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ORIGINAL ARTICLE

Cancer stem cell markers in pediatric sarcomas: Sox2 is associated with tumorigenicity in immunodeficient mice

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Abstract The three most frequent pediatric sarcomas, i.e., Ewing's sarcoma, osteosarcoma, and rhabdomyosarcoma, were examined in this study: three cell lines derived from three primary tumor samples were analyzed from each of these tumor types. Detailed comparative analysis of the expression of three putative cancer stem cell markers related to sarcomas—ABCG2, CD133, and nestin—was performed on both primary tumor tissues and corresponding cell lines. The obtained results showed that the frequency of ABCG2-positive and CD133-positive cells was predominantly increased in the respective cell lines but that the high levels of nestin expression were reduced in both osteosarcomas and rhabdomyosarcomas under in vitro conditions. These findings suggest the selection advantage of cells expressing ABCG2 or CD133, but the functional tests in NOD/SCID gamma mice did not confirm the tumorigenic potential of cells harboring this phenotype. Subsequent analysis of the expression of common stem cell markers revealed an evident relationship between the expression of the transcription factor Sox2 and the tumorigenicity of the cell lines in immunodeficient mice: the Sox2 levels were highest in the two cell lines that were demonstrated as tumorigenic. Furthermore, Sox2-positive cells were found in the respective primary tumors and all xenograft tumors showed apparent accumulation of these cells. All of these findings support our conclusion that regardless of the expression of ABCG2, CD133 and nestin, only cells displaying increased Sox2 expression are directly involved in tumor initiation and growth; therefore, these cells fit the definition of the cancer stem cell phenotype.

Keywords Cancer stem cells · Pediatric sarcomas · Markers · Tumorigenicity · Sox2

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Introduction

Malignancies are the second most frequent cause of death (after injuries) in children under the age of 15 worldwide. For this reason, one of the main goals in pediatric oncology is to understand the biological features of cancers that typically appear during childhood because prompt and precise diagnosis together with specific and effective treatment may lead to a complete cure or to a marked prolongation of life expectancy among these patients.

In this context, cancer stem cells (CSCs) represent a very important research topic in pediatric oncology. In heterogeneous tumor tissue, only CSCs are able to initiate tumor growth after grafting into immunodeficient mice. Therefore, CSCs are undoubtedly key drivers of tumor initiation, progression, metastasizing, and treatment failure [1–3]. Thus, a detailed understanding of the characteristics of particular tumor types and biological features of CSCs may be of great importance for the development of new effective antineoplastic therapies designed specifically for children [4, 5].

Pediatric sarcomas represent a very heterogeneous group of tumors with varying molecular, pathological, and clinical features: osteosarcoma, Ewing's sarcoma, and rhabdomyosarcoma are the most frequent of them. In addition to common stem cell markers, such as Oct3/4, Sox2, and Nanog, special attention is paid to the identification of additional markers that enable the positive detection of CSCs in these tumors. A combination of the cell surface antigens prominin-1 (CD133) and ABCG2 (CD338) together with the intermediate filament protein nestin is the marker expression profile most frequently discussed as a CSC phenotype specific to sarcomas [6–9].

Despite the publication of several studies aimed at the identification and characterization of CSCs using established cell lines and standardized functional assays, little is known about the "previous step" of cancer stem cell biology: which cell subpopulations expressing putative CSC markers predominate after successful derivation of a cell line from a tumor sample. For this reason, our study focused on a detailed comparative analysis of the expression of the most frequently discussed putative CSC markers in pediatric sarcomas, i.e., ABCG2, CD133, and nestin, in both primary tumor tissues and their respective derived cell lines. Three most common pediatric sarcomas, i.e., osteosarcoma, rhabdomyosarcoma, and Ewing's sarcoma, were included in this study: three cell lines derived from three primary tumor samples were analyzed for each of these tumor types. This experimental design provided an important opportunity to compare the pattern of the expression of the markers mentioned above in nine tumor samples paired with nine cell lines. Additionally, in both the cell lines and the tumor samples, special attention was paid to the intracellular localization of CD133 because this characteristic may be relevant to the biological features of tumor cells [10, 11]. Furthermore, all cell lines were tested for tumorigenicity in NOD/SCID mice, and the resulting xenograft tumors were analyzed.

Materials and methods

Tumor samples and primary cell lines

Nine tumor samples collected from patients suffering from pediatric sarcoma and nine corresponding cell lines derived from these tumors were included in this study: a brief description of the cohort is provided in Table 1. The OSA-05 and NSTS-11 cell lines were originally described in our previous studies [12, 13]; all other cell lines were derived using the same procedure to generate primary cultures [14]. The cell lines were maintained under standard conditions as described previously [13]. The Research Ethics Committee of the School of Science (Masaryk University) approved the study protocol, and a written statement of informed consent was obtained from each participant or his/her legal guardian prior to participation in this study.

Immunohistochemistry

Immunohistochemical (IHC) detection was performed on formalin-fixed paraffin-embedded (FFPE) samples of primary or xenograft tumors. The 4 μm thick tissue sections were applied to positively charged slides, deparaffinized in xylene, and rehydrated using a graded alcohol series. For nestin and CD133, antigen retrieval was performed in a calibrated Pascal pressure chamber (Dako, Glostrup, Denmark) by heating the sections in Tris/EDTA buffer (DAKO) at pH 9.0 for 40 min at 97 °C. For ABCG2, the sections were not subjected to any pretreatment. Endogenous peroxidase activity was quenched by incubating the sections in 3 % hydrogen peroxide in methanol for 20 min, followed by incubation at room temperature (RT) with a primary antibody (Table 2). For nestin and ABCG2, the Vectastain Elite ABC kit and the streptavidin-biotin horseradish peroxidase (HRP) detection method were

Table 1 Description of patient cohort, corresponding cell lines, and xenograft tumors

Tumor sample	Gender	Age	Diagnosis	Time of biopsy	Primary cell line	Xenograft tumors		
1	M	17	EWS	DG	ESFT-03	–		
2	M	2	EWS	DG	ESFT-04	–		
3	M	25	EWS/PNET	DG	ESFT-09	–		
4	M	9	OS teleangiectatic	DG	OSA-05	–		
5	F	16	C-OS osteoblastic	DG	OSA-06	–		
6	F	6	C-OS	DG	OSA-13	LTB17	LTB18	LTB19
7	F	16	RMS embryonal	NACHT	NSTS-11	LTB1	LTB2	LTB3
8	F	6	RMS alveolar	DG	NSTS-22	–		
9	M	8	RMS alveolar	PROG	NSTS-28	–		

Gender: *M* male, *F* female. Age at the time of diagnosis: years. Diagnosis: *EWS* Ewing's sarcoma, *PNET* primitive neuroectodermal tumor, *OS* osteosarcoma, *C-OS* conventional osteosarcoma, *RMS* rhabdomyosarcoma. Time of biopsy: *DG* diagnostic, *NACHT* after neo-adjuvant chemotherapy, *PROG* progression of the disease

