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Cancer cell lines represent *in vitro* models for studying malignancies, general cell biology, drug discovery and more. Whether they can be considered as exact representative models of the parental tumors remains uncertain given the acquisition of additional *ex vivo* changes of the cells and the lack of tissue architecture and stroma. Previously, within the EuroBoNeT consortium, we characterized a collection of bone sarcoma cell lines on genomic and proteomic level. Here, we address the phenotypical and functional characterization of the unique set of osteosarcoma cell lines ($n = 19$) *in vitro* and *in vivo*. For functional analysis of differentiation capacity, cells were stimulated towards osteoblasts, adipocytes and chondrocytes. Furthermore, all cell lines were injected subcutaneously and intramuscularly into nude mice to assay their *in vivo* tumor formation capacity as well as for phenotypical analysis of the tumors. All formed tumors were further characterized histologically and immunohistochemically. Out of 19 cell lines, 17 (89%) showed adipogenic differentiation, 13/19 (68%) could differentiate towards osteoblasts and in 6/19 (32%) cell lines chondrogenic differentiation was evident. About half of the cell lines (8/19, 42%) produced tumors *in vivo* after subcutaneous and intramuscular injections. Several cell lines showed invasion into adjacent tissues and one tumor developed several lung metastases. The use of cell lines, especially in cancer research, is of paramount importance. Here, we identify comprehensively characterized osteosarcoma cell lines, which robustly represent clinical osteosarcoma providing researchers useful *in vitro* and *in vivo* models to study the genetics and functional characteristics of this highly malignant neoplasm.
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Tumor cell lines have been considered as instrumental entries into tumor cell biology and often used for studying the mechanisms of carcinogenesis, the functional characteristics of genes and drug discovery, screening and response.[1–8] Nevertheless, concerns have been raised about the use of cell lines that can be divided into two categories of criticism. First, mainly due to poor experimental conduct, false (derived from another cell population within the cell culture than the one intended), cross-contaminated (with other human cells or cells from other species) and/or pathogen- (mycoplasm) contaminated cell lines have been discovered repeatedly.[9–12] Second, the representativeness of cultured cell lines as compared with the original tumors is being questioned as these cells have been cultured in the absence of stroma,[13] hence lacking the proper microenvironment and the original tissue architecture. Moreover during culturing, specific cells are continuously selected based on the *in vitro* conditions and cells might undergo additional *ex vivo* mutations. On the other hand, it has been shown that cell lines adequately represent the tumors they are originating from, especially at the genetic level.[14–19] All in all cell lines appear to be adequate models as long as there are controlled culturing conditions and a good selection process to identify the appropriate ones.[10,11,20] Accordingly, to select osteosarcoma cell lines representative of human osteosarcoma, here we characterize 19 cell lines *in vitro* and *in vivo* by using robust methods subjected to regular quality control.

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Osteosarcoma is a highly malignant tumor, fatal for about one-third of the patients who do not respond to chemotherapy and alternative therapies are still missing. This is mainly due to the rarity and the high genetic heterogeneity of these tumors, which makes it difficult to have patient cohorts that are large enough to compensate for the high genetic variability. One way to bypass this problem is to study osteosarcoma-derived cell lines, which are abundantly used as *in vitro* models because they are highly proliferative and receptive for genetic manipulation by transfection. Recently, through the effort of the EuroBoNet network, a large panel of osteosarcoma cell lines, among other bone sarcoma cell lines, was described genetically.[18] To select the cell lines that are most representative of primary osteosarcoma, here we analyzed their *in vitro* differentiation capacity and their *in vivo* tumorigenicity in nude mice. Furthermore, generated tumors were histologically and immunohistochemically classified.

By this comprehensive study, we were able to identify at least eight cell lines, which convincingly represent primary human osteosarcoma. HOS-143B was discovered to be highly metastatic to the lungs of the animals after subcutaneous and intramuscular xenotransplantations. The cell lines characterized here represent excellent *in vitro* and especially *in vivo* models to obtain a better understanding of osteosarcoma biology, and subsequently the identification of novel targets for specific therapies.

Table 1 Origin and characteristics of the cell lines

Cell line	PD (h)[a]	Subtype[b]	Age[c]	Sex[c]	Reference[d]
HOS	36	N/A	13	F	ATCC—CRL-1543
HOS-143B	36	N/A	13	F	ATCC—CRL-8303
HOS-MNNG	24	N/A	13	F	ATCC—CRL-1547
OSA	24	Fibroblastic	19	M	ATCC—CRL-2098
MG-63	48	Fibroblastic	14	M	ATCC—CRL-1427
Saos-2	48	N/A	11	F	ATCC—HTB-85
U2OS	36	N/A	15	F	ATCC—HTB-96
IOR/MOS	72	Osteoblastic	13	F	Benini *et al*[21]
IOR/OS9	72	Osteoblastic	15	M	Benini *et al*[21]
IOR/OS10	72	Fibroblastic	10	F	Benini *et al*[21]
IOR/OS14	48	Osteoblastic	13	M	Benini *et al*[21]
IOR/OS15	48	Osteoblastic	12	F	Benini *et al*[21]
IOR/OS18	60	Osteoblastic	33	M	Benini *et al*[21]
SARG	72	N/A	25	M	Benini *et al*[21]
KPD	36	Osteoblastic	7	F	Bruland *et al*[22]
OHS	36	Osteoblastic	14	M	Fodstad *et al*[23]
HAL	48	N/A	16	M	Høifødt, Oslo[e]
ZK-58	72	Osteoblastic	21	M	Schulz *et al*[24]
MHM	60	Fibroblastic	41	F	Kjonniksen *et al*[25]

PD, population doubling; NA, not available.

