

New chondrosarcoma cell lines and mouse models to study the link between chondrogenesis and chemoresistance

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Chondrosarcomas are cartilage-forming, poorly vascularized tumors. They represent the second malignant primary bone tumor of adults after osteosarcoma, but in contrast to osteosarcoma they are resistant to chemotherapy and radiotherapy, surgical excision remaining the only therapeutic option. Few cell lines and animal models are available, and the mechanisms behind their chemoresistance remain largely unknown. Our goal was to establish new cell lines and animal cancer models from human chondrosarcoma biopsies to study their chemoresistance. Between 2007 and 2012, 10 chondrosarcoma biopsies were collected and used for cell culture and transplantation into nude mice. Only one transplanted biopsy and one injected cell line has engrafted successfully leading to conventional central high-grade chondrosarcoma similar to the original biopsies. In culture, two new stable cell lines were obtained, one from a dedifferentiated and one from a grade III conventional central chondrosarcoma biopsy. Their genetic characterization revealed triploid karyotypes, mutations in *IDH1*, *IDH2*, and *TP53*, deletion in *CDKN2A* and/or *MDM2* amplification. These cell lines expressed mesenchymal membrane markers (CD44, 73, 90, 105) and were able to produce a hyaline cartilaginous matrix when cultured in chondrogenic three-dimensional (3D) pellets. Using a high-throughput quantitative RT-PCR approach, we observed that cell lines cultured in monolayer had lost expression of several genes implicated in cartilage development (*COL2A1*, *COMP*, *ACAN*) but restored their expression in 3D cultures. Chondrosarcoma cells in monolayer were sensitive to several conventional chemotherapeutic agents but became resistant to low doses of mafosfamide or doxorubicin when cultured in 3D pellets, in parallel with an altered nucleic acid accumulation of the drug. Our results indicate that the cartilaginous matrix produced by chondrosarcoma cells may impair diffusion of several drugs and thus contribute to chemoresistance. Therefore, 3D chondrogenic cell pellets constitute a more relevant model to study chondrosarcoma chemoresistance and may be a valuable alternative to animal experimentations.

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Chondrosarcomas (CSs) are malignant cartilage-forming tumors of the bone. With an estimated annual incidence of 1 in 200 000, they represent the second most common malignant primary bone tumor of adults after osteosarcoma (OS).¹ This pathology affects mostly patients between 50 and 70 years old and shows a high interpatient heterogeneity with

different histological subtypes. More than 80% of total CSs are represented by primary, conventional central CS. This tumor is characterized by a pathological hyaline cartilage formation arising within the medullar cavity and can present low-to-high grades depending on cellularity and aggressiveness.¹ In addition, osseous metaplasia and/or

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calcifications are not unusual.¹ Dedifferentiated CS is a distinct subtype, which only accounts for 10% of the reported cases. It contains a high-grade noncartilaginous sarcoma component and a low-grade well-differentiated cartilaginous tumor, with a clearly defined interface between the two components.¹ Other subtypes named secondary CS represent less than 10% of the patients and derive from benign lesions such as enchondroma (Ollier disease or Mafucci syndrome) or osteochondroma for the peripheral forms.² Gene expression profiling and other phenotypic analyses suggested that CS arise from bone multipotent mesenchymal stem cells (MSCs) driven, more or less, toward chondrogenesis.³

Until recently, CSs were considered to have complex karyotypes with genetic instability without any specific genetic translocation, amplification, or mutation. Indeed, a variety of chromosomal abnormalities and gene mutations can occur in CS, especially with high tumoral grade.⁴ These abnormalities often comprise 12q13 amplification, the region harboring the p53 inhibitor *MDM2* and cyclin-dependent kinase 4 (*CDK4*), but also 9p21 deletion. This chromosomal region includes the *CDKN2A* locus, coding for p16^{INK4A} and p14^{ARF}, which ensures a link between the RB and p53 pathways.⁵ Overall, 96% of high-grade central CS present p53 or RB pathway alterations.⁵ However, specific point mutations in *IDH1*- and/or *IDH2*-encoding isocitrate dehydrogenases were recently identified in 50–90% of enchondroma, conventional central, secondary central and dedifferentiated CS.^{6,7} These somatic mosaic mutations lead to the accumulation of metabolites with potent stabilization of HIF1 α and activation of the hypoxia response pathway. They can be considered as an early event in all cartilaginous tumors with secondary genetic alterations that lead to multiple chromosomal rearrangements.⁸

CSs constitute a serious issue for clinical management of the patients, as they are considered resistant to conventional radiotherapy and chemotherapy.⁹ Wide surgical excision has been established as the best therapeutic option, but local recurrence can occur even 10 years after surgery and no treatment can be proposed to patients affected by an unresectable or metastatic tumor.⁹ With a 10-year survival rate ranging from 30 to 80% depending on the CS subtype and the grade, new therapeutic options are needed, especially for the high-risk patients groups.⁹

Various hypotheses have been put forward to explain the resistance of CS to commonly used chemotherapeutic agents. First of all, it has been suggested that the tumoral environment and the bone niche have a key role in this process.¹⁰ The cartilaginous extracellular matrix and its poor vascularity may also impair the diffusion of the drugs, restraining their access to the tumor cells.^{9,11} Previous works also highlighted a possible role of intrinsic factors in CS chemoresistance. High expression of multidrug resistance (MDR) pumps could be implicated to export the drugs from within the cells, and the role of *ABCB1* gene product P-glycoprotein 1 (also

known as MDR1) in resistance of a CS cell line to doxorubicin (DOX) has been demonstrated.¹² The low proliferation rates of CS cells may also limit the efficacy of chemotherapeutic agents, which target more specifically actively dividing cells.⁹ Moreover, there is evidence that CSs overexpress several anti-apoptotic genes such as *BCL-2* or *Survivin* (also known as *BIRC5*), preventing cell death induced by conventional chemotherapy.^{13,14} Although these few studies lead to interesting hypotheses, the molecular mechanism behind chemoresistance of CS is still a matter of debate.

A reason for the scarcity of studies on CS is that only few cell lines and animal models have been established thus far.^{15–24} This may be related to the low proliferation rates of the tumor cells and the difficulty to reproduce a propitious environment for CS development. Furthermore, the culture conditions to establish a CS cell line in monolayer could occasion a loss of the chondrogenic properties.²⁵ To enrich the present panel of CS models, we have established new CS cell lines and xenograft mouse models from human biopsies. These models were characterized for their genetic, phenotypic, and histological properties and then used to study CS chemoresistance, especially in a three-dimensional (3D) chondrogenic culture model where CS cells produce cartilaginous matrix resembling the original tumor biopsy.

MATERIALS AND METHODS

Tumor Biopsies

Tumor biopsies were collected with informed consent after ethical approval by the Nantes University Hospital Ethics Committee. Donors had not received any chemotherapy or radiotherapy before biopsy collection and were anonymously encoded. Half of each biopsy was frozen at -80°C for future DNA and RNA extraction, the other half being frozen in liquid nitrogen in phosphate-buffered saline (PBS; Lonza, Basel, Switzerland) containing 5% bovine serum albumin (BSA; Sigma-Aldrich, St Louis, MO, USA) and 10% dimethyl sulfoxide (DMSO; Sigma-Aldrich) in the GMP manufacture area of the Unit of Cell and Gene Therapy (UTCG, CHU, Nantes, France), which complies with Biosafety II standards.

Establishment of the Cell Lines

All frozen biopsies were thawed, minced with a scalpel and cultured in either Dulbecco's Modified Eagle Medium (DMEM; Lonza) or MEM- α (Gibco Life-Technologies, Paisley, UK) supplemented with 10% fetal bovine serum (FBS; Lonza) as described previously for metastatic melanoma²⁶ (herein termed standard media). After a few days, adherent cells usually migrate outside the tumor fragments and proliferate. Before confluence, cells were scraped in PBS containing 0.02% EDTA (Lonza), counted using a hemocytometer and reseeded. Several frozen biopsies were also dissociated with 2 mg/ml collagenase A (>0.15 U/mg; Roche Diagnostics, Basel, Switzerland)²⁷ and then cultured as described above. When passage 25 was reached, cells were

considered stable and immortal. A culture sample was then given to our research laboratory (INSERM UMR 957) for genetic and phenotypic characterization and tumorigenicity assays in murine models. From this step, all cell lines were cultured in DMEM (Lonza) supplemented with 10% FBS (Lonza) and 1% penicillin/streptomycin (P/S, 100 U/ml; Lonza).