Table 2 Antibodies and primers used in this study

Primary antibodies						
Antigen	Type/host	Clone (catalog no.)	Manufacturer	Dilution		
				IHC	IF	WB
ABCG2	Polyclonal/Rb	– (bs-0662R)	Bioss	1:400	–	–
ABCG2	Monoclonal/Mo	5D3	BD Pharmingen	–	1:50	–
ALDH1	Monoclonal/Mo	44/ALDH	BD Biosciences	–	–	1:1000
CD133	Monoclonal/Mo	17A6.1	Millipore	1:100	1:100	–
Nanog	Polyclonal/Rb	– (ab21624)	Abcam	–	–	1:100
Nestin	Monoclonal/Mo	10C2	Millipore	1:200	1:100	–
Oct4	Polyclonal/Rb	– (ab19857)	Abcam	–	–	1:500
Sox2	Monoclonal/Rb	EPR3131	Abcam	1:100	1:10	–
Sox2	Monoclonal/Rb	D6D9	Cell Signaling	–	–	1:500
Sox2	Polyclonal/Rb	– (ab137385)	Abcam	–	–	1:1000
α-tubulin	Monoclonal/Mo	TU-01	Exbio	–	1:100	1:500
β-actin	Monoclonal/Mo	AC-15	Sigma	–	–	1:10,000
Secondary antibodies						
Host	Specificity	Conjugate	Manufacturer	Dilution		
				IHC	IF	WB
Goat	anti-Rb IgG	Alexa Fluor 488	Life Technologies	–	1:200	–
Goat	anti-Mo IgG	Alexa Fluor 488	Life Technologies	–	1:200	–
Goat	anti-Rb IgG	HRP	Cell Signaling	–	–	1:5000
Horse	anti-Mo IgG	HRP	Cell Signaling	–	–	1:5000
Primers						
Gene	Gene symbol	Primer sequence				
ABCG2	*ABCG2*	F: 5′-TCACTACTTCCTTCCTTACCCCT-3′ R: 5′-ACAGAAACACAACACTTGGCTG-3′				
CD133	*PROM1*	F: 5′-CCATTGACTTCTTGGTGCTGT-3′ R: 5′-TGGAGTTACGCAGGTTTCTCT-3′				
Nestin	*NES*	F: 5′-AGTGATGCCCCTTCACCTTG-3′ R: 5′-GCTCGCTCTCTACTTTCCCC-3′				
Oct4	*POU5F1*	F: 5′-GCAAAGCAGAAACCCTCGT-3′ R: 5′-ACACTCGGACCACATCCTTC-3′				
Nanog	*NANOG*	F: 5′-AATACCTCAGCCTCCAGCAGAT-3′ R: 5′-TGCGTCACACCATTGCTATTCTTC-3′				
Sox2	*SOX2*	F: 5′-TCCCATCACCCACAGCAAATGA-3′ R: 5′-TTTCTTGTCGGCATCGCGGTTT -3′				
Aldehyde dehydrogenase	*ALDH1A1*	F: 5′- GTCAAAGGCTTCCTGCCCTA-3′ R: 5′- GGTTCTGATAGAGCACTTGGCT-3′				
Heat shock protein HSP 90-beta	*HSP90AB1*	F: 5′-CGCATGAAGGAGACACAGAA-3′ R: 5′-TCCCATCAAATTCCTTGAGC-3′				

Rb rabbit, *Mo* mouse, *HRP* horseradish peroxidase, *F* forward primer, *R* reverse primer

used (Vector Laboratories, Burlingame, CA, USA). For CD133, EnVision+ Dual Link system-HRP without avidin or biotin was applied for detection (Dako). For Sox2, the EXPOSE Rabbit-specific HRP/DAB detection kit (Abcam) was used. 3,3′-diaminobenzidine (DAB) was used as a chromogen. Positive controls were obtained by staining sections of glioblastoma multiforme or breast carcinoma; nestin- or CD133-positive endothelial cells in tumor tissue samples were used as internal positive controls. For Sox2, sections of fetal lung tissue were used as a positive control. Negative controls were prepared by incubating samples in the absence of a primary antibody. Evaluation of all IHC staining results was performed using an Olympus BX51 microscope and an Olympus DR72 camera with uniform settings. All immunostained slides were evaluated at ×400 magnification independently by two observers (IZ and MH). The percentage of positive tumor cells (TC) and the

average intensity of immunostaining (i.e., immunoreactivity, IR) were assessed in at least five discrete foci of neoplastic infiltration.

Immunofluorescence

Indirect immunofluorescence (IF) was performed as previously described [13]. The primary and secondary antibodies used in the experiments are listed in Table 2; mouse monoclonal anti-α-tubulin served as a positive control. An Olympus BX-51 microscope was used for sample evaluation; micrographs were captured using an Olympus DP72 CCD camera and analyzed using the Cell^P imaging system (Olympus). At least 200 cells were evaluated in total within discrete areas of each sample, and the samples were prepared from at least three independent passages of all examined cell lines. The mean percentage of cells showing positivity for the examined antigen and the IR for the antigen were determined. Finally, for each cell line, the total immunoscores were calculated for individual antigens as described previously [15]. The immunoscore values were classified as low (≥100), middle (101–200), or high (201–300).

Western blotting and immunodetection

We also used a previously described procedure [13] to analyze expression of Sox2, Oct4, Nanog, and aldehyde dehydrogenase 1 (ALDH1) in sarcoma cell lines. The primary and secondary antibodies used are listed in Table 2; mouse monoclonal anti-α-tubulin or mouse monoclonal anti-β-actin served as a loading control.

