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ORIGINAL ARTICLE

An orthotopic mouse model for chondrosarcoma of bone provides an in vivo tool for drug testing

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Abstract Chondrosarcoma is a malignant cartilaginous tumor of the bone. Recently, mutations in *isocitrate dehydrogenase-1* (*IDH1*) and *isocitrate dehydrogenase-2* (*IDH2*) were identified in central chondrosarcomas. As chondrosarcomas are notoriously resistant to conventional treatment modalities, the need for model systems to screen new treatment options is high. We used two chondrosarcoma cell lines (CH2879 and SW1353) to generate a bioluminescent orthotopic chondrosarcoma mouse model. Cell lines were stably transduced with a lentiviral luciferase expression vector, and after clonal selection, luciferase-expressing clones were subcutaneously and orthotopically implanted in nude mice. Mice injected with CH2879 cells were treated with doxorubicin over a period of 6 weeks. Both cell lines resulted in tumor growth. CH2879 tumors were consistently larger than SW1353 tumors. No difference in size could be observed between subcutaneous and orthotopic tumors. Tumor growth could be monitored over time through assessment of luciferase activity, without harming the mice. Using this model, we show that doxorubicin does not have a significant effect on in vivo tumor growth. We describe an orthotopic chondrosarcoma mouse model that can be used to test new treatment strategies evolving from in vitro research.

Keywords Chondrosarcoma · Orthotopic mouse model · Luciferase · IDH · Chemoresistance

Introduction

Chondrosarcoma is a malignant cartilaginous tumor, predominantly affecting adults. Several subtypes with different clinicopathological features are recognized, of which conventional central chondrosarcoma is the most common (~90 %). Chondrosarcoma is characterized by the deposition of cartilaginous matrix, which is abundant in low-grade tumors and becomes more myxoid in high-grade chondrosarcomas. High-grade chondrosarcomas also show increased cellularity, associated with increased metastatic behavior [1]. The current therapy strategy for chondrosarcoma is surgical resection. However, due to intrinsic resistance to conventional chemo- and radiotherapy, there is nothing to offer patients with inoperable tumors and metastatic disease [2].

The past decade has brought major advances in the field of gene abnormalities in cartilaginous neoplasms, which has allowed identification of potential targets for therapy. Osteochondroma is a cartilage-capped bony lesion at the surface of the bone, which can progress to secondary peripheral chondrosarcoma. *EXT1* or *-2* alterations have been identified in osteochondromas [3, 4]. Conventional central chondrosarcomas are located in the medulla of bone and a small subset arises in enchondroma, a benign precursor lesion. Recently, *isocitrate dehydrogenase-1* (*IDH1*) or *isocitrate dehydrogenase-2* (*IDH2*) mutations were identified in enchondroma and conventional central chondrosarcoma as well as in dedifferentiated chondrosarcoma [5–7]. Dedifferentiated chondrosarcoma is a high-grade anaplastic

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sarcoma juxtaposed to a conventional chondrosarcoma, which has a poor outcome. The advances in the field also include an expansion in the number of human chondrosarcoma cell lines. At the moment of writing, there is a total of eight conventional chondrosarcoma cell lines [8–13] and four dedifferentiated chondrosarcoma cell lines [8, 13, 14]. Five of these cell lines show mutations in *IDH1* (n=3) or *IDH2* (n=2) [7, 8]. No cell lines derived from peripheral cartilaginous tumors with *EXT1* or *-2* mutations exist. For advancing chondrosarcoma research and to rapidly translate results from basic research to clinical practice, reliable and representative animal models are necessary.

Most current models are based on the subcutaneous xenografting of chondrosarcoma cell lines or human tumor tissue, misrepresenting the natural niche of the tumor in the bone [15]. A swarm rat chondrosarcoma model exists, derived from a tumor tissue line originally isolated from a spontaneously arising tumor in a Sprague-Dawley rat, which has given rise to several different lines of differentiation, each showing unique cytogenetic profiles and tumorigenic properties in vivo [16]. In 2010, an orthotopic chondrosarcoma mouse model derived from the JJ012 cell line was published. Cells were injected in matrigel, and 2/4 intratibial tumors showed spontaneous metastasis formation [17]. Transgenic models for chondrosarcoma have not been developed yet. Mouse models with *ext1* or *-2* mutations show formation of exostoses, but no progression to chondrosarcoma [18–20]. Similarly, transgenic mice carrying *idh1* mutations in neural progenitor cells show a defect in collagen maturation, but no chondrosarcoma formation [21]. Recently, transplantation of a new chondrosarcoma cell line (CH56) with an *IDH2* mutation and *MDM2* amplification led to successful engraftment in paratibial muscle and tumor growth representing a conventional central chondrosarcoma [13].