Short Tandem Repeat (STR) Multiplex Assay

A STR multiplex assay (AmpFISTR SGM Plus PCR Amplification Kit, Applied Biosystems, Courtaboeuf, France) was used on genomic DNA to ascertain the identity of the cell lines when compared with the original patient biopsy.²⁸

Tumorigenicity in Nude Mice

All animals for *in vivo* experimentations were housed under pathogen-free conditions in accordance with the institutional guidelines of the regional Ethical Committee for animal experiments (CEEA Pays de la Loire n°06) and under the supervision of authorized investigators. For subcutaneous transplantation, 6-week-old female Balb/cRj-nude mice (Charles River, Wilmington, MA, USA) were anesthetized by inhalation of an isoflurane-air mixture (2%, 0.2 l/min) and injected with Buprecare (20 µg/kg, Animalcare, York, UK). A slight incision was made into the skin along the right flank of the mouse, to where a 2 × 2 × 2-mm³ fragment of a thawed biopsy was transplanted, and the subcutaneous wounds were then sutured. Animals were followed for more than 4 months and tumor volume was quantified by measuring two perpendicular diameters with a caliper and calculated with the formula $(l^2 \times L)/2$, where l is the smallest and L the largest diameter. When tumors reached 1000 mm³, mice were killed and tumor fragments were collected.

To determine the tumorigenicity of the established cell lines, 4-week-old female Rj:NMRI nude mice (Elevages Janvier, Le Genest Saint Isle, France) were injected with 2×10^6 cells in their left paratibial muscle or footpad as described previously.^{29,30} CH03 cells were also injected together with 1×10^6 human mesenchymal stem cells into the paratibial muscle as described.²⁹

Radiographs of subcutaneous or paratibial tumors were obtained with an MX-20 Digital Radiography System (Faxitron Bioptics, LLC, Tucson, AZ, USA).

Other Cells and Culture Conditions

Human CS cell lines SW1353 (ATCC, Manassas, VA, USA), CAL78 (DSMZ, Braunschweig, Germany) and OUMS27 (HSRRB, Osaka, Japan), human OS cell lines SaOS2, MNNG-HOS (herein termed HOS) and MG63 (all from ATCC), as well as human Ewing sarcoma (ES) cell lines SKES1 (Dr S Burchill, Children's Hospital, Leeds, UK) and TC71 (Dr O Delattre, INSERM U830, Paris, France) were cultured in DMEM or RPMI (Lonza) supplemented with 1% penicillin/streptomycin (P/S, 100 U/ml; Lonza) and 10% FBS (Lonza) as described previously.³⁰

For human MSCs, 10 ml of bone marrow was harvested by iliac crest aspiration from a 39-year-old male donor (after informed consent and ethical approval). MSCs were obtained as previously described³¹ and cultured in a medium composed of MEM- α (Gibco Life-Technologies), 1% P/S, 10% FBS, 1 ng/ml basic fibroblast growth factor (bFGF; R&D systems, Minneapolis, MN, USA) and 2 mM L-glutamine. Adherent cells were frozen at passage 2 after characterization by flow cytometry (CD45⁻, CD34⁻, CD105⁺, CD73⁺ and CD90⁺, purity $\geq 99\%$) before further experiments. In addition, these cells were tested for their ability to differentiate into osteoblast, adipocyte or chondrocyte (see below).

Histology

Human tumor biopsies were fixed, decalcified by nitric acid 10% and embedded in paraffin. Tumors resulting from paratibial cell injections were fixed, decalcified with 4.13% EDTA and 0.2% paraformaldehyde in PBS for 96 h using a KOS microwave histostation (Milestone, Kalamazoo, MI, USA) and embedded in paraffin. Tumors resulting from subcutaneous transplantation of biopsy fragments and chondrogenic cell pellets were fixed and directly embedded in paraffin. Sections (3–4 µm) were stained either with hematoxylin/eosin (HE; Gill2 Hematoxylin, Shandon, Thermo Fisher Scientific, Waltham, MA, USA) or with Alcian blue (pH 2.5; DiaPath, Microstain Division, Martinengo, Italy). All images were captured with a DMRXA microscope (Leica, Nussloch, Germany).

Karyotypes

CH03 (passage 53), CH34 (passage 19) and CH56 (passage 14) CS cells were plated on a 1-well LabTek chamber slide (Nunc, Roskilde, Denmark) and processed for karyotyping in the Department of Medical Genetics (Nantes University Hospital, France).

TP53, MDM2 and CDKN2A Gene Copy Number Analysis

Genomic DNA was extracted using the PureLink Genomic DNA Mini Kit (Invitrogen Life-Technologies, Paisley, UK). Amplification of short sequences of the *MDM2* and *CDKN2A* genes were carried out by real-time quantitative PCR on genomic DNA in SYBR green buffer (Bio-Rad, Hercules, CA, USA) using the CFX96 Real-Time PCR Detection System (Bio-Rad). A 249-bp fragment of the exon 11 region of *MDM2* was amplified with primers MDM2-For 5'-GCTAGC ATTCTGTGACTGAGCAG-3' and MDM2-Rev 5'-CAGGAA CATCAAAGCCCTCTTC-3'. To analyze *CDKN2A* gene copy numbers, two couples of primers were designed: 1 α -For 5'-GAGCAGCATGGAGCCTTC-3' and 1 α -Rev 5'-GCTACCTG ATTCCAATTCCCCT-3' for a 256-bp fragment of the exon 1 α region, 1 β -For 5'-GTGCGTGGGTCCCAGTCTGC-3' and 1 β -Rev 5'-GGGCCTTTTCTACCTGGTCT-3' for a 315 bp of the exon 1 β region. The primers used for targeting a 271-bp fragment of the genomic region surrounding the exon 6 of

TP53 were TP53-For 5'-ACATGACGGAGGTTGTGAGG-3' and TP53-Rev 5'-ACCCAGTTGCAAACCAGAC-3'. Analysis was performed using cytochrome *c*-1 (*CYC1*) or actin β (*ACTB*) as invariant control.

IDH1 and 2 Mutation Analysis

Fragments of exons 4 of *IDH1* and *IDH2*, identified as mutation hot spots in CS,^{6,7} were amplified from genomic DNA by PCR with the following primers: IDH1-F1 5'-CTCAGAGCCTTCGCTTTCTG-3' and IDH1-R1 5'-ATCCCATAAGCATGACGAC-3' spanning IDH1 codons 42 to 137, IDH1-F2 5'-CGGTCTTCAGAGAAGCCATT-3' and IDH1-R2 5'-CACATTATTGCCAACATGAC-3' spanning IDH1 codons 106 to 138, IDH2-F 5'-GGGGTTCAAATTCTGGTTGA-3' and IDH2-R 5'-CTAGGCGAGGAGCTCCAGT-3' spanning *IDH2* entire exon 4 (IDH2 codons 126–178). Sequencing was performed as described previously.³²

TP53 Mutation Analysis

Amplification of the complete coding region of *TP53* was carried out on reverse-transcribed RNA by semi-quantitative PCR with iQ Multiplex Powermix (Bio-Rad) using two couples of primers: primers 1 F 5'-AGACTGCCTTCCGGGTC-3' and 6 R 5'-TAGGGCACCACCACACTATG-3' for a 682-bp fragment related to exons 1–6, and primers 5 F 5'-ATGGCCATCTACAAGCAGTC-3' and 11 R 5'-CAAGAAGTGGAGAATGTCAG-3' for a 721-bp fragment related to exons 5–11. After sequencing of the two PCR products, the data were compared with the reference sequence NM_000546.2 as described previously.³³

Flow Cytometry

MSCs, reference primary bone tumor cell lines and the new CS cell lines CH03 (passage 63) and CH56 (passage 23) were stained at 4 °C for 1 h with mouse anti-human FITC-conjugated CD34 (clone 4H11), PE-conjugated CD45 (clone HI30), PE-conjugated CD105 (clone 43A3) (all from BioLegend, San Diego, CA, USA), PE-conjugated CD44 (clone 515), PE-conjugated CD73 (clone AD2) or FITC-conjugated CD90 (clone 5E10) (all from BD-Pharmingen, Franklin Lakes, NJ, USA) antibodies. Isotypic mouse antibodies (BD-Pharmingen) were used as negative controls. Acquisitions were performed on a FC500 cytometer (Beckman-Coulter, Brea, CA, USA) and the results were analyzed with the CXP Analysis software (Beckman-Coulter).

Differentiation Assays

Differentiation assays were performed in the three mesenchymal lineages on MSCs, CS reference cell line CAL78 and the new CS cell lines CH03 (passage > 65) and CH56 (passage > 25).