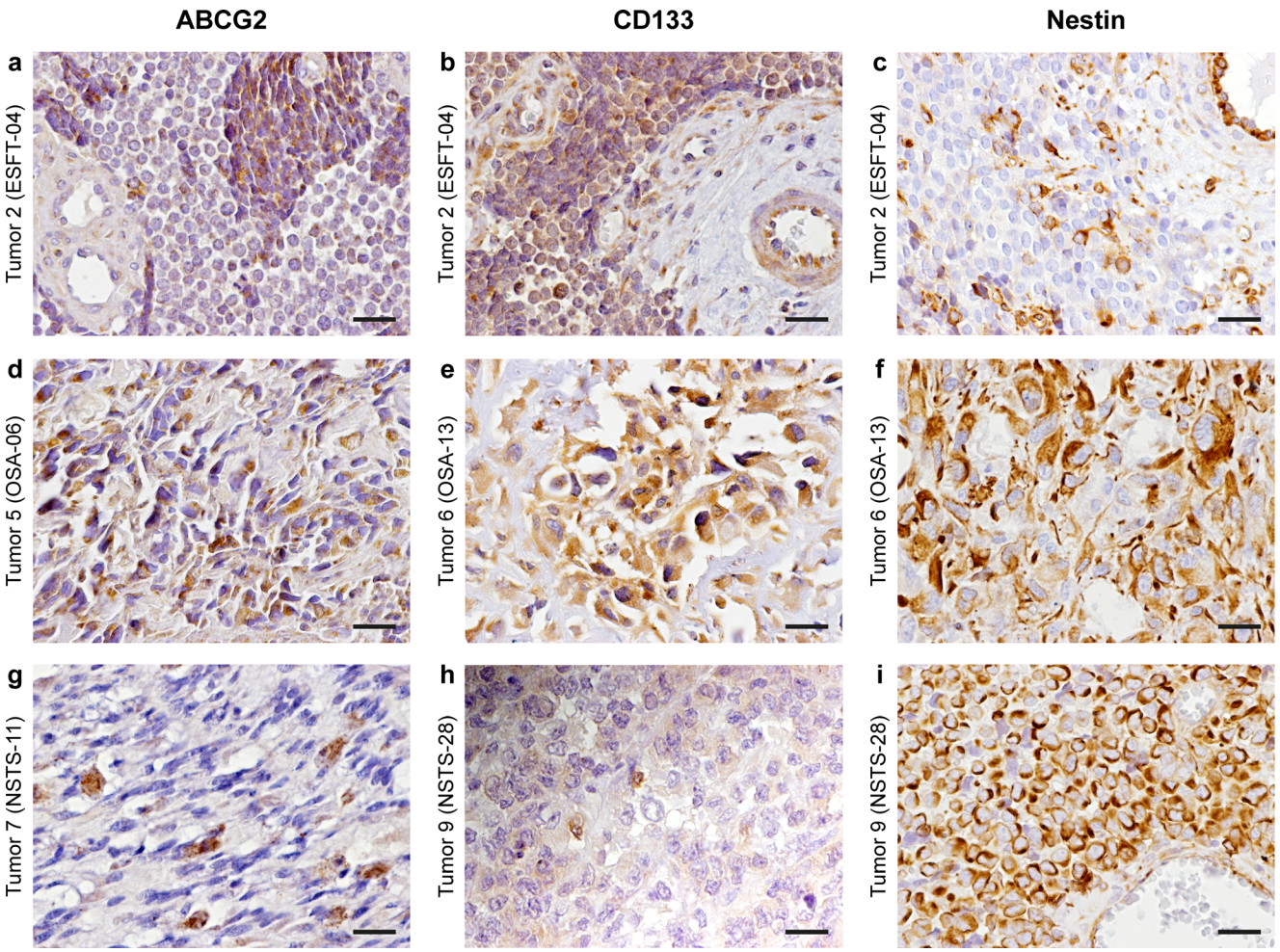

Fig. 1 Expression of ABCG2, CD133 and nestin in tumor tissues as detected by IHC. Ewing's sarcoma (**a**–**c**), osteosarcoma (**d**–**f**), and rhabdomyosarcoma (**g**–**i**) tissue samples were analyzed. Tumor sample and corresponding cell line (in *brackets*) are indicated. *Scale bars*, 50 μm

Real-time quantitative reverse transcription PCR (qRT-PCR)

For qRT-PCR of sarcoma cell lines, total RNA was extracted and reverse transcribed as previously described [13]. Quantitative PCR was performed in a volume of 10 μl using the KAPA SYBR® FAST qPCR Kit (Kapa Biosystems, Wilmington, MA, USA) and 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The data were analyzed by 7500 Software v. 2.0.6 (Applied Biosystems) and relative quantification (RQ) of gene expression were calculated using $2^{-\Delta\Delta CT}$ method [16]; heat shock protein gene (*HSP90AB1*) was used as the endogenous reference control and ESFT-03 cell line served as the arbitrary calibrator. The primer sequences used are listed in Table 2.

In vivo tumorigenicity assay

Enzymatically dissociated cell suspensions of all nine primary cell lines were each injected subcutaneously into three 8-week-old female NOD/SCID gamma mice at a concentration of 1×10^6 cells (for Ewing's sarcoma and osteosarcoma cell lines) or 3×10^5 cells (for rhabdomyosarcoma cell lines) per 100 μl. The mice were examined every 3 days for the presence of subcutaneous tumors. After the appearance of a tumor, the mice were sacrificed, and the tumor tissue was dissected. This study was approved by the Institutional Animal Care and Use Committee of Masaryk University and was registered by the Ministry of Agriculture of the Czech Republic as required by national legislation. Each tumor was divided into two equal portions: one portion was processed for primary culture [14], and the second portion was fixed in 10 % buffered formalin

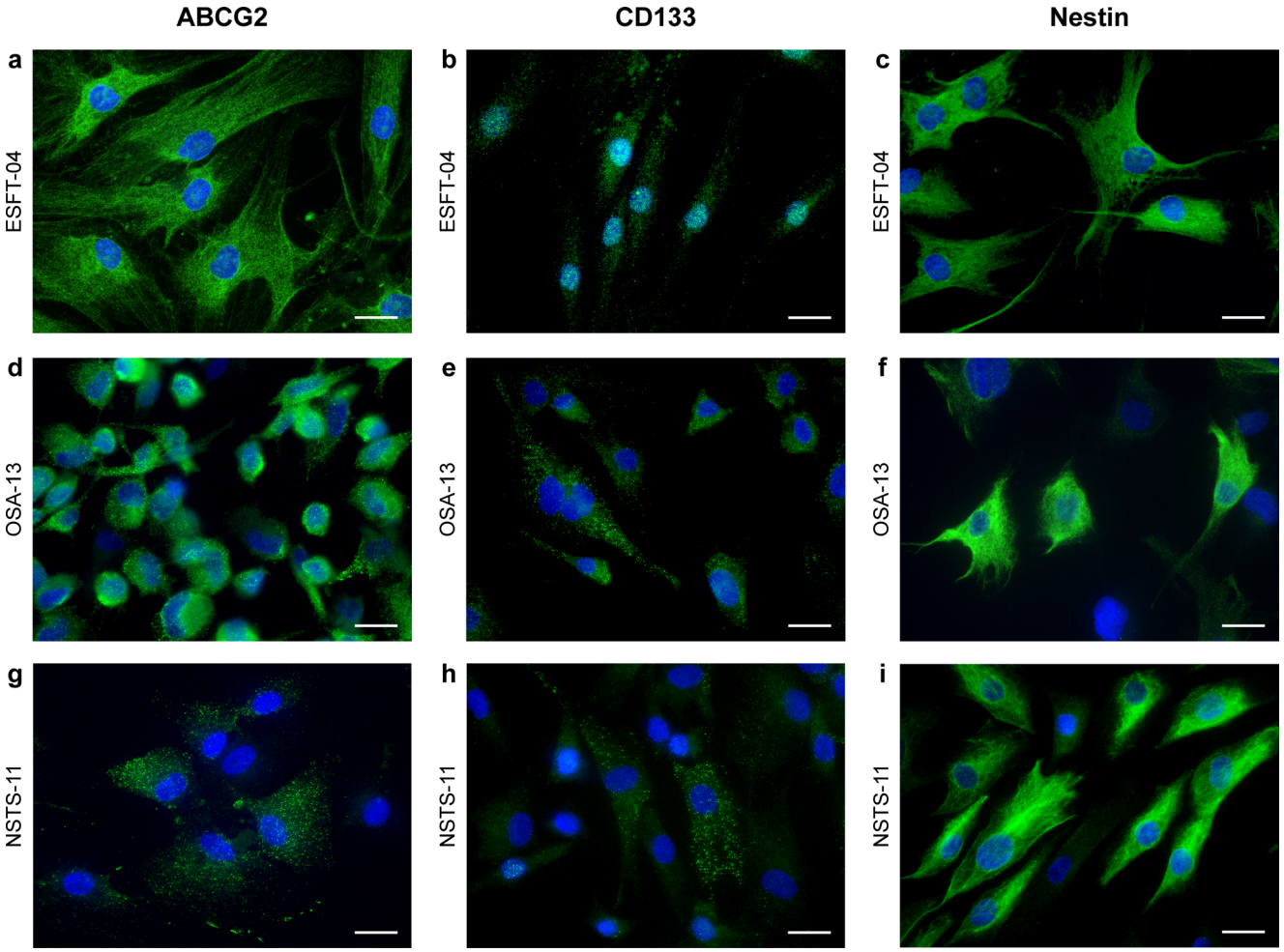

Fig. 2 Expression of ABCG2, CD133 and nestin in sarcoma cell lines as detected by IF. Ewing's sarcoma (**a**–**c**), osteosarcoma (**d**–**f**), and rhabdomyosarcoma (**g**–**i**) cell lines were analyzed. Each marker was visualized by indirect immunofluorescence using Alexa 488-conjugated secondary antibody (*green*); nuclei were counterstained with DAPI (*blue*). *Scale bars*, 25 μm