For each cell line used in this study, the *in vitro* growth speed, the subtype of the originating tumor, the age and the gender of the donor and a reference for the cell line are depicted, respectively.

[a]Average time needed for one PD in hours.

[b]Osteosarcoma subtype of the original tumor from which cells were derived, note that this information is not available for all cell lines, indicated as N/A.

[c]Age and the sex of the patient from whom the tumor originates.

[d]When cell lines are available at the ATCC, the identifiers are given.

[e]Unpublished.

MATERIALS AND METHODS

Cell Lines

Cells were provided by the different partner institutes of the EuroBoNet network[21–25] or derived from ATCC and were grown in RPMI 1640 (Invitrogen, Karlsruhe, Germany) supplemented with 2% L-glutamine (Invitrogen) and 10% fetal calf serum under standard conditions. Control tests for mycoplasm contamination were carried out routinely (two times a month), using a PCR-based commercially available detection kit according to the manufacturer's protocol (VenorGem, Minerva Biolabs, Berlin, Germany). Furthermore, all cell lines and xenografts were genotyped using the Powerplex 1.2 system (Promega, Leiden, the Netherlands), as described previously,[26] to match the cells to their previously published identities and to detect any cross-contaminations.[18] Table 1 summarizes the characteristics and the culturing conditions of the cell lines.

In Vitro Differentiation

Before differentiation, alkaline phosphatase (ALP) activity was measured for all cell lines to determine their basal ALP activity. Differentiation studies were performed as described previously:[27] cells were induced to differentiate into mature mineralizing osteoblasts over a 3-week culture period by plating 10^4 cells in 500 μl medium per well of a 24-well plate (for ALP measurement) and by plating 2×10^4 cells in 1 ml medium per well of a 12-well plate (for mineralization). Ascorbic acid (50 μg/ml) was added to the medium starting on day 4 and β-glycerolphosphate (5 mM) on day 11. Osteoblastic differentiation was quantified as described previously,[28] with some modification. In short, cells were lysed in ALP lysis buffer (10 mmol/l glycine, 0.1 mmol/l $MgCl_2$, 10 μmol/l $ZnCl_2$, 0.1% Triton X-100) and 25 μl was used to determine ALP activity using 6 mmol/l *p*-nitrophenylphosphate as a substrate and measuring absorbance at 405 nm on an ELISA reader. For comparisons, ALP activity was calculated per μl per minute until saturation. The presence of mineralization was assessed by staining with Alizarin Red S (20 mg/ml, pH 5.5). Adipogenic differentiation was induced by culturing 10^4 cells in 500 μl medium per well of a 24-well plate for 2 weeks. FBS was replaced by 10% charcoal-stripped FBS in the basal medium and indomethacin (50 μM) was added starting on day 4. Adipocytes containing lipid droplets were stained with Oil Red O (3 mg/ml). For chondrogenic differentiation, cells were cultured as pellets (2×10^5 cells per

pellet) in U-shaped 96-well plates containing 200 μl DMEM supplemented with P/S (1%), pyruvate (100 μg/ml), transferrin selenite (10 μl/ml) and proline (40 μg/ml). During the first 2 weeks, ascorbic acid (50 μg/ml), TGF-β3 (10 ng/ml) and dexamethasone (10^{-7} M) were added to the medium. Starting at the third week, ascorbic acid (50 μg/ml), BMP6 (500 ng/ml) and β-glycerolphosphate (5 nM) were added. After 5 weeks, pellets were fixed in formalin and embedded in paraffin and sections were stained with toluidine blue to identify chondrogenic matrix.

In Vivo Tumor Formation

After trypsinization, cells were counted and dilutions of 2×10^6 cells in 10 μl phosphate-buffered saline were prepared. Each cell line was injected into one nude mouse at three locations: two subcutaneous injections on the back (upper left and lower right corner) and one intramuscular injection (upper part of the hind left paw). After the injections, tumor growth was screened twice a week by observation and palpation. Animals were killed when tumors reached approximately 1 cm in diameter or no sign of tumor formation was detected after 6 months or when any kind of animal suffering was detected. All cell lines that did not produce tumors were re-injected into a second mouse (again at three locations) to validate this observation. After being killed, all tumors were surgically removed and archived by freezing as well as by fixing in formalin and embedding in paraffin. Furthermore, a complete autopsy was performed to detect invasion, angiogenesis and/or metastases of the tumors. Growth into adjacent tissues was labeled as 'invasion' only when after subcutaneous injection cells invaded underlying tissues (mostly muscle) and only when this was confirmed not to be caused by direct injection into those tissues. (Neo)-angiogenesis was considered when blood vessels were identified inside the tumor's mass at locations where they were not anatomically expected and/or when vessel subcutaneously grew towards the tumors. All animal experiments were performed according to the Dutch and Spanish animal experiments guidelines, and approved by the Valencia University Animal Experiments Committee.

