Parental cells	CSCs-enriched populations
MG63	+	+++
A549	++	+++
T24	+	+++
CaCo-2	++	+++

Original magnification was 200x for parental cells and 400x for CSC-enriched cells.

Figure 2e

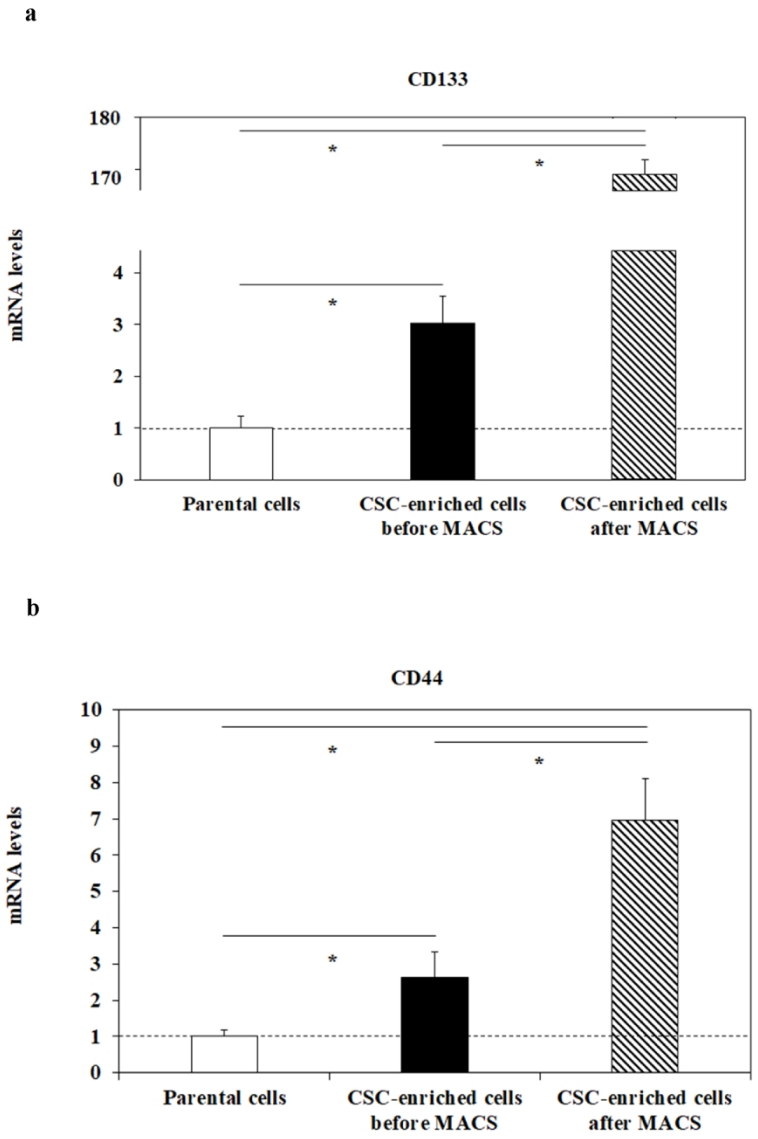
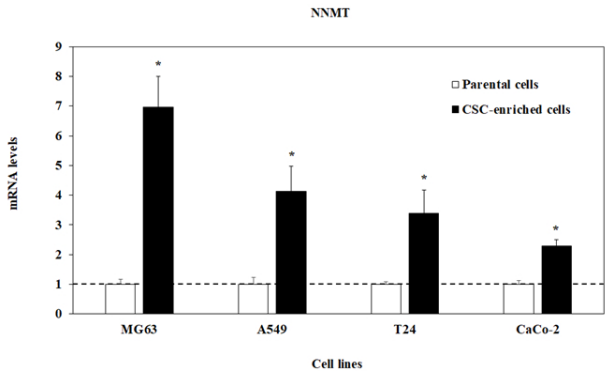

a
CD133
mRNA levels
180
170
4
3
2
1
0
*
*
*
Parental cells
CSC-enriched cells before MACS
CSC-enriched cells after MACS
b
CD44
mRNA levels
10
9
8
7
6
5
4
3
2
1
0
*
*
*
Parental cells
CSC-enriched cells before MACS
CSC-enriched cells after MACS