Table 3 Analysis of ABCG2, CD133, and nestin expression in tumor samples and corresponding cell lines

Tumor sample	Cell line	ABCG2				CD133				Nestin			
		IHC (tumor)		IF (cell line)		IHC (tumor)		IF (cell line)		IHC (tumor)		IF (cell line)	
		% TC	IR	% PC	I-sc	% TC	IR TC	% PC	I-sc	% TC	IR TC	% PC	I-sc
Ewing's sarcoma													
1	ESFT-03	+/−	+	93 %	201.00	+	+	67 %	106.00	−	−	45 %	83.00
2	ESFT-04	+	+	95 %	235.00	+++	+	97 %	174.00	+	++	65 %	131.00
3	ESFT-09	++	++	92 %	197.00	+++	+	70 %	115.00	−	−	53 %	98.00
Osteosarcoma													
4	OSA-05	+/−	+	98 %	249.09	+++	++	56 %	99.90	+++	+++	51 %	93.50
5	OSA-06	+++	++	97 %	231.58	+++	++	86 %	149.87	+++	+++	94 %	230.00
6	OSA-13[a]	+/−	+	86 %	199.25	+++	++	94 %	215.65	+++	+++	74 %	160.00
Rhabdomyosarcoma													
7	NSTS-11[a]	+/−	++	50 %	82.32	++	+	51 %	88.52	+++	+++	67 %	141.67
8	NSTS-22	+	+	47 %	72.00	+	+	50 %	94.09	+++	+++	65 %	164.79
9	NSTS-28	+/−	++	44 %	69.55	+++	+	54 %	100.08	+++	+++	61 %	101.77

The percentage of positive tumor cells (% TC) was categorized into five levels as follows: − (0 %), +/− (1–5 %), + (6–20 %), ++ (21–50 %), and +++ (51–100 %). The immunoreactivity of tumor cells (IR TC) was graded as − (none), + (weak), ++ (medium), and +++ (strong). For cell lines, the mean percentage of cell positive for the respective antigen (% PC) is given. Immunoscores (I-sc) were determined by multiplying the percentage of positive cells by the respective immunoreactivity

IHC immunohistochemistry, *IF* immunofluorescence

[a] Primary cell lines proved to be tumorigenic in NOD/SCID gamma mice

for 24 h, routinely processed for histological examination and embedded in paraffin. Tissue sections of FFPE samples were stained with hematoxylin-eosin and examined. Alternatively, IHC detection was performed as previously described.

Results

In general, comparison of the results from IHC staining of the primary tumor samples (Fig. 1) and from IF of the corresponding cell lines (Fig. 2) showed a selection of cells expressing ABCG2, CD133 and nestin under in vitro conditions. All cell lines included in this study showed at least approximately 50 % positive cells for each of these markers, although the respective immunoscore values varied from low to high (Table 3).

ABCG2 was found relatively rarely and at a low intensity in all tumor samples independent of the sarcoma type (Table 3, Fig. 1a, d, g), although the Ewing's sarcoma (Fig. 2a) and osteosarcoma (Fig. 2d) cell lines showed strong expression of this molecule in almost all cells. However, in the rhabdomyosarcoma cell lines (Fig. 2g), only approximately half of the cells were positive for ABCG2 and ABCG2 IR was scored as middle (Table 3).

CD133 was more frequently detected in the tumor samples of all sarcoma types, but the intensity of immunostaining was weak in Ewing's sarcomas and rhabdomyosarcomas and was middle in osteosarcomas (Table 3, Fig. 1b, e, h). In all examined cell lines, the frequency of CD133-positive cells was greater than 50 %, and the IR appeared to be higher than that in the primary tumors (Table 3, Fig. 2b, e, h). The atypical nuclear localization of CD133 was observed in some tumor samples and in all cell lines at various frequencies (Table 4, Fig. 3a–c), but the most surprising result was in the ESFT-04 cell line, in which absolute nuclear positivity for CD133 was observed (Fig. 3d, e). These cells clearly exhibited a strong selection advantage because nuclear positivity for CD133 was found only sporadically in the corresponding primary tumor tissue (Fig. 3f).

Quite different results were achieved for nestin: although it was expressed very intensively in all osteosarcoma and rhabdomyosarcoma tumor samples (Table 3, Fig. 1f, i), one Ewing's sarcoma tumor sample showed a markedly low proportion of nestin-positive cells (Fig. 1c), and the other two Ewing's sarcoma tumor samples were nestin-negative (Table 3). In contrast, cell lines, including those derived from Ewing's sarcomas, contained more than 50 % nestin-positive cells and displayed medium or high IR (Table 3, Fig. 2c, f, i).

In all cell lines, the expression of ABCG2, CD133 and nestin was further examined at the transcriptional level by qRT-PCR (Fig. 4a). For nestin, the mRNA levels nearly completely correlated with the immunoscore as calculated

Table 4 Analysis of the subcellular localization of CD133 in tumor samples and corresponding cell lines

Tumor sample	Cell line	Localization	IHC (tumor)		IF (cell line)	
			% TC	IR TC	% PC	I-sc
Ewing's sarcoma						
1	ESFT-03	Me | Cy	− | +	− | +	67.00	106.00
		Nu	−	−	11.20[b]	11.20
2	ESFT-04	Me | Cy	− | +++	− | +	97.00	155.00
		Nu	+/−	+	97.00[b]	232.00
3	ESFT-09	Me | Cy	− | +++	− | +	70.00	115.00
		Nu	−	−	53.00[b]	53.00
Osteosarcoma						
4	OSA-05	Me | Cy	+ | +++	++ | ++	56.00	99.90
		Nu	++	+	10.66[b]	10.66
5	OSA-06	Me | Cy	+/− | +++	+ | ++	86.00	149.87
		Nu	+/−	+	16.91[b]	16.91
6	OSA-13[a]	Me | Cy	+++	++	94.00	215.65
		Nu	−	−	50.33[b]	50.33
Rhabdomyosarcoma						
7	NSTS-11[a]	Me | Cy	− | ++	− | +	46.40	83.92
		Nu	−	−	4.60	13.80
8	NSTS-22	Me | Cy	− | +	− | +	44.00	88.09
		Nu	+/−	+	6.00	18.00
9	NSTS-28	Me | Cy	− | +++	− | +	48.80	94.88
		Nu	−	−	5.20	15.60

Localization of CD133 was classified as membranous (Me), cytoplasmic (Cy) or nuclear (Nu). The percentage of positive tumor cells (% TC) was categorized into five levels: − (0 %), +/− (1–5 %), + (6–20 %), ++ (21–50 %), and +++ (51–100 %). The immunoreactivity of tumor cells (IR TC) was graded as − (none), + (weak), ++ (medium), and +++ (strong). For cell lines, the mean percentage of cell positive for the membranous / cytoplasmic or nuclear localization (% PC) is given. Immunoscores (I-sc) were determined by multiplying the percentage of positive cells by the respective immunoreactivity

IHC immunohistochemistry, *IF* immunofluorescence

[a] Primary cell lines proved to be tumorigenic in NOD/SCID gamma mice

[b] Cells were not exclusively positive for nuclear localization but represented a subset of membranous/cytoplasmic positive cells

for individual cell lines. However, no such trends were observed for ABCG2 or CD133.