Tissue Array Construction

Tissue cores from formalin-fixed and paraffin-embedded (FFPE) tumor areas selected by two pathologists (IM and ALLB) on the basis of a hematoxylin and eosin (H&E)-stained slide were taken from each specimen (Beecher Instruments, Silver Springs, MD, USA). The cores (2 mm diameter) were arrayed on a recipient paraffin block using a tissue arrayer from Beecher Instruments.[29] At least three cores from each tumor were sampled to outweigh intratumoral heterogeneity.[30] Each tissue array contains additional cores from other tissue types both as internal controls for immunohistochemistry (IHC) as well as for orientation purposes.

Staining

IHC was performed on FFPE tissue array sections as described previously.[31] Next to the H&E, Periodic acid-Schiff and Masson's trichrome stainings to assay the histology of the xenografts, the slides were stained with all antibodies listed in Table 3, which also summarizes the IHC conditions. All stainings in this study were independently evaluated by two pathologists (IM and ALLB) as previously described in detail.[32,33] Intensity and percentage of positive neoplastic cells were evaluated. Cellular localization of immunopositivity (nuclear, cytoplasmic or membranous) was also recorded. Conflicting assessments were reviewed until final agreement was achieved. A final immunopositivity score was indicated per sample as negative (−), weakly positive (+), moderately positive (++) or strongly positive (+++).

HOS-143B

To study the progression of the only metastatic cell line, HOS-143B, cells were injected two more rounds in 5 and 10 animals, respectively. In addition from one of the primary tumors, a piece was subcutaneously transplanted to another recipient nude mouse, and this was repeated three times. Both from subcutaneous and intramuscular tumors, lung metastases were analyzed for p53 protein expression and mutation state as described previously.[18,34,35]

RESULTS

Differentiation Capacity of Osteosarcoma Cell Lines

Differentiation towards at least one of the three lineages was detected in all cell lines, 14 out of 19 cell lines could differentiate towards at least two lineages and three cell lines (OSA, IOR/OS9 and IOR/OS18) differentiated into all three lineages (Table 2). In 17/19 (89%) cell lines, adipogenic differentiation was observed, 13/19 (68%) could differentiate towards osteoblasts and in 6/19 (32%) cell lines chondrogenic differentiation was evident (Figure 1).

Cell Line-Derived Tumors Represent Osteosarcoma

Eight out of 19 osteosarcoma cell lines tested produced tumors *in vivo* after subcutaneous and intramuscular injections (Figure 2a–h), intramuscular tumors formed on average twice as fast compared with the tumors produced by the same cells injected subcutaneously. All tumors were characterized as high-grade sarcomas and, although not always abundant, tumor cells produced osteoid that is characteristic of osteosarcoma. The tumor produced by one of the oldest human-derived cell lines, U2OS, showed abundant osteoid production (Figure 2i) and infiltrating immune cells were detected (Figure 2n). The IOR/OS9 cells showed differentiation *in vivo* (Figure 2e, j and o). Furthermore, six cell lines were shown to invade into adjacent tissues and in all recipient animals angiogenesis was found. The HOS-143B cell line was tumorigenic and metastatic (Figure 3).

Table 2 *In vitro* functional analysis and *in vivo* tumor progression

	In vitro differentiation[a]			*In vivo* tumor growth					
Cell line	Osteo	Chondro	Adipo	Follow up[b]	1st injection[c]	2nd injection[c]	Invasion	Angiogenesis	Meta[t]
HOS	Yes	No	Yes	6	No	No	N/A	N/A	No
HOS-143B	No	No[e]	Yes	1	Yes	N/A	Yes	Strong	Yes
HOS-MNNG	No	No	Yes	1	Yes	N/A	Yes	Weak	No
OSA	Yes	Yes[f]	Yes	1	Yes	N/A	No	Weak	No
MG-63	No	Yes	No	6	No	No	N/A	N/A	N/A
SaOS2	No	Yes[f]	Yes	6	No	No	N/A	N/A	N/A
U2OS	No	No	Yes	3	No	Yes	Yes	Weak	No
IOR/MOS	Yes	No	Yes	6	No	No	N/A	N/A	N/A
IOR/OS9	Yes	Yes	Yes	3	Yes	N/A	Yes	Weak	No
IOR/OS10	No	No	Yes	6	No	No	N/A	N/A	N/A
IOR/OS14	Yes	No[e]	Yes	1	Yes	Yes	Yes	Strong	No
IOR/OS15	Yes	No[e]	Yes	6	No	No	N/A	N/A	N/A
IOR/OS18	Yes	Yes	Yes	6	No	No	N/A	N/A	N/A
SARG	Yes	No[e]	Yes	6	No	No	N/A	N/A	N/A
KPD	Yes	No[e]	Yes	6	No	No	N/A	N/A	N/A
OHS	Yes	No[e]	Yes	1	Yes	N/A	No	Moderate	No
HAL	Yes	No	Yes	6	No	No	N/A	N/A	N/A
ZK-58	Yes	No[e]	Yes	6	No	No	N/A	N/A	N/A
MHM	Yes	Yes	No	3	No	Yes	Yes	Moderate	No

N/A, Not available.

This table summarizes whether cells could differentiate *in vitro* and specifies the lineage(s) of differentiation. On the right part is indicated whether a cell line was tumorigenic *in vivo* under the described circumstances, and if Yes, the occurrence and extent of invasion to adjacent tissues and angiogenesis are indicated. Please Note that injected animals were observed for 6 months if tumors were Not found before.

[a]Whether cells could be pushed towards osteogenic, chondrogenic and/or adipogenic differentiation.

[b]Time in months from injection to killing of the mice.

[c]Whether tumor formation was seen after a first or second round of injection of the cells.