We here show successful tumor engraftment of luciferase-expressing chondrosarcoma cell lines in the tibia of mice, enabling in vivo monitoring of tumor growth. Using CH2879, we were able to confirm the resistance to doxorubicin. Thus, our model can be used for pre-clinical therapy testing.

Methods

Cell lines

The chondrosarcoma cell lines CH2879 and SW1353 were cultured in RPMI 1640 medium (Invitrogen) supplemented with 10 % heat-inactivated fetal bovine serum (Lonza, Breda, the Netherlands), 1 % glutaMAX (Invitrogen, Bleiswijk, the Netherlands), and 50 U/mL penicillin with 50 μg/mL streptomycin (MP Biomedicals, Eindhoven, the Netherlands). CH2879 is known to be wild type for *IDH1* and *2* and *TP53*, while SW1353 contains a mutation in *IDH2* (R172S), *TP53* (V203L), and *NRAS* (Q61K) [7, 22]. Hek293t cells were cultured in DMEM (Invitrogen, Bleiswijk, the Netherlands) supplemented with 10 % heat-inactivated fetal bovine serum (Lonza) and 100 U/mL penicillin with 100 μg/mL streptomycin.

Production of lentiviral particles in Hek293t cells

The self-inactivating lentiviral vector, pLV.CMV.luc.bc.PURO, and "helper" vectors, pMD 2\VSV-G, pMD L\pRRE, and pRSV-rev, were kindly provided by Dr. Eric Kaijzel (LUMC). Briefly, the lentiviral vector together with the three helper vectors was cotransfected overnight into Hek293t cells using Lipofectamine 2000 (Invitrogen, Bleiswijk, the Netherlands) in OptiMem (Invitrogen, Bleiswijk, the Netherlands) after which medium was replaced by fresh culture medium. Viral supernatants were harvested 48 h after transfection, filtered through a 0.45-μm filter, and stored at −80 °C until transduction in chondrosarcoma cell lines.

Generation of clonal luciferase expressing chondrosarcoma cell lines

The chondrosarcoma cells were transduced with the lentiviral supernatant in the presence of 1 μg/mL dextran (Sigma-Aldrich, Zwijndrecht, the Netherlands) for 4 h. After transduction, cells were selected using 2 μg/mL puromycin (Sigma-Aldrich, Zwijndrecht, the Netherlands). Following antibiotic selection, single-cell-derived cultures were obtained using limited dilution and screened for luciferase activity. As estrogen signaling was shown to be active in cartilaginous tumors [23], clones were selected using TaqMan gene expression arrays for *ESR1*, *CYP19A1*, and *AR* according to manufacturer's protocol (Applied Biosystems, Bleiswijk, the Netherlands). All cultures selected for in vivo implantation were tested for the presence of HIV p24.

Animals

All procedures were approved by the Leiden University animal experimental committee and Local Government (Animal protocols 08158 and 10019), performed in accordance with the national legislation of the Netherlands and in compliance with the "Code of Practice Use of Laboratory Animals in Cancer Research" (Inspectie W&V, July 1999). Athymic mice (BALB/c *nu*/*nu* 6 weeks old) were acquired from Charles River (Charles River, L'Arbresle, France), housed in individually ventilated cages, and food and water was provided ad libitum. For all in vivo experiments, a total of 45 mice were used.

CH2879 and SW1353 tumor cell injection in mice

For both CH2879 and SW1353 cell lines, three single-cell-derived luciferase-expressing clones were used for both subcutaneous and orthotopic implantation into mice. Twelve mice were subcutaneously injected, four with the different CH2879 clones, four with the different SW1353 clones, and four with non-transduced cell lines to control for interference with tumorigenicity by the luciferase construct.