For osteoblastic differentiation, 10⁴ cells per well were plated in a 24-well plate and grown in DMEM supplemented with 10% FBS (Hyclone Perbio, Bezons, France), 1% P/S, 100 nM dexamethasone (Sigma-Aldrich), 10 nM vitamin D3

(Roche Diagnostics), 50 μ g/ml ascorbic acid and 10 mM β -glycerophosphate (Sigma-Aldrich). The medium was changed three times a week for 2 weeks. The cells were fixed with 70% ethanol for 1 h at 4 °C and stained for 10 min with alizarin red S (40 mM, pH 7.4; Sigma-Aldrich).³¹

For adipogenic differentiation, 2 \times 10⁴ cells were plated per well of a Millicell EZ SLIDE four-well glass (Millipore, Merck, Darmstadt, Germany) and grown in DMEM supplemented with 10% FBS, 1% P/S, 1 μ M dexamethasone, 500 μ M 3-isobutyl-1-methylxanthine and 60 μ M indomethacine (Sigma-Aldrich). The medium was changed three times for 1 week. The cells were fixed with 4% paraformaldehyde for 15 min at RT and intracytoplasmic lipid droplets and nuclei were stained in the dark with 0.1 mg/ml Nile Red (Sigma-Aldrich) for 15 min and DAPI (Invitrogen Life-Technologies) for 5 min at RT.³¹

For chondrocytic differentiation, 2.5 \times 10⁵ cells were placed in a 15-ml polypropylene tube (Corning, Capitol Scientific, Austin, TX, USA) and centrifuged. The pellet was cultured in 500 μ l standard medium or ready-to-use complete chondrogenic differentiation medium (following the manufacturer's recommendations, Lonza) containing FBS, dexamethasone, ITS (insulin transferrin selenium) and 10 ng/ml freshly added transforming growth factor β 3 (TGF- β 3, Peprotech, Rocky Hill, NJ, USA). The medium was carefully removed to avoid pellet re-suspension and replaced every 2–3 days for 21 days. Then, the pellet was washed in PBS (Lonza), fixed at 4 °C with 4% formaldehyde and embedded in paraffin. Sections (3–4 μ m) were then stained with Alcian blue as described above.

Following the same protocol, chondrogenic differentiation was also assayed with a homemade medium composed of DMEM High Glucose, Glutamax, Pyruvate (Gibco Life-Technologies) supplemented with 350 nM L-proline (Sigma-Aldrich), ITS 1 \times (Gibco Life-Technologies), 100 nM dexamethasone, 170 nM L-ascorbic acid 2-phosphate (Sigma-Aldrich), P/S 1 \times and 10 ng/ml TGF- β 3 (Peprotech).

TaqMan Low-Density Array (TLDA) Analysis

Total RNA was extracted from SW1353, OUMS27, CH03 (passage > 65), CH34 (passage 23) and CH56 (passage > 25) cell cultures using Tri Reagent (Molecular Research Center, Cincinnati, OH, USA). The same protocol was used for total RNA extraction from CH34 and CH56 original tumor fragments and chondrogenic 3D cell pellets of CH03 and CH56 freshly grinded with an Ultra Turrax Disperser DI25 (IMLAB sarl, Lille, France). First-strand cDNA synthesis was performed from 500 ng to 1 μ g total RNA using ThermoScript RT-PCR System (Invitrogen Life-Technologies). A custom TLDA (Applied Biosystems Life-Technologies) was designed on a 384-well microfluidic card (190 genes in duplicates) based on several commercial Gene Signature Plates, covering different biological processes such as mesenchymal differentiation, stemness, cell proliferation, apoptosis or chemoresistance. Experimental procedures were performed

according to the Applied Biosystems TLDA user bulletin. Analysis was performed using *CYCI* and hypoxanthine-guanine phosphoribosyltransferase (*HPRT*) as invariant controls and results were expressed as $2^{-\Delta Ct}$. Principal component analysis (PCA) was performed using R console (<http://www.r-project.org/>), and hierarchical clustering was carried out using the centroid linkage method with Cluster v3.0 software.³⁴ Functional enrichment analysis was performed with DAVID v6.7 to identify biological processes in which genes are implicated (<http://david.abcc.ncifcrf.gov/>).

Real-Time PCR

Total RNA were extracted from indicated biopsies or cell lines and reverse-transcribed as indicated above. The real-time PCR (qPCR) mixture contained 10 ng reverse-transcribed total RNA, 200–300 nM previously described primers,^{27,30} and SYBR Green PCR MasterMix (Bio-Rad). Analysis was performed using *CYCI* and *HPRT* as invariant controls. Results were expressed as $2^{-\Delta Ct}$.

Chemosensitivity Assay

Reference OS, ES, CS, CH03 (passage > 65) and CH56 (passage > 25) cell lines were plated in 96-well plates ($N = 3$) as monolayer and exposed for 72 h to increasing concentrations of DOX (Sigma-Aldrich), cisplatin (CIS; Sigma-Aldrich), or mafosfamide (MAF; Baxter, Deerfield, IL, USA). The number of viable cells was estimated with an XTT assay (Roche) using a light spectrometer (Victor², 1420 Multilabel Counter; Perkin Elmer, Waltham, MA, USA) and the half maximal inhibitory concentrations (IC50) were estimated.

3D cell pellets of CH03 and CH56 were cultured for 21 days in standard or complete chondrogenic differentiation medium. From days 18 to 21, they were exposed to increasing concentrations of DOX, MAF, or CIS. The pellets were then treated with a 2-mg/ml Collagenase A (Roche) solution in DMEM + 1% P/S. The cell suspensions were washed with complete medium and cultured in 24-well plates. After 3 days of culture, the adherent cells were trypsinized and viable cells were quantified with trypan blue staining.

To compare cells in monolayer with cell pellets, CH03 and CH56 cells cultured as monolayer were plated in 24-well plates ($N = 2$) and exposed to increasing concentrations of DOX, MAF, or CIS. After 3 days of culture, the adherent cells were trypsinized and viable cells were quantified with trypan blue staining.

The intranucleic incorporation of DOX in paraffin-embedded CH03 3D standard and chondrogenic cell pellets treated or not with DOX was assessed on 3- μ m sections by fluorescence microscopy with a DMRXA microscope associated with a HBO 100 mercury lamphouse (Leica), the nuclei being counter-stained with DAPI (Invitrogen Life-Technologies). Sections of CH03 3D standard and chondrogenic cell pellets were also stained with Alcian blue to verify the presence of a cartilaginous matrix.

Statistical Analysis

Results were analyzed with unpaired *t*-test or Mann–Whitney followed by Fisher's post-hoc test using GraphPad InStat v3.02 software. Results are given as mean \pm s.d. and results with $P < 0.05$ were considered significant.

RESULTS

Establishment of New CS Cell Lines and Tumorigenicity in Nude Mice

Between December 2007 and January 2012, 10 patients diagnosed with CS at the Nantes University Hospital (France) were included in this study entitled Bone Cell Supplier (BCS; Table 1). Several high-grade tumors (grade II–III) were analyzed for tumorigenicity in nude mice, especially those giving rise to a stable cell line in culture (see below). Only the subcutaneous transplantation of the BCSC01 patient biopsy produced tumor development (Table 1; 4 out of 5 transplantations gave a cancer xenograft). This patient had a very bulky grade II conventional central CS of the sternum with incomplete resection (with macroscopic residues, scored R2) and died only 2 months after diagnosis (Table 1 and Figure 1a), suggesting a link between cancer volume/resection/survival and tumorigenicity in mice. The xenografted tumors presented massive cancer nodules with tumor cells engulfed in a cartilaginous matrix. The cancer nodules were poorly vascularized, but surrounded by stromal areas with numerous blood vessels. This histological profile was identical to the grade II CS features identified during the diagnosis of the corresponding patient's biopsy (Figure 1a). Subsequent transplantation of the BCSC01 xenograft tumor in other nude mice also resulted in a tumoral development, indicating that the tumor could be maintained and propagated *in vivo* (data not shown).

Out of the 10 CS biopsies, 2 immortal cell lines could be established. These cultures reached passage 25 and their identity with the original biopsy was certified by STR genotyping (Supplementary Table 1). Similar results were obtained using either frozen or fresh biopsies, or using dissociation with collagenase A in place of a mechanical mincing (data not shown). The BCSC03 (CH03) cell line originated from a high-grade dedifferentiated CS of the femur presenting high cellularity and few cartilaginous zones (Figure 1c). The second cell line, BCSC56 (CH56), was isolated from a grade III conventional central CS of the humerus, with large cartilaginous areas, zones of high tumor cellularity and cellular atypia with vascular vessels, osseous metaplasia and evidence of bone erosion (Figure 1b). Both osteolysis and calcifications were observed in patient radiographs. Cancer cells could be isolated from another grade III conventional central CS, BCSC34 (CH34), with similar histological and radiographic features (Figure 1d). Although CH03 and CH56 cells could be maintained more than 1 year in continuous culture (more than 70 and 35 passages, respectively), and are therefore considered stable and

immortal, CH34 cells stopped growing and eventually died around passage 28 (Table 1), precluding their complete characterization (see below).