[d]Whether metastases were found upon autopsy of the animal.

[e]Cells could Not form a pellet.

[f]Weakly positive.

Protein Expression Profiles by Immunohistochemistry

Because of the unavailability of specific markers for osteosarcoma, tissue arrays from primary xenografts and metastases were stained for a panel of proteins described to be associated with the tumor in different processes (Table 3):

- differential differentiation: osteonectin (ON), osteocalcin (BGLAP), vimentin (VIM), cytokeratin (CK), epithelial membrane antigen (EMA), CD34, smooth muscle actin (SMA), desmin (DES) and transcription factor SOX9 (SOX9);
- oncogenesis: CD99, B-cell lymphoma protein 2 (BCL2), C-Kit (KIT), caveolin 1 (CAV1), Ki-67, TP53 (p53), CDKN2A (p16), CDKN1A (p21), epidermal growth factor receptor (EGFR), HER2, tumor protein D52 (TPD52) and S100; and
- invasion/migration: ezrin (EZR), e-cadherin (E-Cad) and CD31.

The immunohistochemical expression profiles of xenografts from different cell lines show similar patterns of protein expression reminiscent of primary human osteosarcoma as known from the literature. This pattern is maintained in the metastases as well, indicating a homogeneous cell population. The expression of the proteins described here did not correlate with differentiation capacity or tumorigenicity of the cell lines.

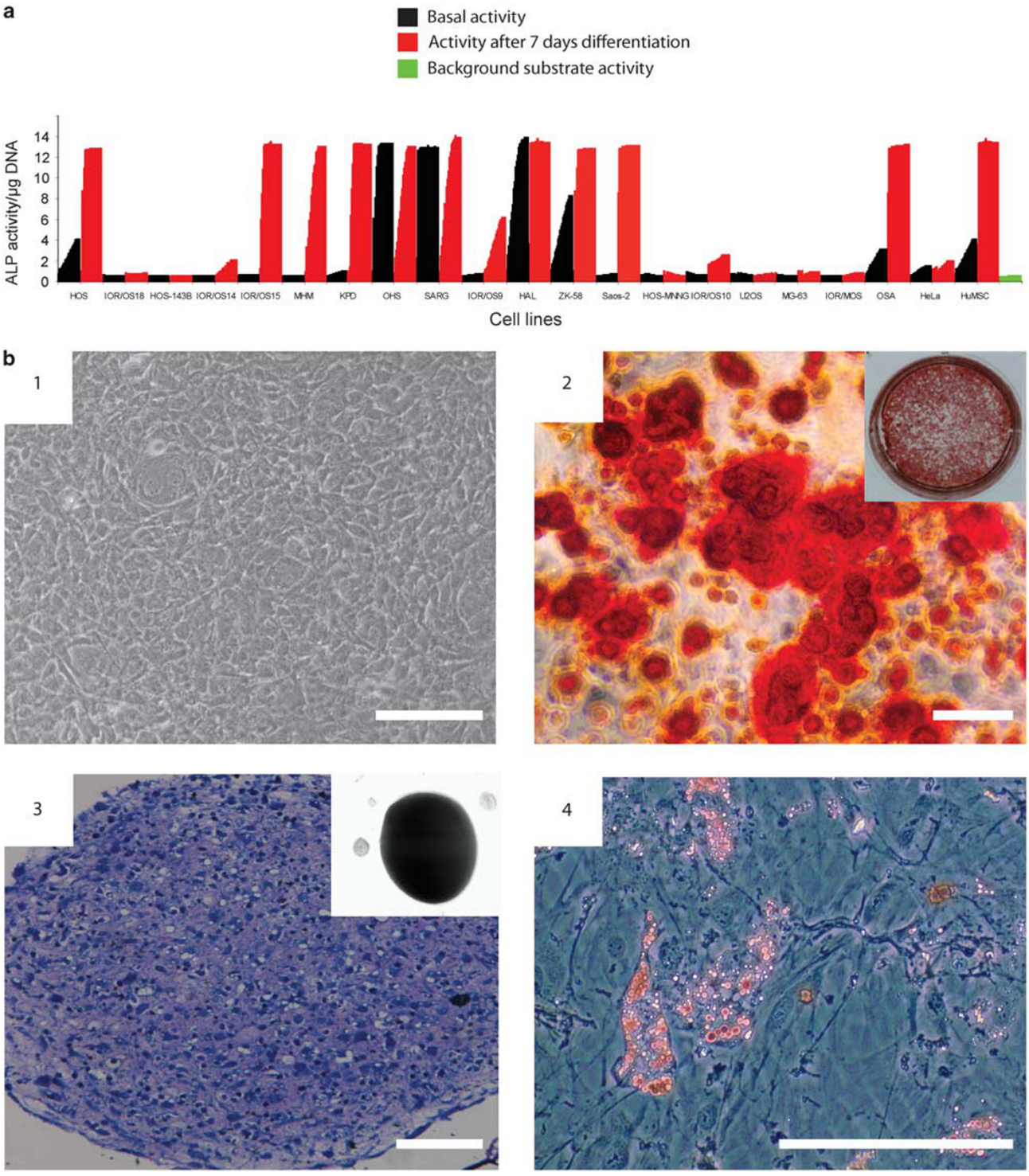
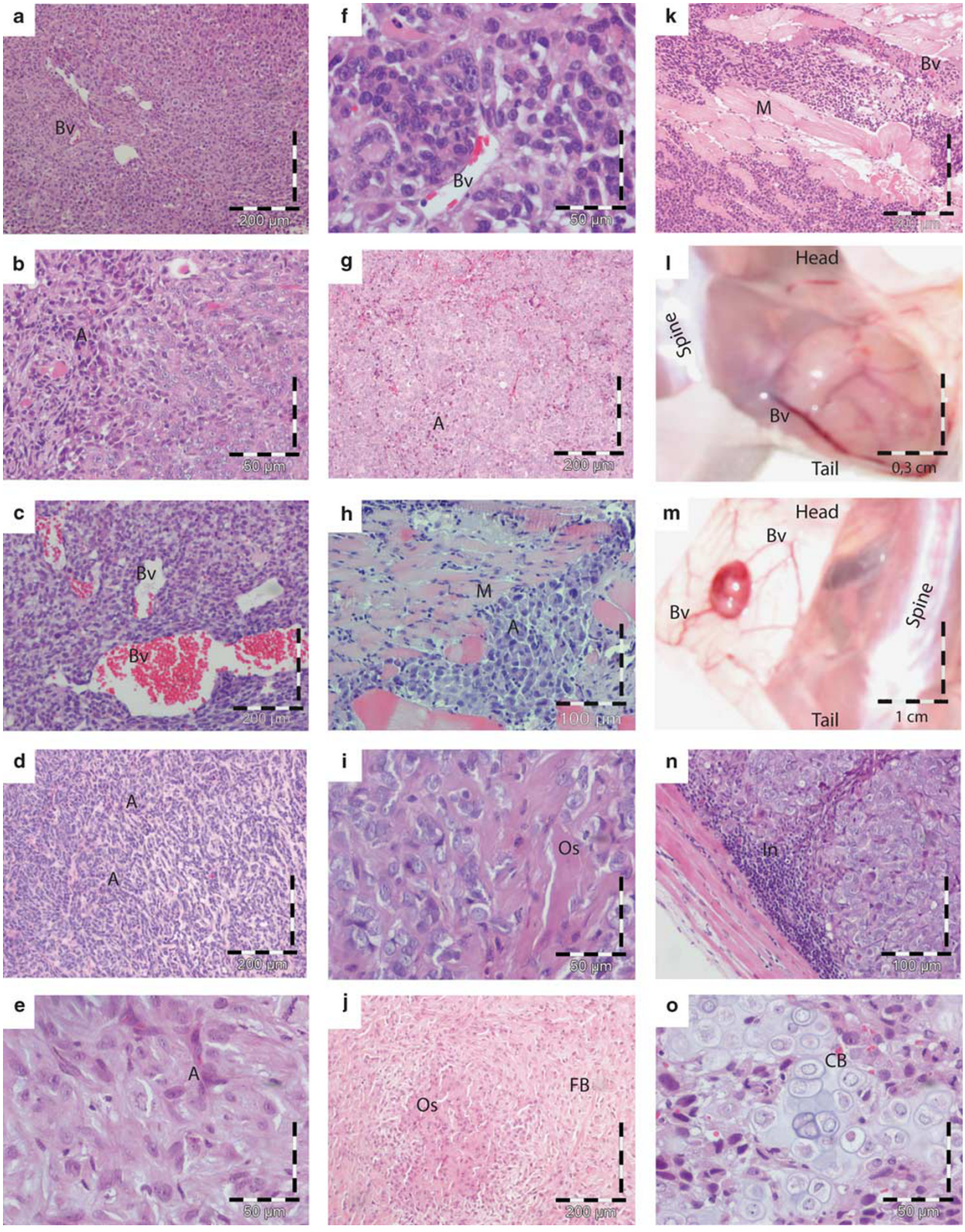