Orthotopic injection of SW1353 or CH2879 was performed in 33 mice using two different methods. Regardless of the injection method, mice were anesthetized by isoflurane prior to subcutaneous or orthotopic injection in the tibia with luciferase-expressing cells (1×10^6 cells in 40 μL PBS or 2.5×10^5 cells in 10 μL PBS, respectively). Eighteen mice (nine with SW1353 LUC clones and nine with CH2879 clones, three mice per clone) were injected with a single-cell suspension of luc+cells into the right tibiae as described previously [24]. In brief, two small holes (~0.35 mm each) 4–5 mm apart were created in the bone cortex of the upper right tibiae using a dental drill, and reservoir for the cells was created by flushing out the bone marrow from the proximal end of the shaft. After inoculation with a 30-gauge needle through the lower hole, the skin wound was sutured. Alternatively, 15 mice were injected orthotopically with a single-cell suspension of CH2879 LUC 10 cells directly into the tibia, without prior creation of a reservoir (Sup Fig. S1).

The progression of cancer cell growth was monitored weekly by optical imaging. After the experimental period or 6 weeks after start of signal detection for mice not on treatment regime, the animals were sacrificed and tumors were collected for histological assessment.

In vivo treatment of CH2879 orthotopic tumors

The 15 mice injected immediately without prior creation of a reservoir were divided into two groups, in order to investigate the effect of doxorubicin on tumor growth. Seven mice were treated with 12 mg/kg doxorubicin (obtained from the in-house hospital pharmacy in a 0.9 % NaCl solution) during 6 weeks as a single intraperitoneal (i.p.) dose every 2 weeks, while the control group of eight mice was monitored for 6 weeks. Treatment was started as soon as tumors were detected using the IVIS 100 (Caliper LifeSciences, Hopkinton, MA), i.e., bioluminescent (BLI) signals of 10^5 P/s/cm^2 (~0.6 cm^3). Mice with a bioluminescent signal $\geq10^9$ P/s/cm^2 (~1 cm^3) were considered to have too severe tumor burden and were sacrificed. Mice were sacrificed when treatment was completed or when they showed severe clinical signs as a result of tumor burden or treatment. Tibiae were collected for histological assessment. Lungs were harvested to investigate possible metastases.

In vivo imaging

To monitor luciferase activity, mice were anesthetized using isoflurane. Images were acquired 5 min after i.p. injection of D-luciferin (150 mg/kg) using 30-s exposure time. Tumor take was monitored using the IVIS 100 (Caliper LifeSciences, Hopkinton, MA), and bioluminescent signals were quantified using Living Image 3.0 (Caliper LifeSciences, Hopkinton, MA).

Tissue embedding and staining

Lungs were fixed in 4 % paraformaldehyde and embedded in paraffin. Tibiae were decalcified in 0.4 M EDTA/PBS after fixing in 4 % paraformaldehyde. After decalcification with EDTA, tibiae were embedded in paraffin and 5-μm sections were stained with hematoxylin and eosin (H&E) for morphology or 0.08 % toluidine blue (Brocacef Holding, Maarssen, The Netherlands) to assess matrix formation.

Results

Transduction of CH2879 and SW1353 cell lines

Based upon expression of estrogen signaling markers (results not shown), three representative clones were selected for each cell line. SW1353 clones 1, 4, and 8 most closely resembled the non-transduced cell line while for CH2879 this was the case for clones 4, 7, and 10. All clones showed luciferase expression and were negative for HIV p24.

Tumor growth after injection of luciferase-transduced SW1353 and CH2879 chondrosarcoma cell lines

Six-week-old Balb/C nude mice were injected with either SW1353 or CH2879. Tumor growth was observed at 1 week through emission of a bioluminescent signal by both subcutaneous (Fig. 1a) and orthotopic (Fig. 1b–d) grafts. Subcutaneous injection of SW1353 clones 1 and 4 resulted in tumors in 4/4 mice and of clone 8 in 1/4 mice. Orthotopic injection of SW1353 clones resulted in tumor growth in all nine mice. CH2879 clones 4 and 7 resulted in tumor growth in 3/4 mice and of clone 4 in 4/4 mice, while orthotopic injection of CH2879 clones resulted in tumor growth in all nine mice. No BLI signals were observed in the lungs and histological examination also provided no evidence of metastases.