When injected into the paratibial muscle or footpad of nude mice, repeated injections of CH03 cells, alone or in association with MSCs (to enhance the tumoral development) did not produce a xenograft. In contrast, injection of CH56 cells in the paratibial muscle led to a tumoral development in two of the four nude mice (Table 1). In radiographs, extensive bone remodeling with bone erosion and calcifications was observed. In histological sections, tumor areas with cartilaginous matrix, high cellularity, and osseous metaplasia were identified (Figure 1b). This radiological and histological profile strongly correlated with the corresponding patient's tumor (Figure 1b).

Genetic Characterization of the New CS Cell Lines

Karyotype of CH56 cells presented constant triploidies for chromosomes 1, 2, 3, 4, 7, 10, 16, 18, and 20, and tetraploidies could be observed for chromosomes 19, 21, and 22. Moreover, a major rearrangement occurred on chromosome 13 and one of the two gonosomes was often missing (Figure 2a). CH34 cells presented a similar karyotype as CH56, with fewer rearrangements. Frequent triploidies were notably noticed for chromosomes 5, 7, 20, and 22, as well as a possible translocation between chromosomes 1 and 10. Loss of gonosomes was also reported (data not shown). CH03 cells harbored an even higher genetic instability with a general hypertriploidy and strongly altered chromosomes that could not be identified. On average, 96 chromosomes were numbered.

Sequencing of *IDH1* and *IDH2* identified point mutations at characteristic CS hot spots^{6,7} in the two conventional central CH34 and CH56 CS cells, CH34 cells also harboring a referenced silent *IDH1* polymorphism.³⁵ The dedifferentiated CS cell line CH03 did not show any *IDH1* or *IDH2* mutations, but presented a deletion in *TP53*-coding sequence (Figure 2b). Note that the sequence of *TP53* could present some smaller peaks corresponding to minor amplicons generated by alternative splicing. The p.R312C point mutation for *IDH1* in CH34 cells appeared less than heterozygous, which could be explained by triploidy of chromosome 2 in certain cells.

Homozygous deletion of *CDKN2A* (exons 1 α and 1 β coding for p16^{INK4A} and p14^{ARF}, respectively) was observed in CH34 and CH56 cells, whereas CH03 cells had lost only one copy of p16^{INK4A}-coding exon. *MDM2* gene amplification, although at low level (2.5 \times), was significant in CH34 and CH56 but not in CH03 cells, and therefore co-occurred with *CDKN2A* deletion (Figure 2c). There was no gross *TP53* gene deletion in CH03, CH34 or CH56 cells (Figure 2d). All genetic characterization data are gathered in Table 2.

Expression of Mesenchymal Cell Surface Markers and Differentiation Abilities

CS cells are expected to express mesenchymal membrane markers and to produce a cartilaginous matrix, at least when they are cultured in a chondrogenic medium.¹³ Because OS-derived cell lines are able to differentiate into the osteoblastic or adipocytic lineage when cultured in corresponding differentiation medium,³⁶ we hypothesized that CS cell lines may have the same properties.

Table 1 Summary of patients clinical data, derived cell lines and tumorigenicity in nude mice

Patient	Gender	Age (years)	Subtype (history)	Location (recurrence)	Grade	Resection	Survival (months)	Passage	Tumorigenicity
BCSCH01	M	83	Conv	Sternum	II	R2	2	2	Yes ^a , grade II
BCSCH03	F	74	Dedifferentiated	Femur (1st)	High	R0	16	>70	No ^{a, b, c, d}
BCSCH06	M	57	Conv	Sacrum (4th)	II	R0	>44	5	NP
BCSCH30	M	46	Secondary (MO)	Femur (1st)	I	R0	>30	0	NP
BCSCH34	M	73	Conv	Tibia	III	R0	>26	28	No ^a
BCSCH36	F	28	Secondary (OD)	Scapula (1st)	I	Curettage	>23	5	NP
BCSCH37	F	41	Secondary (MO)	Ischion	II	R1	>22	3	NP
BCSCH45	F	64	Conv	Femur	II	R0	>23	2	NP
BCSCH56	F	64	Conv	Humerus	III	R0	>14	>40	No ^a ; Yes ^c , grade III
BCSCH59	F	67	Conv	Femur	I	Curettage	>7	0	NP

Conv, conventional central CS; MO, multiple osteochondroma; NP, not performed; OD, Ollier Disease (multiple enchondroma), R0, complete removal; R1, microscopic residues; R2, macroscopic residues.

^aAfter biopsy subcutaneous transplantation in Balb/c Nude mice.

^bAfter paratibial and intrafootpad cell injection in Balb/c Nude mice.

^cAfter paratibial cell injection in NMRI nude mice.

^dAfter paratibial cell co-injection with human mesenchymal stem cells in NMRI Nude mice.