To detect a possible relationship between the expression of these markers and tumorigenic potential, all cell lines were tested using an in vivo tumorigenicity assay. Surprisingly, only two cell lines—OSA-13 and NSTS-11—were able to form tumors in immunodeficient mice (Table 3, Fig. 5a–c, g–i). Furthermore, the detected tumorigenicity of these cell lines did not correspond to any comparable change in the expression of the markers described above or to the atypical nuclear localization of CD133 (Table 4). Only qRT-PCR showed increased transcriptional levels of ABCG2 and CD133; this pattern was not observed at the protein levels, as detected by IF (Fig. 4a, Table 3).

For this reason, we performed additional qRT-PCR experiments to evaluate the levels of common stem cell markers (Oct4, Nanog, Sox2, and ALDH1) to identify possible changes associated with the tumorigenicity of OSA-13 and NSTS-11 cell lines. Among these markers, only Sox2 showed elevated mRNA levels in the tumorigenic cell lines but not in the other cell lines (Fig. 4b, Table 5). Further analysis at the protein level showed identical results: Sox2 was highly expressed exclusively in the two tumorigenic cell lines as detected by Western blotting, whereas no differences in expression of Oct4, Nanog and ALDH1 were observed between tumorigenic and non-tumorigenic cell lines (Fig. 4c). Furthermore, IF analysis showed the highest immunoscores of Sox2 in the two tumorigenic cell lines (Table 5, Fig. 6d–f), and the expression of Sox2 was validated by Western blotting using two independent antibodies (Fig. 6g).

In the last step of our study, we analyzed all primary tumor samples and all xenograft tumors for Sox2 expression via IHC staining. Among the primary tumors, the

highest proportion of Sox2-positive cells was found in the tumor sample from which the tumorigenic NSTS-11 cell line was derived, and these Sox2-positive cells were typically accumulated in small distinct clusters or striations (Fig. 6c). A rare incidence of Sox2-positive cells was identified in two additional tumor samples, but their corresponding cell lines were non-tumorigenic (Table 5). Finally, IHC analysis of all xenograft rhabdomyosarcoma and osteosarcoma tumors showed a marked increase in the frequency of Sox2-positive cells in all xenograft rhabdomyosarcoma and osteosarcoma tumors (Fig. 5d–f, j–l) compared with the corresponding primary tumor samples.

Discussion

The initial aim of our study was to analyze the changes in the expression of ABCG2, CD133, and nestin as putative CSC markers in pediatric sarcomas, in both primary tumors and corresponding cell lines derived from these tumors. We intended to elucidate the selection process for these three markers during the derivation process under in vitro conditions because the findings published in this field bring to date had reported partly contradictory results [6].

ABCG2, a plasma membrane ATP-binding cassette (ABC) transporter responsible for the multidrug resistance of tumor cells, was reported to be a specific marker of CSCs in osteosarcoma cell lines, as only this ABC transporter family member was detected in sarcospheres formed during a functional assay of the CSCs [17, 18]. However, the expression of other ABC transporters was described in several osteosarcoma and Ewing's sarcoma cell lines, specifically in side populations (SPs) detected within these cell lines [19, 20]. Our results are in accordance with these findings: although ABCG2 expression was weak and infrequent in the primary tumors, the immunoscore for ABCG2 was markedly increased in all six cell lines derived from Ewing's sarcoma or osteosarcoma. In contrast, the expression of ABCG2 remained weak in all three rhabdomyosarcoma cell lines under in vitro conditions. Other research groups also reported the low expression of ABCG2 in rhabdomyosarcomas [21]; however, increased ABCG2 immunoreactivity was found in the embryonal subtype compared with the alveolar subtype of rhabdomyosarcoma [22].

CD133 is a pentaspan transmembrane glycoprotein with unclear biological functions. The AC133, i.e., glycosylated epitope of CD133 is widely discussed to be a putative "universal" marker of CSCs in various human malignancies [23]. Among our nine tumor samples, six of them showed a high frequency of CD133-positive cells, but CD133 IR was only weak to medium in all samples. Nevertheless, these cells apparently maintain their selection advantage under in vitro conditions because all cell lines contained at least 50 % CD133-positive cells and because the immunoscore values were middle or high. These results are in accordance with previously published findings on rhabdomyosarcomas as well as on rhabdomyosarcoma and osteosarcoma cell lines [12, 13, 24]. Conversely, only low levels (up to 7.8 %) of CD133-positive cells were reported in four cell lines derived from