Figure 1 Functional characterization of the cells by multi-lineage differentiation assays. (**a**) The quantitative alkaline phosphatase (ALP) activity per μg DNA is depicted. For each cell line, the activity was measured before, to assay basal activity, and after 7 days of differentiation. Enzyme activity was recorded during 3 h, in general showing increase at the initial phase after which saturation and stable activity followed. HeLa and a primary human mesenchymal stem cell culture (HuMSC) were used as positive and negative controls, respectively. On the basis of the ALP activity, cell lines can be divided into three groups: (1) cells that do not show basal activity before differentiation and which does not increase after 7 days of differentiation; (2) cells that do not show basal activity, but this does increase significantly after differentiation and (3) cells that show high ALP activity before and after differentiation. (**b**) Representative pictures of cell cultures showing the morphology of IOR/OS18 cells at confluence before differentiation (1), mineral staining of HOS cells by Alizarin Red S (2), a paraffin section of MG-63 pellets induced to differentiate towards chondrocytes and stained by toluidine blue (3) and adipocytes, differentiated from HOS-MNNG cells, containing lipid droplets stained by Oil Red O (4). Bars represent 100 μm, and in the insets, the wells from the same samples are depicted.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HOS-143B: A Metastasizing Cell Line

After autopsy of the mouse injected subcutaneously and intramuscularly with HOS-143B cells, multiple lung metastases were detected. Subsequently, five mice were injected only subcutaneously (at two locations making a total of 10 injections) and an additional 10 mice only intramuscularly, of which, respectively, 3 and 10 again rapidly (3–6 weeks after detection of the primary tumor) developed lung metastases. Moreover in one animal, which received an intramuscular injection of 2×10^6 HOS-143B cells, brain metastases were detected (Figure 3). Detailed examination of the mice and the tumors confirmed that the metastases were not caused by accidentally intravenous injections. Furthermore, a piece of one of the tumors was xenografted into another nude mouse; this process was repeated three times, that is, passaged three times *in vivo*, after which the tumor was still metastatic. The xenografts and the metastases were shown to have high expression of the p53 protein in line with the previously detected p53 mutation in the originating HOS-143B cell line.[18] Here p53 mutation analysis showed that this *p.Arg156Pro* mutation was also present in the metastases.