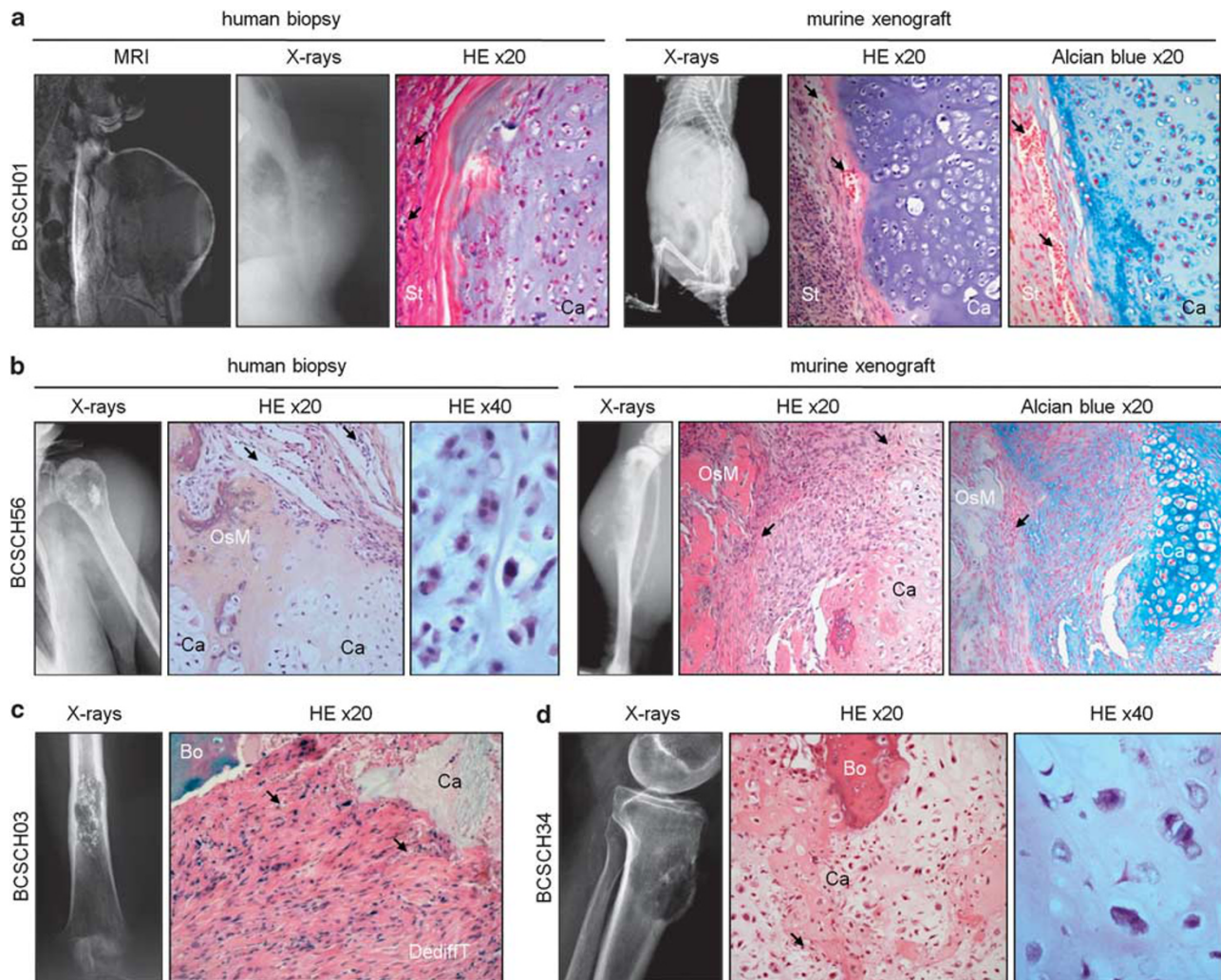


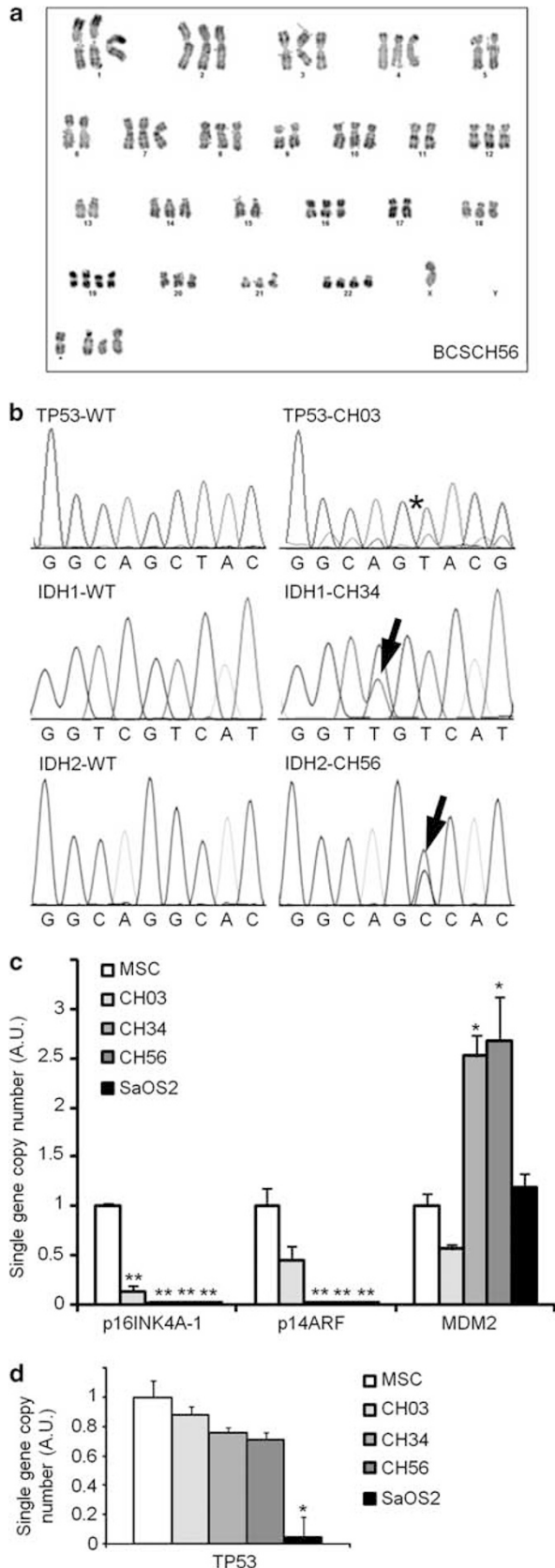
Figure 1 Characterization of chondrosarcoma (CS) subtypes. **(a)** Human and murine BCSC01 tumors. Human tumor was analyzed *in situ* by MRI and radiography, and a biopsy was analyzed by hematoxylin/eosin (HE) staining (original magnification $\times 20$). Subcutaneous xenografts in nude mice were analyzed by radiography, HE and Alcian blue staining ($\times 20$). **(b)** Human and murine CH56 tumors. The human tumor was analyzed *in situ* by radiography, and a biopsy was analyzed by HE staining. Paratibial tumors in nude mice were analyzed by radiography, HE, and Alcian blue staining ($\times 20$). **(c,d)** Human CH03 and CH34 tumors. The human tumor was analyzed *in situ* by radiography, and a biopsy was analyzed by HE staining ($\times 20$ and $\times 40$). St, stroma; Ca, cartilaginous area; DediffT, dedifferentiated tumor; OsM, osseous metaplasia; Bo, bone; arrows, blood vessels.

Flow cytometry analysis revealed that CH03 and CH56 cells expressed the four main mesenchymal markers, CD44, 73, 90, 105, and did not express the hematopoietic markers CD34 and 45 (Figures 3a and b). Other well-referenced CS and OS cell lines showed the same expression pattern, all with a lower level of CD90 compared with MSCs. In contrast, ES cell lines presented lower expressions of CD44 and 73 compared with MSCs, CS, or OS cells (Figure 3b).

When placed in a chondrogenic medium as 3D pellet cultures for 21 days, CH03 and CH56 cells were able to synthesize a cartilaginous matrix resembling high-grade CS (Figure 4). Alcian blue staining revealed large amounts of glycosaminoglycans, although at a lower level than in MSCs pellets. When cultured as 3D pellets in standard medium (no chondrogenic agents added), CH56 cells produced a similar

cartilaginous matrix but CH03 produced less matrix and it was Alcian blue negative (Figure 4), indicating that these two cell lines had, in standard medium, a different differentiation status. In contrast, OS cells HOS could not produce such a cartilaginous matrix, even in a chondrogenic medium, whereas other CS cells CAL78, SW1353, OUMS27, or Swarm rat CS produced pellets that were too small or spontaneously disintegrated in these culture conditions (data not shown). Cartilaginous matrix production by CH03 and CH56 cells was much less effective in another homemade chondrogenic differentiation medium (see Materials and Methods; data not shown).

Unlike MSCs or CAL78 cells, CH03 or CH56 cells were not able to produce a mineralized matrix when placed in osteogenic medium. When placed in adipogenic medium, all CS



cells accumulated small cytoplasmic lipid droplets (Figure 4; compared with MSCs having larger lipid droplets), but similar results were obtained in standard medium.

Thus far, our results demonstrated that CH03 and CH56 cells possess the key characteristics of CS, especially when they were cultured as 3D pellets in a chondrogenic medium.

Transcriptomic Analysis of Cell lines, Biopsies and Chondrogenic 3D Pellets

To better characterize these phenotypic differences, we next performed a high-throughput quantitative RT-PCR analysis of five CS cells cultured in monolayer (CH03, CH34, CH56, SW1353 and OUMS27), two chondrogenic 3D pellets (CH03 and CH56) and two original patient's biopsies (CH34 and CH56; CH03 biopsy could not be analyzed due to insufficient material). Expression of 190 genes was analyzed, encompassing master transcription factors and key markers of chondrogenesis, osteogenesis, adipogenesis, stemness, cell proliferation, cell death, or chemoresistance. PCA performed on all genes showed that CS biopsies and pellets form a distinct group from cells in monolayer, with the exception of CH56 cells, which were more similar to biopsies and pellets than the other cell lines cultured in monolayer (Figure 5a). Indeed, 64 genes were found overexpressed by more than twofold in biopsies and pellets compared with the five cell lines cultured in monolayer (only 35 genes with $P < 0.05$), whereas 14 genes were underexpressed (expression level < 0.6 , only two genes with $P < 0.05$; Figure 5b and Supplementary Table 2). Hierarchical clustering using these 78 genes confirmed that biopsies and pellets formed a distinct group from cells cultured in monolayer (Figure 5b). According to the functional enrichment analysis, overexpressed genes were mainly involved in skeletal development ($P = 0.009$), especially cartilage development ($P = 0.016$), whereas underexpressed genes were involved in cell cycle ($P = 0.028$) and apoptosis ($P = 0.05$; Supplementary Table 2). When taking into account only the 37 genes with a significant differential expression, only the biological processes of skeletal and cartilage development were found enriched (not shown). Although only two genes were significantly underexpressed, expression of *BIRC5* (apoptosis inhibitor), *BAX* (apoptosis activator) and/or *E2F1* (proliferation and apoptosis activator), for instance, was significantly lower in

Figure 2 Genetic characterization of CH03, CH34, and CH56 cell lines. (a) CH56 karyotype showed generalized triploidy and chromosomal translocations (13 to 14). (b) *TP53*, *IDH1*, and *IDH2* mutations were identified by direct sequencing. Mutated sites, confirmed in the reverse sequence, are indicated with an arrow. (c,d) Single-gene copy numbers of indicated genes were estimated by quantitative PCR on genomic DNA. Results are expressed relative to mesenchymal stem cells (MSCs), error bars indicate s.d. Experiments were carried out twice, and one representative experiment is presented here. Statistical analysis was performed using *t*-test (compared with MSCs, * $P < 0.05$, ** $P < 0.01$). A.U., arbitrary units.

CH56 or CH34 pellets and/or patient's biopsies than in corresponding cells in monolayer. Inversely, expression of *XIAP* (apoptosis inhibitor) was higher in CH56, CH34, and CH03 pellets or patient's biopsies (Figure 5c). When including additional CS, OS, or ES biopsies and cell lines, the main chondrocytic genes *COL2A1*, *COMP*, and *ACAN* were all significantly more expressed in CS biopsies and pellets than in CS cell lines (except CH56 cells that maintained a high expression of *COMP* and *ACAN*), OS (except *ACAN*, which was highly expressed in OS biopsies) or ES samples (Figure 5d).