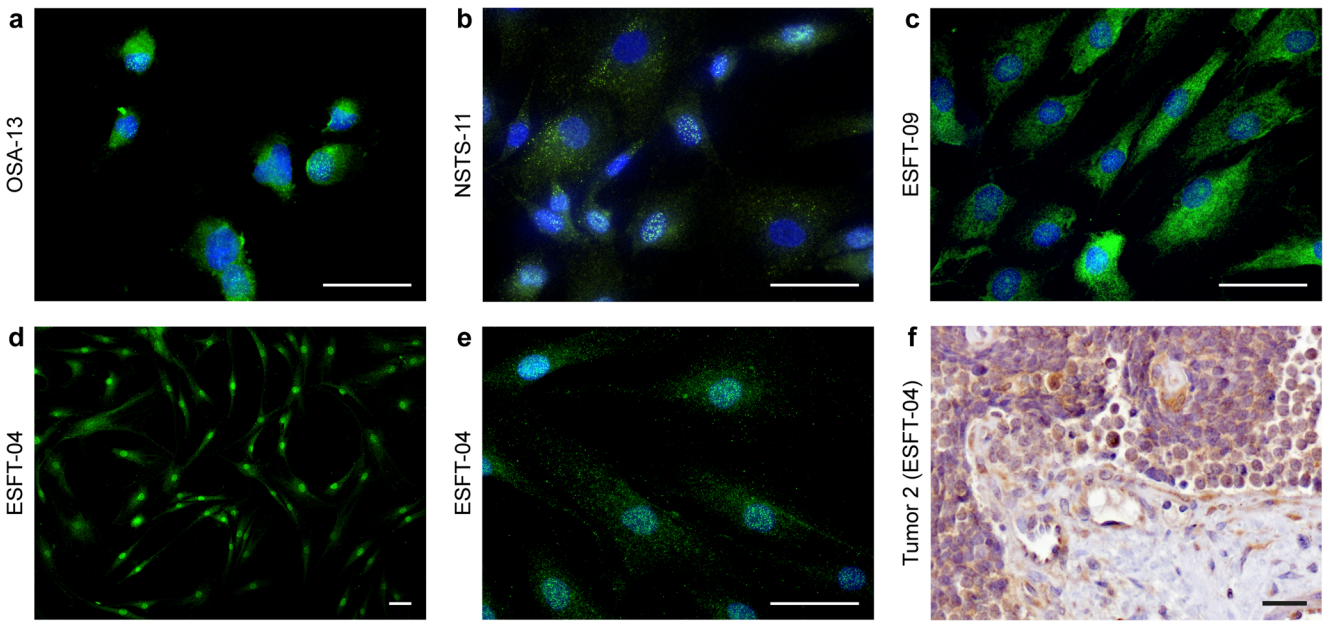

Fig. 3 Alterations in the CD133 subcellular localization in sarcoma cell lines and tumor tissues. (**a**–**e**) IF revealed nuclear localization of CD133 (*green*) in cell lines derived from all three types of sarcoma; nuclei were counterstained with DAPI (*blue*). (**d**–**e**) Nuclear positivity was detected absolutely in the ESFT-04 cell line but sporadically in the corresponding tumor (**f**). *Scale bars*, 50 μm

osteosarcomas and chondrosarcomas and in the corresponding primary tumors [25]. This discrepancy could be explained by either the use of different antibodies for CD133 detection or by variances in the subcellular localization of CD133.

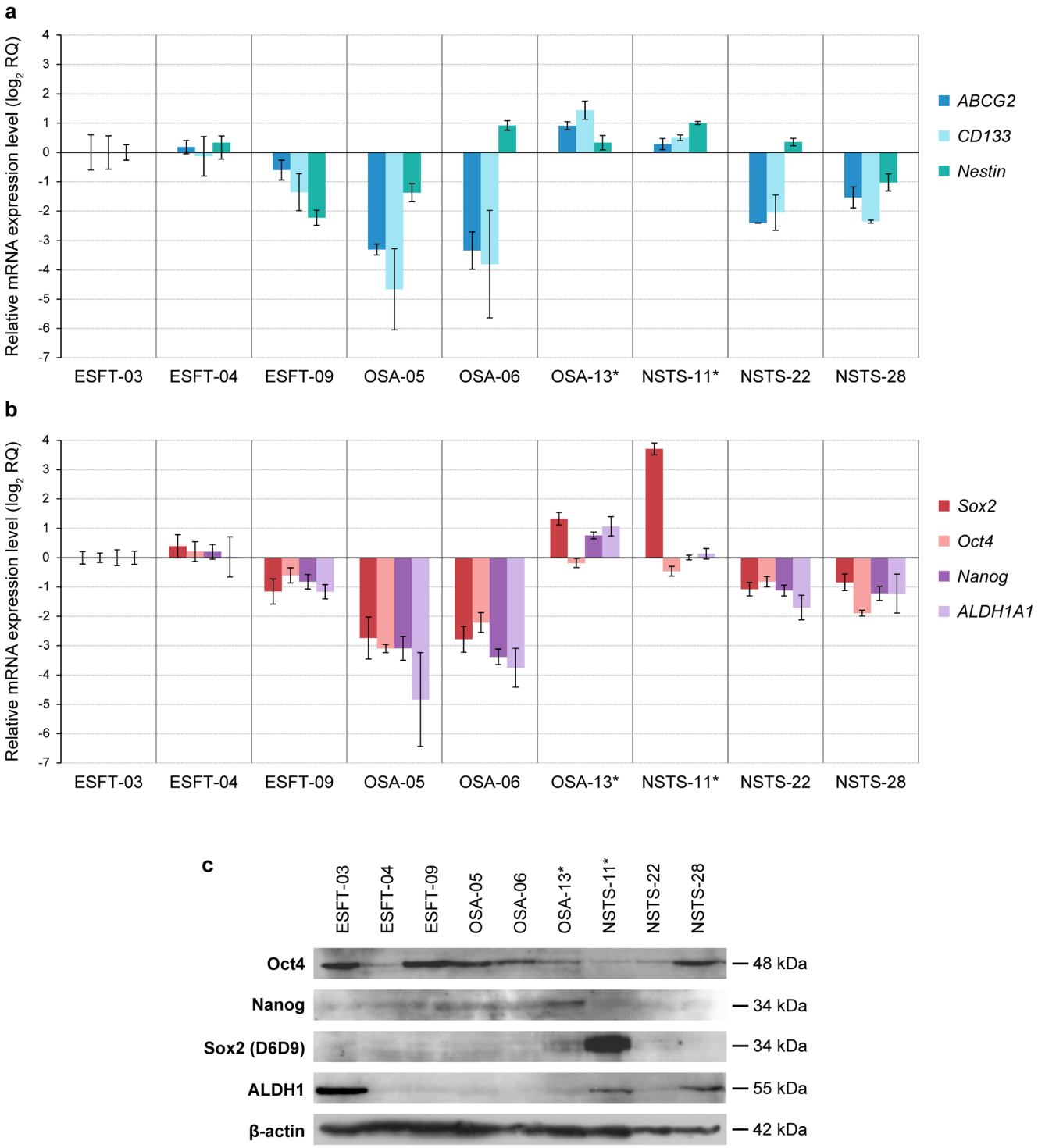

Fig. 4 qRT-PCR and Western blot analysis of CSCs markers expression in sarcoma cell lines. Gene expression levels of sarcoma-specific CSC markers (**a**) and common stem cell markers (**b**) were determined using qRT-PCR. Only tumorigenic cell lines (indicated by *asterisks*) but not non-tumorigenic cell lines expressed Sox2 at mRNA level. The ESFT-03 cell line served as the arbitrary calibrator; the data are presented in log2 scale. The *error bars* indicate the calculated maximum (RQMax) and minimum (RQMin) expression levels that represent the standard error of the mean expression level (RQ value). (**c**) Western blotting of common stem cell markers confirmed upregulation of Sox2 exclusively in tumorigenic cell lines (indicated by *asterisks*). β-actin served as a loading control

Based on other recent studies, CD133 is also clearly detectable in the cytoplasm of tumor cells, where it could be involved in signal transduction, specifically in the canonical Wnt pathway or the PI3K/Akt pathway [26–29]. Very recently, the nuclear localization of CD133 in a stable proportion of cells in rhabdomyosarcoma cell lines was clearly established [11]. For this reason, we considered cells displaying apparent cytoplasmic and/or nuclear positivity for CD133 as CD133-positive; thus, the frequency of these cells must be higher than the reported frequency of CD133-positive cells as detected by flow cytometry. Nevertheless, our results clearly showed that neither cytoplasmic nor nuclear localization of CD133 is clearly associated with the tumorigenic potential of these cells: the tumorigenic NSTS-11 cell line contained only up to 5 % of cells displaying nuclear positivity for CD133, whereas the non-tumorigenic ESFT-04 cell line displayed nuclear positivity for CD133 in nearly all cells.