BLI signals of CH2879 tumors continued to increase during the course of 7 weeks, indicative of progressive tumor growth, whereas signal strength of SW1353-derived tumors did not increase suggesting growth arrest. CH2879-derived tumors were consistently larger than SW1353-derived tumors, as indicated by stronger BLI signals (Fig. 1a, b). Orthotopic

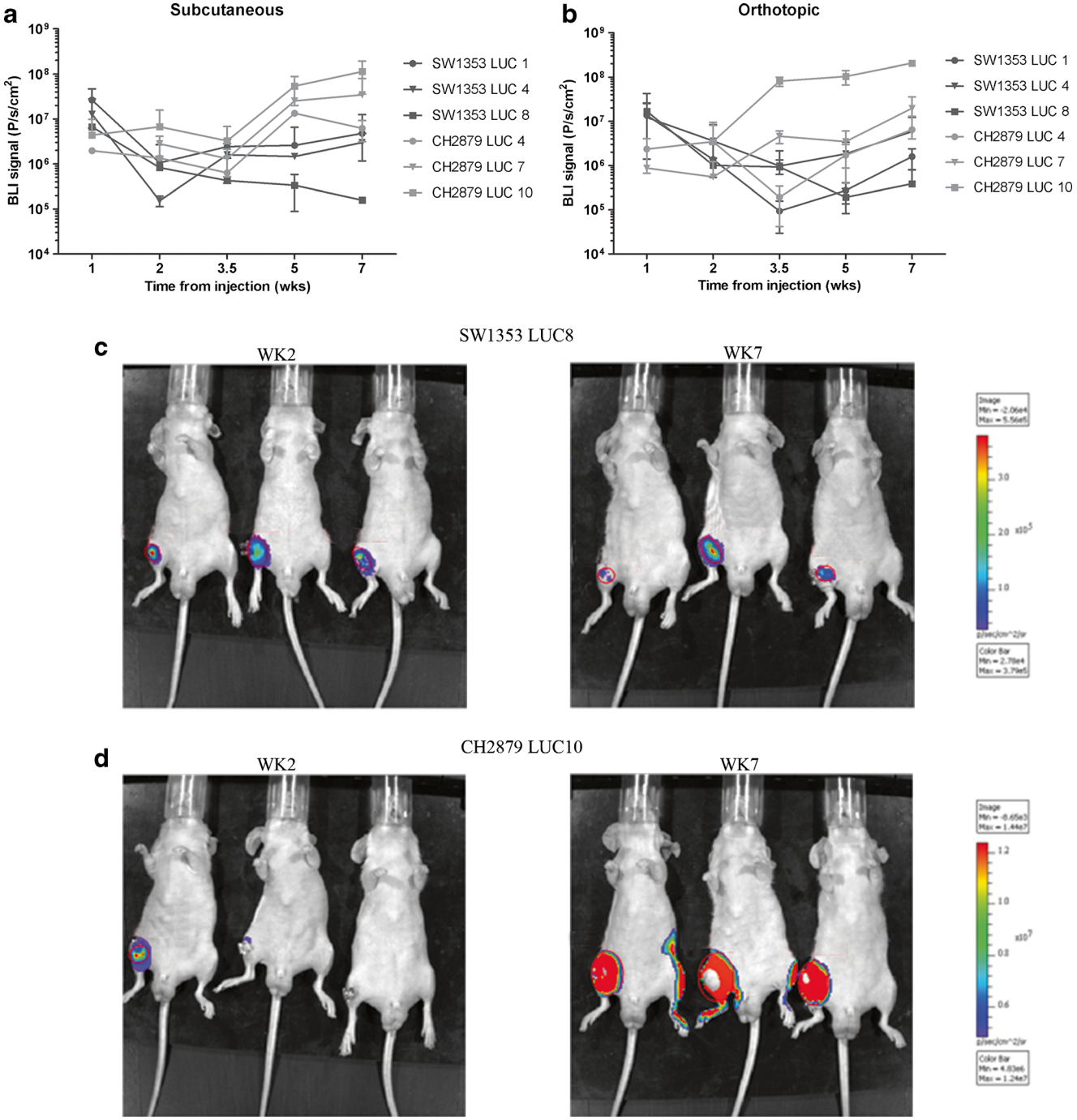

Fig. 1 Generation of chondrosarcoma mouse model. **a** Subcutaneous injection of SW1353 and CH2879 cells led to tumor growth in 4/4 mice. Standard deviation indicates variation in measurements between the 4 injected mice. **b** Orthotopic injection of SW1353 and CH2879 cells led to tumor growth in 3/3 mice, CH2879 clone luc 10 resulted in larger tumors than other clones. Standard deviation indicates variation in measurements between the 4 injected mice. **c** Each SW1353 clone was injected orthotopically in the left tibia of 3 mice. SW1353 clone 8 is shown with BLI signals at week 2 (*left panel*) and week 7 (*central panel*) with luminescence scale (*right panel*). Luciferase signals for this clone did not show an increase during the 7-week observation time. Standard deviation indicates variation in measurements between the 9 injected mice. **d** Each CH2879 clone was injected orthotopically in the left tibia of 3 mice. CH2879 clone LUC 10 is shown with BLI signals at week 2 (*left panel*) and week 7 (*central panel*) with luminescence scale (*right panel*). This clone showed the strongest increase in luciferase signals during the 7-week observation time, as evidenced by the strong *red signal*. Standard deviation indicates variation in measurements between the 9 injected mice

and subcutaneous SW1353 and CH2879 grafts emitted comparable BLI signals. CH2879 LUC 10 showed the strongest BLI signal (Fig. 1b, d) and was selected for further experiments. Orthotopic grafts after creation of a cell reservoir

resulted in tumor growth within 1 to 2 weeks, while immediate injection of cells resulted in a detectable BLI signal only after 4 weeks. However, as tumor growth was obtained after immediate orthotopic injection and is also considered less painful, we used this approach in subsequent experiments.

CH2879 tumors resemble high grade chondrosarcoma

Orthotopic CH2879 LUC 10 cell grafts showed diaphyseal localization of tumor cells, which grew circumferentially and expanded through the cortex (Fig. 2a, e). Histologically, tumor