Table 2 Summary of the genetic characterization of CH03, 34 and 56 cells

Cell line	BCSCH03	BCSCH34	BCSCH56
Karyotype	Hypertriploidy	Triploidy + translocations	Triploidy + translocations
IDH1	WT	P.R132C ^a	WT
IDH2	WT	WT	P. R172S
TP53	c.318delC ; P.Y107TfsX15 ^b	WT	WT
p16 ^{INK4A}	Deletion	Deletion	Deletion
p14 ^{ARF}	Normal	Deletion	Deletion
MDM2	Normal	Amplification	Amplification

WT, wild type.

^aP. G105G polymorphism.

^bP. R72P polymorphism.

Chemoresitivity of Cell Lines and Chondrogenic 3D Pellets

Viability assays with XTT reagent were carried out on CH03 and CH56 cells in monolayer in standard culture conditions alongside reference CS, OS and ES cell lines to evaluate their sensitivity to three conventional chemotherapeutic agents. All CS cell lines were as sensitive to MAF (the active compound of ifosfamide) and CIS as OS or ES cell lines (Figure 6a and Table 3). After DOX treatment, the three CS cell lines SW1353, CAL78, and CH56 had IC50s similar to OS or ES cell lines, but the two CS cell lines OUMS27 and CH03 showed a significantly higher IC50 (Figure 6a and Table 3), indicating that they may have an intrinsic relative resistance to this drug.

When cultured in chondrogenic medium as 3D pellets, followed by cell dissociation and analysis of cell viability using trypan blue exclusion, CH03 cells appeared less sensitive to DOX (IC50 = 231 nM) compared with CH03 cells in standard medium (in monolayer or 3D pellets; IC50 = 30 and 32 nM, respectively; Figure 6b). Similar results were observed with MAF, ie cells in chondrogenic 3D pellets were less sensitive (IC50 = 32 μM) than cells in standard medium (in monolayer or 3D pellets; IC50 = 2.8 and 3.9 μM, respectively; Figure 6b). However, there was no or only a small effect of chondrogenic differentiation on the sensitivity of CH03 to CIS (IC50 = 4.5 μM in chondrogenic 3D pellets *versus* 1.8 μM in monolayer and 4.2 μM in standard 3D pellets; Figure 6b). In CH56 cells, we also observed a decreased sensitivity to DOX in chondrogenic 3D pellets compared with cells in monolayer (IC50 = 199 and 11 nM, respectively), cells in standard 3D pellets having an intermediary sensitivity (IC50 = 69 nM; Figure 6c).

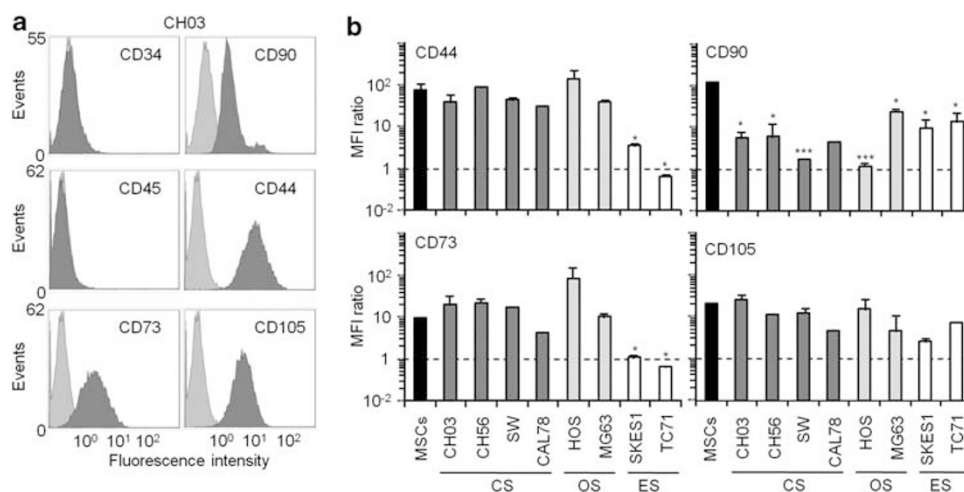


Figure 3 Cell surface mesenchymal marker expression in chondrosarcoma, osteosarcoma and Ewing sarcoma. (a) Expression of four mesenchymal (CD44, 73, 90, and 105) and two vascular/hematopoietic (CD34 and 45) markers was estimated in CH03 cells by flow cytometry (light gray: isotypic control; dark gray: marker-specific antibody). (b) Similar analyses were performed on indicated cell lines. Results are expressed as mean fluorescence intensity ratio (marker-specific antibody/isotypic control) ± s.d. (n = 2–3 for each cell line). Values at the « 1 » threshold correspond to no expression. Statistical analysis was performed using *t*-test (**P* < 0.05 compared with mesenchymal stem cells (MSCs)).

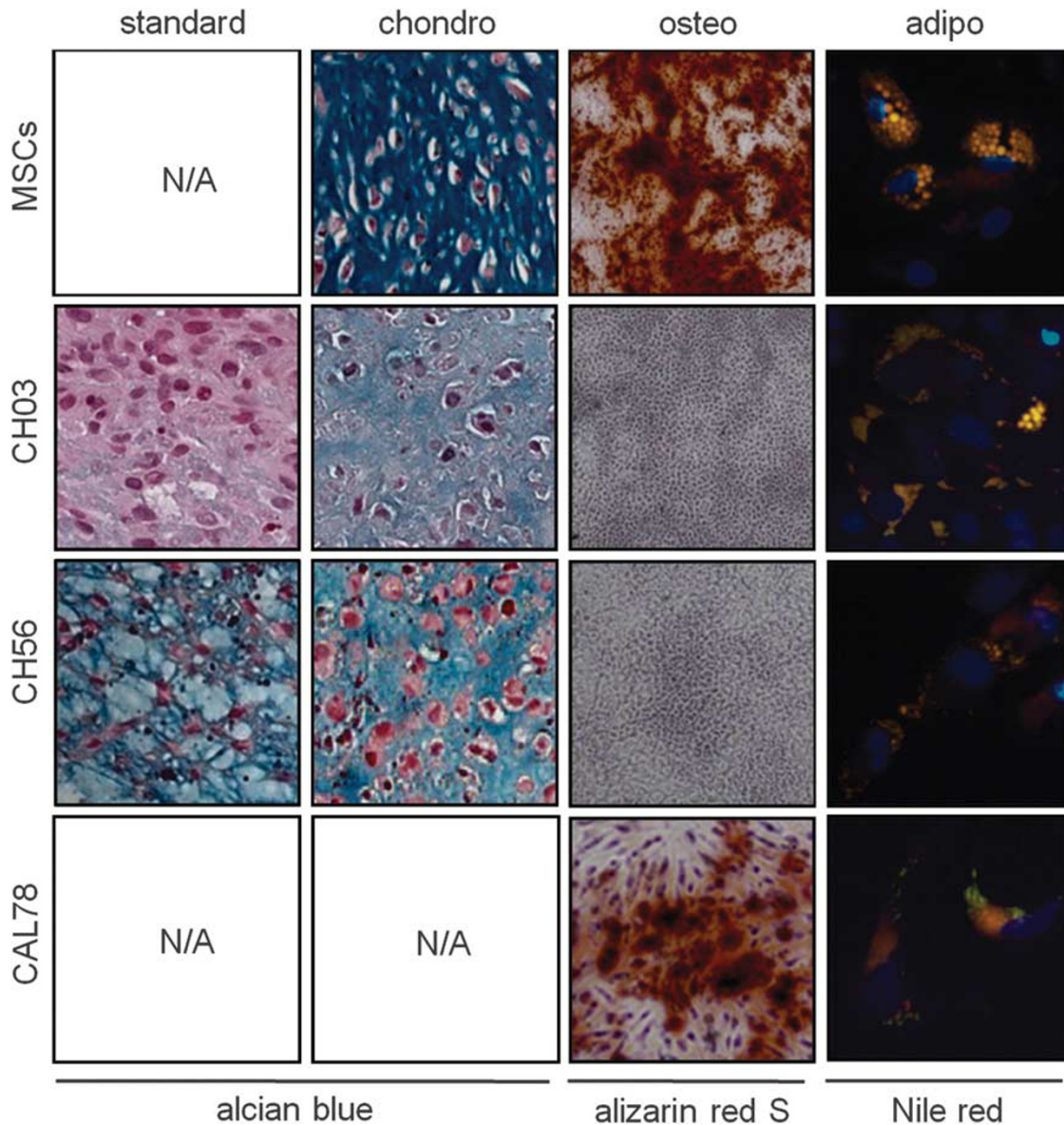


Figure 4 Differentiation abilities of the new chondrosarcoma (CS) cell lines. CH03, CH56, CAL78 cells, and mesenchymal stem cells (MSCs) were cultured in standard, chondrogenic (both as 3D pellets), osteogenic or adipogenic (both as cell monolayer) medium. Their differentiation status was then determined by specific staining: Alcian blue on paraffin-embedded pellets for production of cartilaginous matrix by chondrocytic cells, alizarin red S for matrix mineralization by osteoblastic cells and Nile red to reveal lipid droplets in adipocytic cells (original magnification $\times 20$, $\times 10$ and $\times 40$, respectively). Chondro, chondrogenic differentiation; osteo, osteoblastic differentiation; adipo, adipogenic differentiation; N/A, not available, pellets spontaneously disintegrated.