DISCUSSION

Osteosarcoma is a malignant disease of the childhood and improvements in the survival rates have reached a plateau phase. This is partly due to a lack of good understanding of the biology of this complex tumor given its rareness and the high genetic heterogeneity and instability at diagnosis. To overcome this problem, the establishment of novel osteosarcoma models and a good characterization of the existing models is essential. Tumor-derived cell lines could be used as excellent *in vitro* and *in vivo* models as long as they are representative of the original tumor. For this we assayed here all, genetically characterized, osteosarcoma cell lines to identify the ones most representative for clinical osteosarcoma that could be used to establish valid *in vitro* and *in vivo* models.

Although it has been reported that cell lines represent most of their parental tumors characteristics, they are questioned because of the additional (*in vitro* obtained) genetic alterations[8,14] and to what extent the cell lines are equal to the original tumors. Here, we show that a number of osteosarcoma cell lines can produce growing and in one case even metastasizing tumors under technically feasible circumstances. We hypothesize that after years of cultivation, the cultures become more homogeneous based on clonal selection, slowly resulting into more stabilized genetics, and in some cases resuming in the loss of tumorigenic potential. The main aim of this study was to identify cell lines that are both known to be genetically stable providing useable models and still able of producing tumors representative of human osteosarcoma.

Eight cell lines were identified, which gave rise to tumors after injection into immune-deficient mice. Given their histology and osteoid production, the tumors were shown to be representative of osteosarcoma. Furthermore, a spectrum of different important features of tumorigenesis was assessed in the xenografts, identifying proper cell lines to model immune attraction (U2OS), angiogenesis (IOR/OS-14 and HOS-143B), invasion to adjacent tissues (MHM), *in vivo* differentiation (IOR/OS9) and metastasis (HOS-143B). Here we focused on HOS-143B as metastatic osteosarcoma models are exceptional,[36] and a metastatic human-derived osteosarcoma cell line could model the metastasizing process of osteosarcoma, which is the main clinical issue for the patient. This cell line was already reported to be metastatic in an orthotopic model;[37] however, its rapid metastasizing potential in a technically more manageable setting, that is, subcutaneous injection as shown here, is new. Moreover, even after transplanting a piece of the primary xenograft into a new recipient and repeating this up to three times, still metastases rapidly could be detected in the lungs of the animals. This indicates metastatic potential and excludes that the lung metastases are a result of migrating cells after injection of a loose cell mass into the mice. The availability of HOS-143B non-tumorigenic parental cell line HOS and its tumorigenic, but non-metastatic progeny cell line HOS-MNNG,[38] makes these three cell lines excellent models to comparatively study osteosarcoma progression and metastasis, respectively. For example, the *TP53* mutation found in all three cell lines might suggest that p53 has a role in tumor initiation, but not that much in progression and metastasis, and that additional events (HOS-MNNG was generated by the chemical agent *N*-methyl-*N*′-nitro-*N*-nitroguanidineare and HOS-134B via a Ki-ras oncogene transformation) are needed. This might explain the lack of association between *TP53* mutation and metastasis or outcome in osteosarcoma patients.[39,40]

Figure 2 *In vivo* characterization of the cell lines. Hematoxylin and eosin (H&E)-stained sections of the tumors resulting from subcutaneous and intramuscular injections of cell lines into nude mice are shown. (**a**–**h**) Primary xenografts of the eight tumorigenic cell lines: HOS-143B (**a**), HOS-MNNG (**b**), OSA (**c**), U2OS (**d**), IOR/OS9 (**e**), IOR/OS14 (**f**), OHS (**g**) and MHM (**h**) are represented, respectively. (**i**) A section of the tumor produced by U2OS cells (one of the oldest human-derived cell lines), which shows abundant osteoid (Os) production. (**j**, **o**) Two sections from a single IOR/OS9 originating tumor, which are next to the osteoblastic areas (**e**) illustrate fibroblastic and chondroblastic differentiation, respectively, showing *in vivo* differentiation of the cells. (**k**) After subcutaneous injection of and tumor formation by IOR/OS14 cells, this picture shows aggressive invasion of the tumor cells into the adjacent tissues. (**l**, **m**) Macroscopical pictures of intramuscular (HOS-MNNG) and subcutaneous (OHS) tumors, respectively. Please note the formation of big blood vessels towards the tumors indicating angiogenesis. In (**m**), the skin of the mouse is cut and flipped open to the side, which shows that the tumor is isolated in the subcutane and that injection did not accidentally penetrate the underlying tissues. (**n**) Picture is showing a part of the U2OS tumor where repeatedly infiltrating native immune cells were identified. Abbreviations: osteoid (Os), atypical tumor cells (**a**), blood vessels indicating neo-vascularization (Bv), muscle (M), infiltrating cells (In), fibroblastic matrix (FB) and chondroblastic matrix (CB).