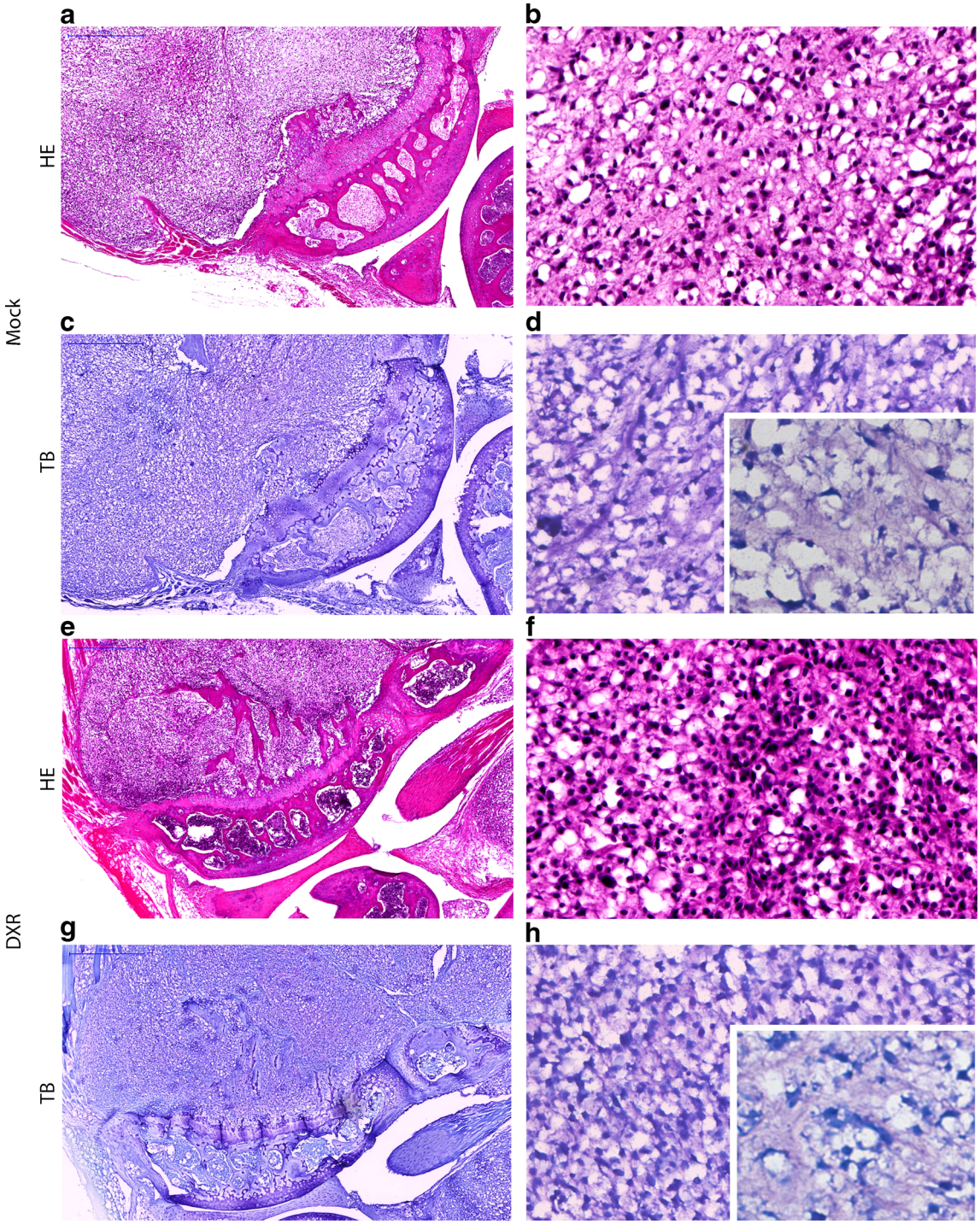

Fig. 2 Orthotopic CH2879 tumor resemble chondrosarcoma morphology with matrix deposition. Control mouse (*Mock* **a**–**d**) and mouse treated with doxorubicin (*DXR*, **e**–**h**) using H&E staining (**a**, **b**, **e** , **f**) and toluidine blue staining (**c** ,**d**, **g**, **h**) showing *purple coloration* where proteoglycans are produced

cells resembled high-grade chondrosarcoma (Fig. 2b, d, f, h). Tumor cells filled the entire tibial medullary cavity and displaced the endosteum (Sup Fig. S2). Large tumors showed either total absorption of the tibial bone (Sup Fig. S2 A, Ai) or focal perforation of the cortex (Sup Fig. S2 B, Bi). Tumors were located close to but did not infiltrate the growth plate (Fig. 2a, c, e, g). Toluidine blue staining confirmed the deposition of proteoglycans (Fig. 2c, d, g, h).

Doxorubicin treatment does not influence chondrosarcoma tumor growth

Mice orthotopically injected with CH2879 LUC 10 cells were treated with doxorubicin 12 mg/kg for 6 weeks once tumors reached ~0.6 cm^3. Mean BLI starting values for mice on doxorubicin (n=7) were 8.4×10^6 P/s/cm^2 ($\pm3.5\times10^6$) with end BLI values 1.9×10^8 P/s/cm^2 ($\pm1.5\times10^8$). For the control