Histological analyses performed on 3D pellets revealed that the fluorescent drug DOX at 100 nM accumulated in the nuclei of CH03 cells when cultured in standard medium but did not when cultured in chondrogenic medium (Figure 6d). At a higher concentration (1000 nM), DOX accumulated in both culture conditions. Therefore, there was a good correlation between DOX nuclear accumulation and reduced cell viability, and in presence of a cartilaginous matrix, CS cells were significantly protected from low doses of DOX or MAF but not CIS.

DISCUSSION

In this study, two new cell lines were established, one from a grade III primary central CS and one from a dedifferentiated CS. After genetic and phenotypic characterization, we have designed a 3D culture model that induces or sustains the chondrogenic differentiation of these CS cells. This culture condition was particularly relevant to study the link between chondrogenesis and chemoresistance of CS and may therefore represent an alternative to animal models that are difficult to obtain from biopsy transplantation or cell line injection.

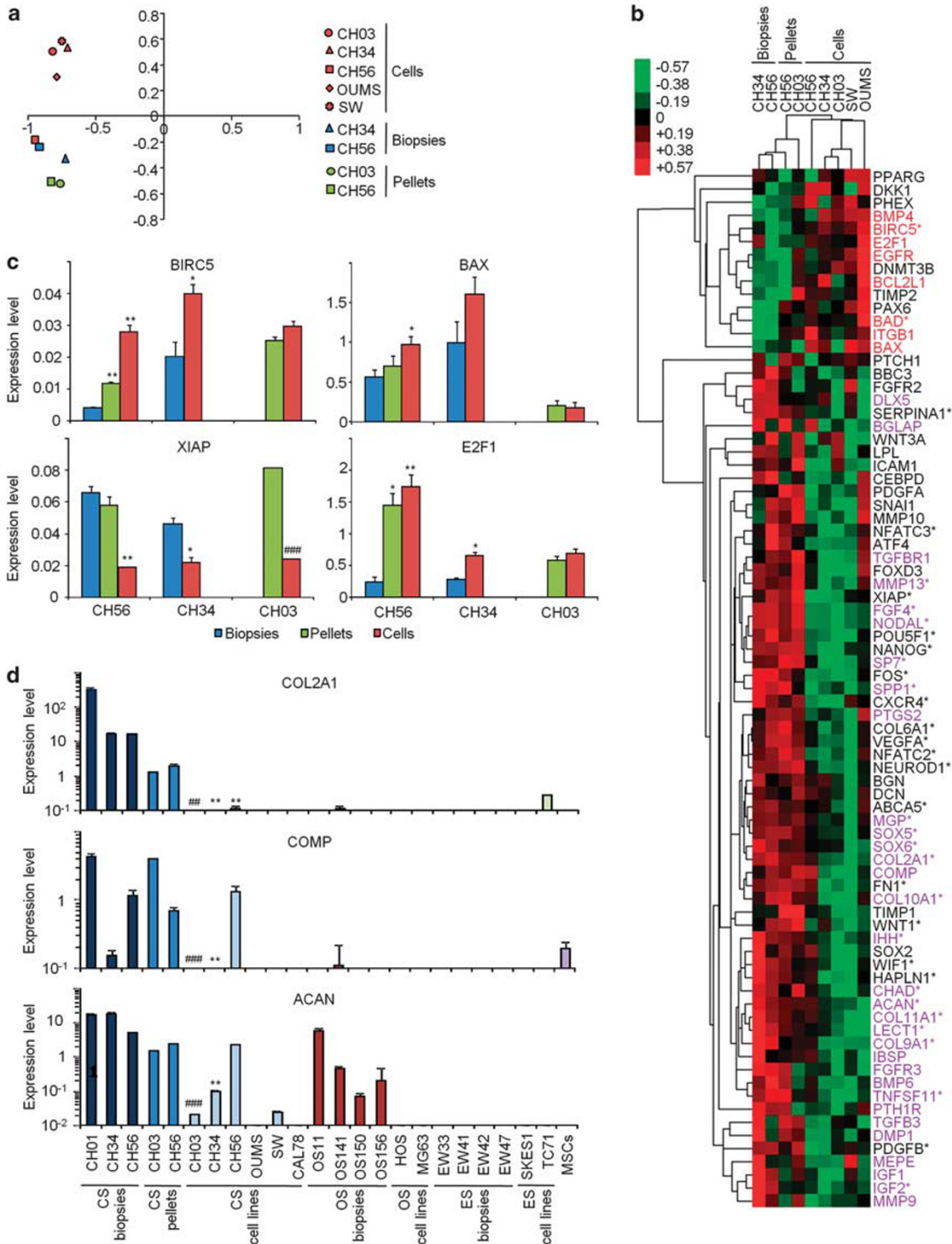


Figure 5 Transcriptomic analysis. **(a)** Principal component analysis comparing the gene expression profiles of two chondrosarcoma (CS) biopsies, two chondrogenic 3D pellets and five cell lines in monolayer was performed using 190 genes of interest. **(b)** Hierarchical clustering analysis (centroid linkage method, uncentered correlation) was performed using a subset of 78 genes differentially expressed between CS cell lines and the group containing CS biopsies and chondrogenic 3D pellets. Genes for which the difference was significant are indicated (* $P < 0.05$; Mann–Whitney test). **(c)** Expression levels of indicated genes were confirmed in CH03, CH34, and CH56 biopsies, chondrogenic 3D pellets or cells in monolayer. Statistical analysis was performed using t -test (for CH34 and CH56 pellets and cells, * $P < 0.05$ and ** $P < 0.001$ compared with the original biopsy; for CH03 cells, #### $P < 0.001$ compared with CH03 pellet). **(d)** The expression levels of *COL2A1*, *COMP*, and *ACAN* were determined in indicated tumor biopsies, cell lines in monolayer or chondrogenic 3D pellets as well as normal human mesenchymal stem cell (MSC). Statistical analysis was performed using t -test (for CH34 and CH56 pellets and cells, ** $P < 0.01$ compared with the original biopsy; for CH03 cells, ## $P < 0.01$ and #### $P < 0.001$ compared with CH03 pellet).