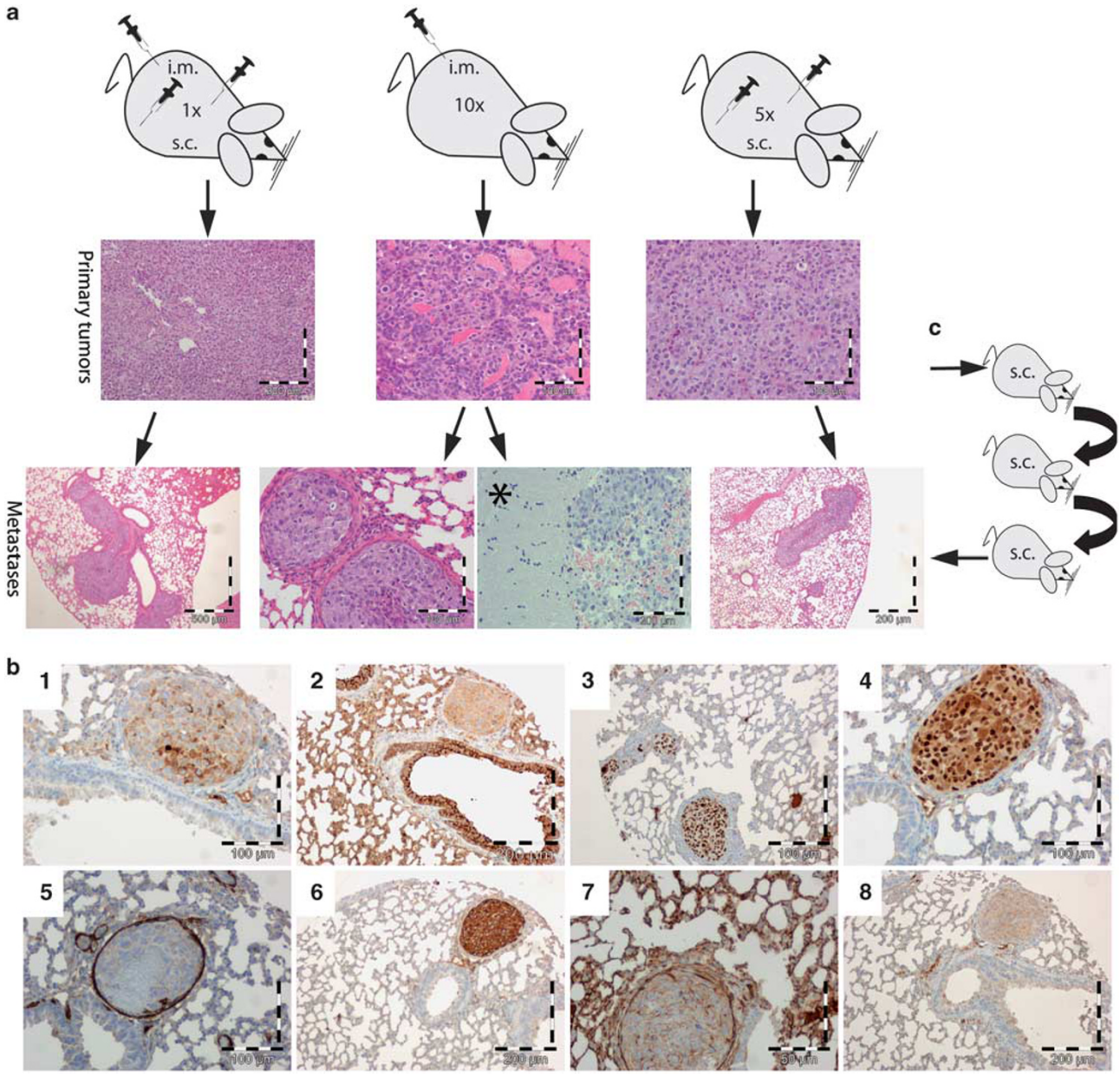

Figure 3 Metastatic HOS-143B cell line. (**a**) After subcutaneous (s.c.) and intramuscular (i.m.) injection of HOS-143B cells into one nude mouse, lung metastases were detected. To determine whether i.m. or s.c. tumors or both were metastatic, respectively, 10 and 5 animals were solely i.m. and solely s.m. injected. The primary tumors after these injections in the animals again led to the formation of multiple lung metastases and in one case a brain metastasis was found as indicated by *. (**b**) Sections 1–8 show representative images of the immunohistochemical staining of HOS-143B lung metastases. Pictures, respectively, depict: osteonectin, ezrin, Ki-67, p53, smooth muscle actin, vimentin, caveolin 1 and CD99. (**c**) To confirm that the HOS-143B-derived tumors are actively metastasizing after the primary tumor is formed and that lung and brain metastases are not only a result of migrating cells just after injection, a small piece of one of the tumors was subcutaneously transplanted into a new recipient mouse. After the tumor again grew up to 1 cm, the procedure was repeated. Even after three subsequent repetitions, metastases were still detected in the lungs of the animal.