Nestin, a class VI intermediate filament protein, is widely described as an important marker of CSCs, especially in tumors of neurogenic origin [9, 30]. However, our previous

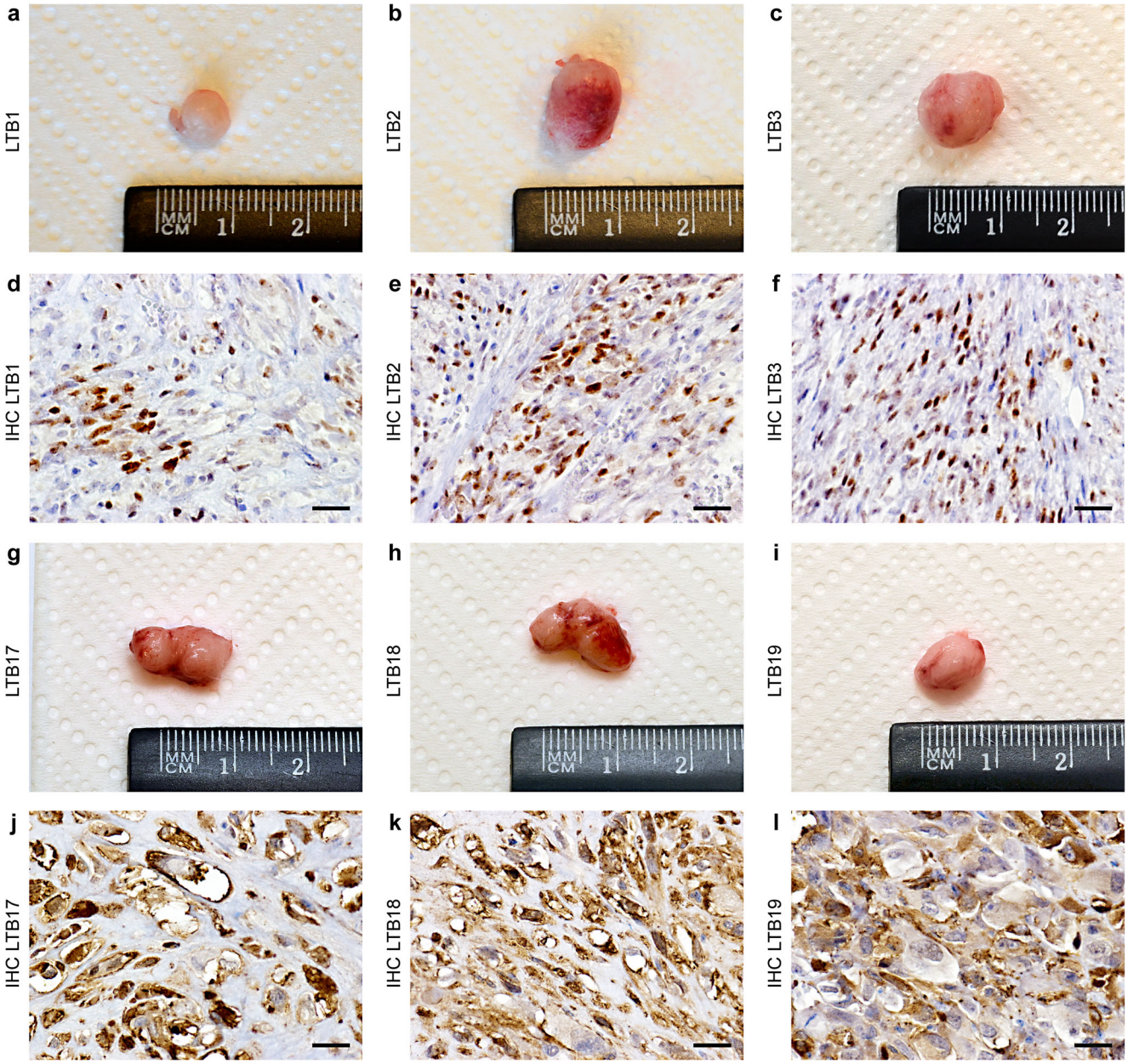

Fig. 5 In vivo tumorigenicity assay and IHC analysis of Sox2 expression in the resulting xenograft tumors. Only NSTS-11 (**a**–**c**) and OSA-13 (**g**–**i**) cells formed xenograft tumors in NOD/SCID gamma mice. (**d**–**f**, **j**–**l**) Markedly enhanced Sox2 expression was detected in xenograft tumors by IHC. *Scale bars*, 50 μm

Table 5 Analysis of Sox2 expression in tumor samples and corresponding cell lines

Tumor sample	IHC − tumor		Primary cell line	IF − cell line	
	% TC	IR TC		% PC	I-sc
Ewing's sarcoma					
1	−	−	ESFT-03	95.00 %	95.00
2	+/−	+	ESFT-04	94.00 %	94.00
3	−	−	ESFT-09	94.00 %	94.00
Osteosarcoma					
4	−	−	OSA-05	71.00 %	71.00
5	−	−	OSA-06	78.00 %	78.00
6	−	−	OSA-13[a]	94.00 %	145.00
Rhabdomyosarcoma					
7	+	+	NSTS-11[a]	76.00 %	146.00
8	−	−	NSTS-22	25.00 %	25.00
9	+/−	+	NSTS-28	10.00 %	10.00

The percentage of tumor cells (% TC) positive for Sox2 was categorized into five levels: − (0 %), +/− (1–5 %), + (6–20 %), ++ (21–50 %), and +++ (51–100 %). The immunoreactivity of tumor cells (IR TC) was graded as − (none), + (weak), ++ (medium), and +++ (strong). For IF analysis, the mean percentage of cell positive for the Sox2 (% PC) is given. Immunoscores (I-sc) were determined by multiplying the percentage of positive cells by the respective immunoreactivity

IHC immunohistochemistry, *IF* immunofluorescence

[a] Primary cell lines proved to be tumorigenic in NOD/SCID gamma mice

studies reported a variable proportion of nestin expression in high-risk osteosarcomas and corresponding cell lines, although high levels of nestin tended to indicate a worse clinical outcome in these patients [12, 31]. In contrast, rhabdomyosarcoma primary tumors showed high levels of nestin expression, but cell lines derived from these tumors contained only up to 10 % of nestin-positive cells [13]. Our recent results showed strong expression of nestin in tumor tissue of both osteosarcomas and rhabdomyosarcomas, but cell lines derived from these tumors were primarily assigned a middle immunoscore. Furthermore, nestin expression appears not to be associated with the tumorigenicity of these cell lines. These findings are in accordance with the previously published studies on bone sarcoma cell lines, in which no clear relationship between nestin expression and sarcosphere-forming capacity was found [17, 25]. The weak or absent expression of nestin in Ewing's sarcomas is also in agreement with other studies of this tumor type, which have reported negativity for nestin or low expression levels of nestin [32, 33]. However, another research group found 54 % positivity for nestin in tumor samples from their cohort [24].

In summary, our results showed that the frequency of putative CSC markers apparently changed after explantation of the tumor tissues and their transfer to cell cultures. Although the frequency of cells positive for ABCG2 and CD133 predominantly increased in the respective cell lines, the high levels of nestin expression were reduced in both osteosarcomas and rhabdomyosarcomas under in vitro conditions. These findings suggest the selection advantage of cells expressing ABCG2 or CD133, but the in vivo functional tests did not confirm the tumorigenic potential of the cells harboring this phenotype.