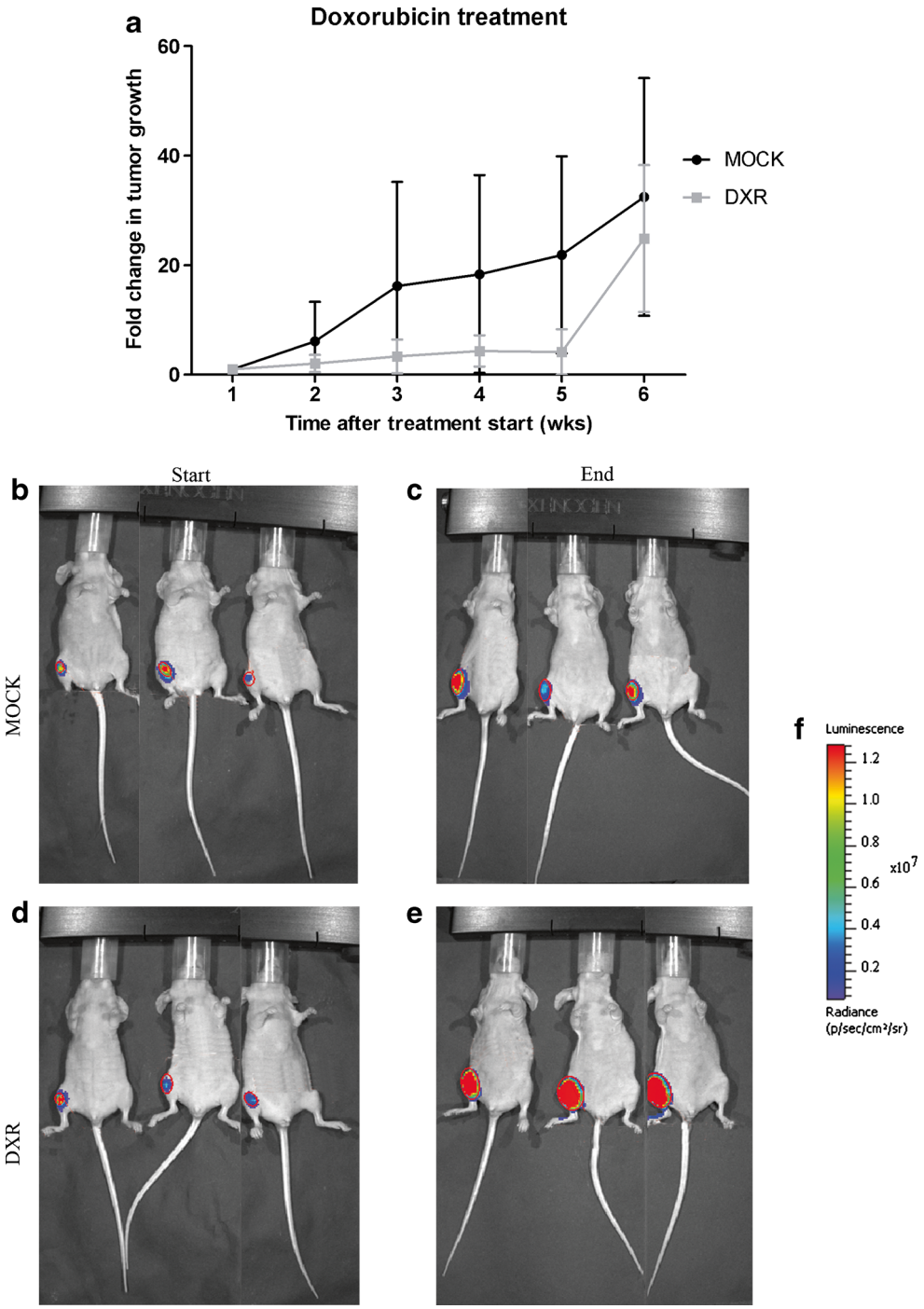

Fig. 3 Orthotopic CH2879 tumors are resistant to doxorubicin. **a** Mice were followed for bioluminescent signal indicating tumor presence from injection of tumor cells. At treatment, start fold change of tumor growth was set at 1 and change in tumor growth monitored per week. Treated mice (*DXR*) showed a short lapse in tumor growth but start to catch up with untreated (*MOCK*) mice in tumor size at week 6. Standard deviation indicated variation in measurements of 8 untreated mice and 7 doxorubicin treated mice. **b**, **c** Bioluminescent signals for 3 untreated mice at start of signal detection (**b**) and after 6 weeks (**c**). **d**, **e** Bioluminescent signals for 3 mice treated with doxorubicin at start of signal detection and doxorubicin treatment (**d**) and after 6 weeks at completion of treatment cycle (**e**). **f** Luminescence scale indicating the strength of BLI signals (in p/s/cm^2/sr), based on the premise that a larger tumor will emit more luciferase and therefore a stronger signal will be detected, represented in the *red spectrum* on the scale. The increase in tumor size from **b**, **c** to **d**, **e** in control and doxorubicin treated mice is observed by the increase in *red signal*