The system used here, subcutaneous and intramuscular injections of human osteosarcoma cell lines into immune-deficient mice, did not result in tumor formation in 11 out of 19 cell lines. A second attempt of injecting these 11 cell lines in new recipient mice confirmed their inability of generating tumors under these circumstances. This might indicate that these cell lines have lost their tumorigenicity because of long-term selection for other characteristics important to survive the *in vitro* culturing conditions or that the lines are originating from other cell populations in the initial heterogeneous cell culture than the osteosarcoma cells. Alternatively, this might reflect the dissimilar microenvironment used here (under the skin and inside the muscle) compared with the intramedullar locations where mostly

Table 3 Immunohistochemical analysis of primary the xenografts and metastases

Antibodies				Primary xenografts								Metastases			
Protein	Company	Dilution	AR	HOS-143B	HOS-MNNG	OSA	U2OS	IOR/OS9	IOR/OS14	OHS	MHM	1	2	3	4
OSN	Novocastra	1:50	Citrate	+++	+++	+++	++	+++	++	+++	+++	+++	+++	+++	+++
VIM	Novocastra	1:200	Citrate	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
PLAP	Dako	1:10	Citrate	+	+	+	−	+	−	−	+	−	++	+	+
CK	Dako	1:50	Citrate	+++	+++	−	−	−	+++	−	+++	++	+++	+++	+++
EMA	Dako	1:200	Citrate	−	−	−	−	−	−	−	++	+	+	+	−
CD34	Novocastra	1:50	Citrate	−	−	−	−	−	−	−	−	−	−	−	−
SMA	Novocastra	1:200	Citrate	−	−	−	−	+++	−	−	−	−	−	−	−
DES	Dako	1:100	Citrate	−	−	−	−	−	−	−	−	−	−	−	−
CD 99	Dako	1:50	Citrate	++	++	+	−	++	++	+	+++	−	++	+++	+++
BCL-2	Dako	1:50	Citrate	−	−	−	−	−	+	+	−	−	++	−	−
KIT	Dako	1:50	Citrate	−	−	−	−	−	−	−	−	−	−	−	−
CAV1	Santa Cruz	1:200	Citrate	++	+++	++	++	+	+++	−	+++	+++	+++	+++	+++
Ki67	Dako	1:50	Citrate	+++	+++	+++	++	+	+++	+++	+++	+++	+++	+++	+++
P53	Novocastra	1:50	Citrate	+++	+++	+	−	−	+++	+++	+++	+++	+++	+++	+++
P16	Santa Cruz	1:100	Citrate	−	−	+++	−	−	−	+++	−	−	−	−	−
P21	Dako	1:25	Citrate	+++	+	++	+	+++	++	++	++	++	++	++	++
EGFR	Dako	1:100	EDTA	−	−	−	−	−	−	−	+	−	+	+	+
HER2	Novocastra	1:50	Citrate	−	−	−	−	−	−	−	−	−	−	−	−
TPD52	Australian	1:100	Citrate	−	−	−	++	−	−	+++	+	−	−	+	−
S100	Dako	1:200	Citrate	−	−	−	−	++	−	−	−	−	−	−	−
SOX-9	Santa Cruz	1:100	Citrate	−	−	−	−	−	−	−	−	−	−	−	−
EZR	Santa Cruz	1:250	Citrate	++	+++	+++	+	++	++	+++	+++	++	+++	+++	+++
E-CAD	Novocastra	1:20	Citrate	−	−	−	−	−	−	−	+	−	+	+	+
CD31	Dako	1:20	Citrate	−	−	−	−	−	−	−	−	−	−	−	−

−, negative; +, weakly positive; ++, moderate positive; +++, strongly positive.

The first four columns are depicting the antibodies' characteristics and the immunohistochemical staining conditions. Furthermore, the staining patterns of the xenografts and four HOS-143B metastases are shown.

osteosarcoma is found, indicating that we cannot exclude the tumorigenicity of these cell lines by other techniques as orthotopic injections. Therefore, this study was especially useful for selecting cell lines that are tumorigenic under simplified conditions to identify technically practical models. Moreover, the limited *in vivo* lineage-specific differentiation of the cell lines underlines the importance of (stromal or micro-environmental) stimulation for this process as most cell lines do differentiate *in vitro*.

Recently, the use of cell lines has been questioned again as reports show high incidence of cross-contaminations between cell lines with all disastrous consequences,[41] for which the ATCC has developed a *cell line identification standard*.[42] One advantage of the cell lines series used here is that they all were previously characterized at genetic level,[18] thereby allowing for checking cell identities and excluding cross-contaminations. In this study, all cell lines were genotyped at the end of the experiments to match with their initial profiles and all xenografts were genotyped to match with the profiles of the originating cell lines. From our extensive experience, especially with these highly proliferative cells, and the ongoing debate in literature, we strongly recommend regular-based DNA profiling of cell lines. Any cell line, as long as it is in culture, should regularly undergo quality checks to confirm its identity and pathogen-free state, as cross-contamination with more aggressive cell lines, like HeLa and HOS, and animal cell lines can happen even in best hands.

For the past 30 years, study of osteosarcoma has widened our knowledge about this aggressive malignancy. The high genetic instability of the primary tumor, the rareness of the

disease and poor access to primary patient material due to intensive treatment regimens hamper biological studies. Therefore, multiple representative models are needed to get more insight into different processes involving osteosarcoma initiation, progression and treatment. Next to novel models that could shed light to osteosarcoma initiation, we hypothesized that after extensive biological and genetical characterization, osteosarcoma cell lines could provide good models to study osteosarcoma progression and treatment. We were able to show (multi-lineage) differentiation capacity of nearly all osteosarcoma cell lines. This might indicate the stemness of these tumors providing more knowledge about their origin and its useful information to investigate the role of differentiation in tumorigenesis. After identifying tumorigenic cell lines under simplified conditions, we could pinpoint a number of cell lines that could be used as models for specific research questions.

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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