In contrast, the most important finding of our study was the evident relationship between the expression of the transcription factor Sox2, as demonstrated by qRT-PCR and Western blot analysis, and the tumorigenicity of the OSA-13 and NSTS-11 cell lines. To confirm this interesting result at the protein level, we performed further analysis of Sox2 expression in cell lines via IF. Similarly, the Sox2 levels were highest in the two tumorigenic cell lines, although the immunoscore values did not display the same profile as the qRT-PCR results. Subsequent analysis of Sox2 expression in primary tumors via IHC staining confirmed the presence of Sox2-positive cells in the tumor from which the NSTS-11 cell line was derived. Interestingly, these Sox2-positive cells tended to be accumulated in small areas of the tumor tissue. This finding implies morphological similarity among a stem cell niche. Moreover, IHC analysis of the xenograft tumors showed a substantial increase in the frequency of Sox2-positive cells in all tissue samples.

Our findings are in full accordance with the results reported for human and murine osteosarcoma cell lines [34]. Increased Sox2 levels were also detected in sarcospheres derived from osteosarcoma [35] and rhabdomyosarcoma cell lines [36], although the correlation of Sox2 expression with tumorigenic potential was not reported in these studies. Other recent studies showed that Sox2 expression is required for self-renewal and tumorigenicity of CSCs in other tumor types, including glioblastoma [37, 38], melanoma [39], ovarian carcinoma [40], cervical carcinoma [41], prostatic carcinoma [42], lung carcinoma [43, 44], and squamous-cell carcinoma of the skin [45, 46]. Finally, the involvement of Sox2 in sarcoma tumorigenesis was indirectly illustrated via the targeting of Sox2 by miR-126, which acts as a tumor suppressor in osteosarcomas [47].

All of these findings support our conclusion that cells displaying elevated expression of Sox2 are key mediators of sarcoma tumorigenesis. Although the experimental data on these tumor types remain limited, our results provide the first evidence that increased Sox2 expression is associated with the tumorigenic potential of not only osteosarcomas but also rhabdomyosarcomas. Regardless of the expression of ABCG2, CD133, and nestin, only cell lines displaying increased Sox2 expression were tumorigenic, and the xenograft tumors showed apparent accumulation of Sox2-positive cells.

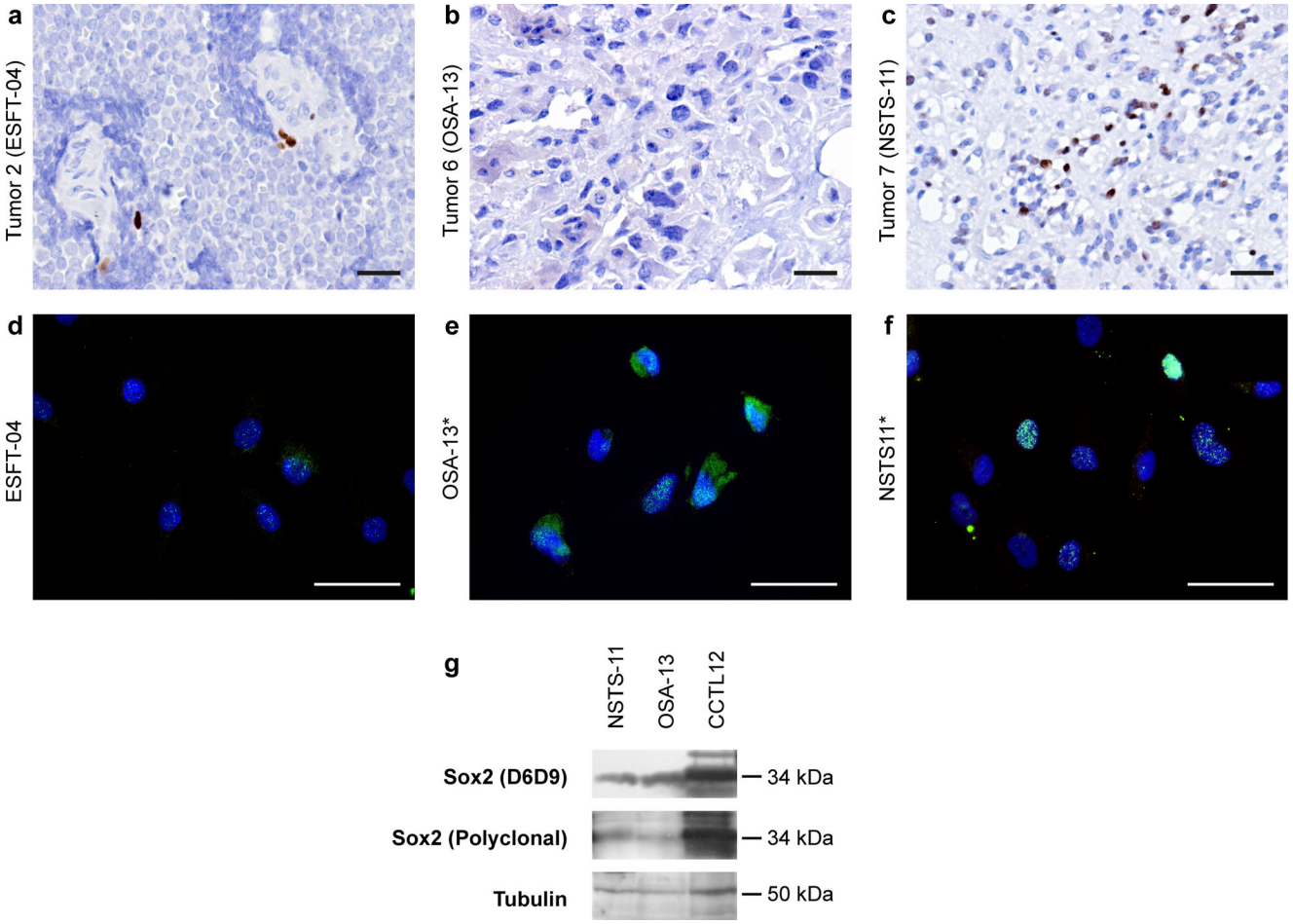

Fig. 6 Expression of Sox2 in sarcomas and derived cell lines. **a**–**c** IHC analysis revealed the presence of Sox2-positive cells in tumor tissues; corresponding cell lines are indicated in brackets. **d**–**f** Elevated Sox2 expression was detected using IF in tumorigenic cell lines (indicated by *asterisk*). **g** Western blotting using two different antibodies confirmed the expression of Sox2 in tumorigenic cell lines as detected by IF. Protein lysate of human embryonic stem cell line CCTL-12 [48] was used as positive control. *Scale bars*, 50 μm

Taken together, sarcoma cells displaying high levels of Sox2 are undoubtedly directly involved in tumor initiation and growth; therefore, these cells fit the definition of the CSC phenotype. Thus, the Sox2 pathway could be considered as a target for new anticancer drugs or immunotherapies based on up-to-date approaches such as chimeric antigen receptors or dendritic cell vaccines.

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Compliance with ethical standards The Research Ethics Committee of the School of Science (Masaryk University) approved the study protocol, and a written statement of informed consent was obtained from each participant or his/her legal guardian prior to participation in this study. This study was approved by the Institutional Animal Care and Use Committee of Masaryk University and was registered by the Ministry of Agriculture of the Czech Republic as required by national legislation.

Conflicts of interest None

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