Figure 3

a



b

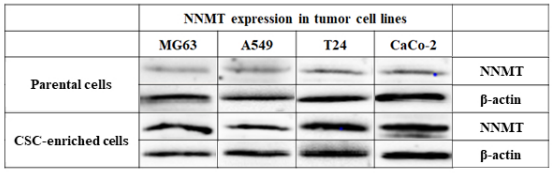
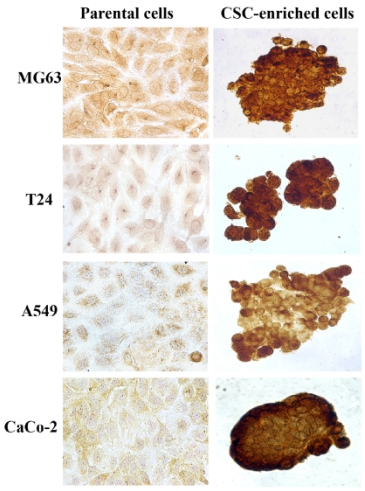


Figure 4ab revised

190x338mm (96 x 96 DPI)

c



Cell Lines	NNMT expression	
	Parental cells	CSCs-enriched populations
MG63	++	+++
A549	+	+++
T24	+	+++
CaCo-2	+	++

Original magnification was 200x for parental cells and 400x for CSC-enriched cells.

Figure 4c

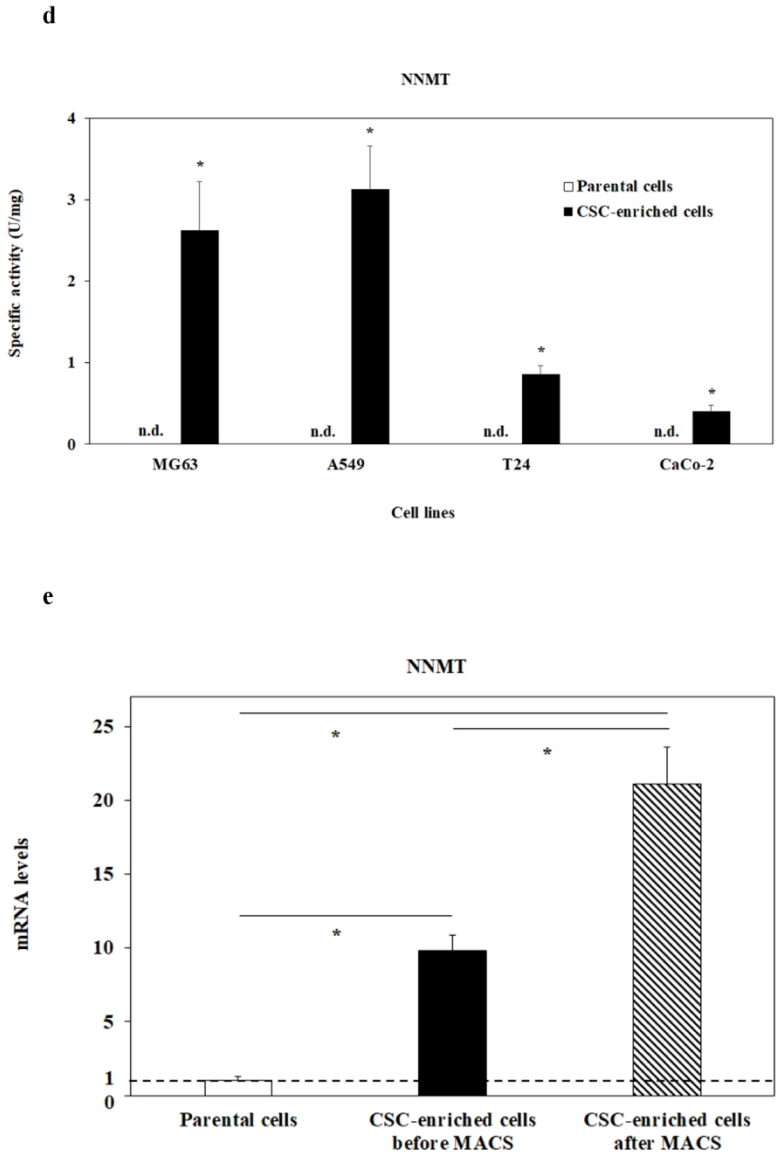

d
NNMT
Specific activity (U/mg)
Parental cells
CSC-enriched cells
n.d.
MG63
A549
T24
CaCo-2
Cell lines
e
NNMT
mRNA levels
Parental cells
CSC-enriched cells before MACS
CSC-enriched cells after MACS

Figure 4de