group ($n=8$), starting BLI values were 4.1×10^6 P/s/cm^2 ($\pm5.3\times10^6$) with end BLI values 5.9×10^7 P/s/cm^2 ($\pm2.1\times10^7$). Mice receiving doxorubicin showed slower tumor growth than control mice (Fig. 3a) but during the last cycle of doxorubicin tumor growth strongly increased (Fig. 3a), suggesting resistance to doxorubicin. Figure 3b–e shows BLI intensities at the start (Fig. 3b) and the end of treatment (Fig. 3c) as well as BLI intensities of three mice treated with doxorubicin at the start of treatment (Fig. 3d) and at the end of treatment (Fig. 3e) along with the luminescence scale (Fig. 3f). Tumors in the doxorubicin-treated group showed stronger BLI intensity at the end of 6 weeks of treatment but also at the start, and the calculated fold change (Fig. 3a) indicates that the relative signal increase was similar. Statistical analysis of both BLI signals and fold change shows that doxorubicin treatment did not significantly influence tumor growth (two-tailed independent t test for fold change $p=0.143$; for BLI signals $p=0.2$). Mouse weight was stable during the entire experiment.

Discussion

Chondrosarcoma is notoriously resistant to conventional chemotherapy and identification of new targets is crucial. New targets for therapy are the Bcl-2 family members, the inhibition of which renders chondrosarcoma cells chemosensitive [25–29], survival pathways including HIF1α [30–32], Src [33–35], PI3K [22, 33, 36], and the mTOR [37, 38] pathways. The presence of adequate animal models to test candidate targets for therapy before proceeding to clinical evaluation is invaluable, since for rare tumors, it is difficult to set up clinical trials.

Chondrosarcoma represents a heterogeneous group of tumors and we have shown that also central chondrosarcoma cell lines are heterogeneous [22]. In terms of gene abnormalities, subsets of chondrosarcoma contain mutations in *IDH1* or *-2*, *NRAS*, and *COL2A1* as well as in genes involved in Hh, Rb, and p53 signaling [22, 39]. Any model will therefore not reflect the full spectrum of chondrosarcoma heterogeneity.

Most published chondrosarcoma mouse models are subcutaneous [15], whereas human chondrosarcoma typically occurs either in the medulla of the bone or on the bone surface [40]. A mouse model mimicking the human situation is preferable, especially since extracellular matrix of a tumor and its blood supply purportedly play a role in chemoresistance [34]. Subcutaneous xenografted chondrosarcoma mouse models are valuable and have been used to assess targeting of the hedgehog signaling pathway in chondrosarcoma [41]. A spontaneously metastatic mouse model based upon orthotopic xenografting of JJ012 chondrosarcoma cells has been developed [17] but we were unable to grow tumors using JJ012 cells. This might be explained by the fact that we did not use matrigel when we xenografted JJ012 cells. We successfully engrafted CH2879 and SW1353 and therefore selected these for our experiments. The main advantage of our model is the possibility to closely monitor tumor growth within the bone by bioluminescence imaging, which is particularly useful when drug response is studied. We found direct injection of tumor cells in the bone to result in more efficient grafting than subcutaneous injection, presumably as the bone microenvironment more closely resembles the tumor microenvironment. Even though grafting of the cells in a hole drilled in the tibia led to earlier onset of tumor growth than immediate injection into the tibia, the latter approach was successful and furthermore considered to be less painful for the mice. Our model of direct injection of luciferase transduced cells in the tibia results in tumors, growing in the medullary cavity and producing proteoglycans similar to conventional human central chondrosarcoma and detectable by bioluminescence imaging.

Conclusions

We developed an orthotopic chondrosarcoma mouse model using luciferase-transduced human chondrosarcoma cells, which allows live imaging of tumor growth. This model might contribute to bridging the gap between pre-clinical research and clinical implementation of new therapy strategies.

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Conflict of interest The authors have no competing interests to declare.

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