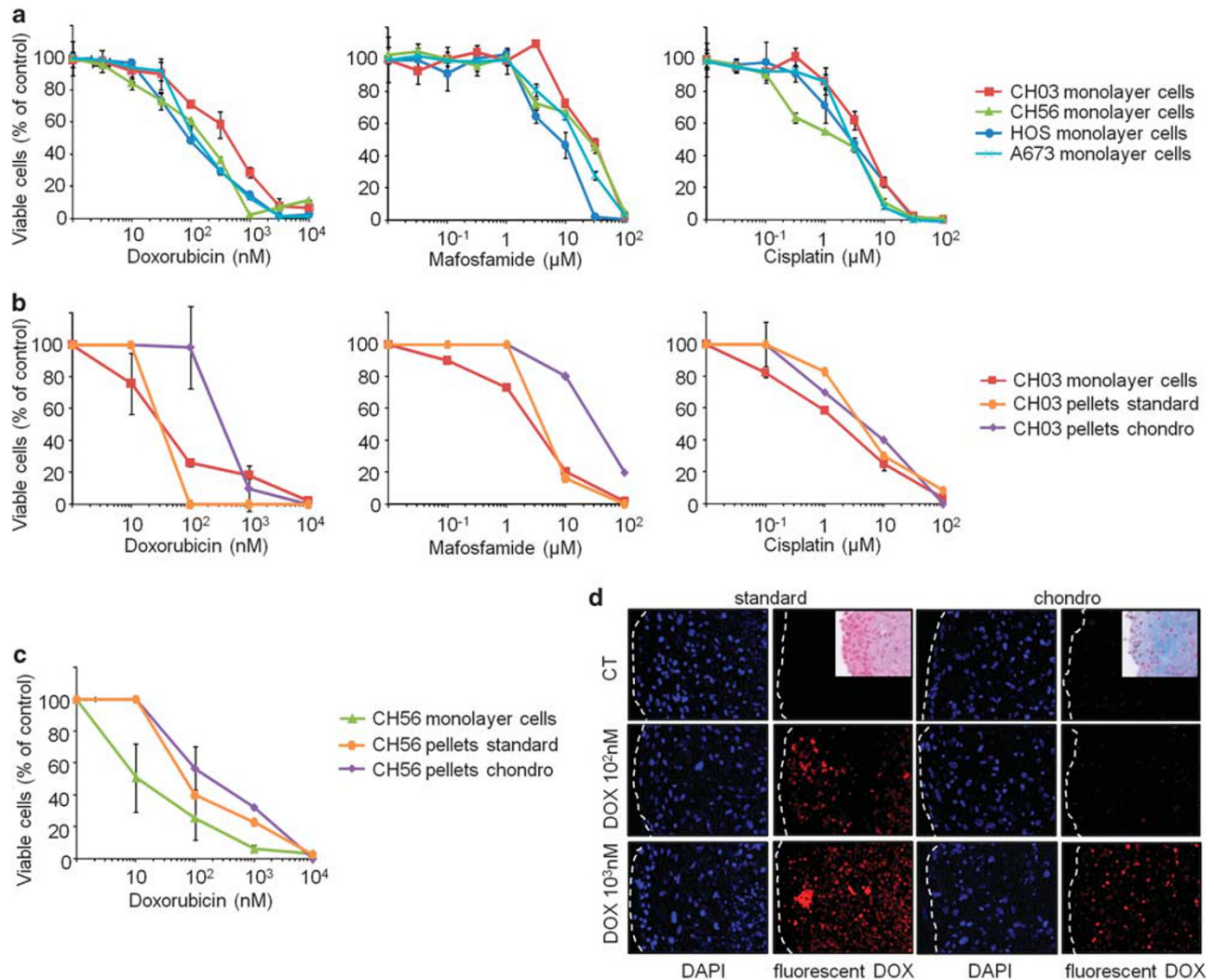


Figure 6 Chemosensitivity of chondrosarcoma (CS) cell lines. **(a)** HOS, A673, CH56, and CH03 cells in monolayer were treated with increasing concentrations of DOX, MAF, or CIS for 3 days. Viable cells were then quantified by XTT assay. All experiments were carried out at least three times in triplicate. Graphs show data from one representative experiment, error bars indicate s.d. **(b)** CH03 cells were cultured as 3D pellets in standard or chondrogenic medium and then treated with DOX, MAF, or CIS for 3 days. After dissociation, cells were cultured for 3 additional days as monolayer in standard medium (without drug) and viable cells were then quantified by trypan blue staining. In parallel, CH03 cells in monolayer were treated with the drugs for 3 days and viable cells were then quantified by trypan blue staining. Experiments with 3D pellets and cells in monolayers were performed twice. Results are expressed as the mean \pm s.d. of these two experimental data. CH03 pellets chondro, CH03 3D chondrogenic pellets. **(c)** CH56 cells were treated with DOX as in **(b)**. CH56 pellets chondro, CH56 3D chondrogenic pellets. **(d)** CH03 cells were cultured as 3D pellets in standard or chondrogenic medium and then treated with DOX for 3 days. Accumulation of DOX was analyzed by fluorescence microscopy, nuclei were visualized after DAPI staining and cartilaginous matrix formation after Alcian blue staining (inset, original magnification $\times 20$). Experiments were performed twice, one representative experiment is presented here. Chondro, chondrogenic 3D pellets; DOX, doxorubicin.

Indeed, in our hands, CS biopsy transplantations in immunocompromised mice gave rise to only one tumoral development. This xenografted tumor presented the same histological subtype as the original patient affected by a primary central grade II CS and could be propagated to other mice after further transplantations, thus is a valuable representative model of the pathology. Besides, this patient had a very bulky tumor, and died only 2 months after surgery, suggesting a link between tumorigenicity in nude mice and patient survival. However, the tumor of another CS patient with a poor survival did not engraft (CH03) and after

cell injection, only one cell line was tumorigenic in nude mice; yet, the corresponding patient was still alive 14 months after surgery (CH56). In the literature, the disease outcome is rarely indicated but we found another cell line (ch-3573), derived from a grade II CS with lung metastasis and poor survival, which is tumorigenic in nude mice (Supplementary Table 3).²⁰ Similar results were also obtained in a large cohort of breast cancer patients where tumor engraftment was a prognostic indicator of disease outcome, independently of the grade or cell line establishment.³⁷ A study on a larger cohort of CS patients is necessary to

Table 3 Sensitivity of malignant primary bone tumor cell lines to mafosfamide, cisplatin and doxorubicin

Drugs		Mafosfamide	Cisplatin	Doxorubicin
Tumor type	Cell line	IC50 (μ M)	IC50 (μ M)	IC50 (nM)
Reference OS	SaOS2	22 \pm 3	7 \pm 0.9	119 \pm 78
	HOS	7 \pm 3	3 \pm 0.2	147 \pm 37
Reference ES	SKES1	17 \pm 7	3 \pm 0.6	47 \pm 14
	A673	36 \pm 19	3 \pm 0.6	150 \pm 36
Reference CS	SW1353	20 \pm 7	5 \pm 0.7	140 \pm 44
	CAL78	46 \pm 12	7 \pm 3	80 \pm 38
	OUMS27	31 \pm 6	6 \pm 2	2591 \pm 1851
CS cell line	CH03	28 \pm 13	5 \pm 1	360 \pm 55
	CH56	34 \pm 13	9 \pm 4	184 \pm 54

Results are expressed as mean(IC50) \pm s.d. $N = 3-4$ independent experiments in triplicate.

ascertain this possibility, which is complicated by the rarity of this disease.

The three cell cultures that reached passage 25 correspond to the CS patients with the highest tumoral grades (conventional central grade III or dedifferentiated CS), irrespective of the patient survival. Other published CS cell lines are also derived from high-grade tumors, usually with recurrence or metastasis (Supplementary Table 3), suggesting that the establishment of CS cell lines is facilitated by the genetic alterations that characterize the most aggressive tumors. The *IDH1/IDH2* mutation is now considered as an early oncogenic event in cartilaginous tumors with secondary genetic alterations, affecting, for instance, the p53 pathway, which ultimately lead to genome instability and complex karyotypes.^{8,38} Genetic characterization of our CS cells revealed a predominant triploidy with many chromosome rearrangements. A complete or partial deletion of the p16^{INK4A}-coding region of the *CDKN2A* gene was observed in our three CS cells similar to what has been described for the majority of the previously published CS cell lines (Supplementary Table 3). It co-occurs with low level of *MDM2* amplification in CH34 and 56 cells as well as *TP53* point mutation in CH03 cells. Although *IDH1* or *IDH2* are mutated in CH34 and 56 cells, CH03 cells have wild-type *IDH* genes. When considering the five dedifferentiated CS cell lines published thus far (Supplementary Table 3), only one cell line has a mutated *IDH* (L2975).²¹

Establishment of a solid tumor cell line in culture is usually performed in monolayer. By doing so, the 3D environment is lost with potential deleterious side effects on differentiation. Chondrogenesis is especially sensitive to the 3D environment, primary chondrocytes in monolayer being well known to lose their capacity to produce a cartilaginous matrix.²⁵ When cultured as monolayer, CH56 and CH03 cells

expressed characteristic extracellular membrane markers of mesenchymal cells (such as MSCs, OS and to a lesser extent ES cell lines). They were not able to synthesize mineralized matrix in osteoblastic conditions, but accumulate small cytoplasmic lipid droplets even in standard monolayer culture condition. This observation suggests that CS cells are already engaged in the adipocytic differentiation pathway or that CS cells have a particularly high metabolic activity allowing abnormal lipid accumulation.

When cultured in 3D pellets, CH56 cells spontaneously produced a cartilaginous matrix with high cellularity resembling the original conventional central grade III CS. In contrast, CH03 cells in standard 3D pellets did not produce a cartilaginous matrix and thereby is similar to the high-grade anaplastic areas of the original dedifferentiated CS. On addition of chondrogenic agents, CH03 3D pellets looked like the cartilaginous areas of the original dedifferentiated CS. These results indicate that CH03 and CH56 cells have intrinsically in culture a different differentiation status, in relation with the original cancer subtype.

At the transcriptome level, CH03 cells cultured in monolayer poorly expressed chondrocytic markers, but their expression was enhanced in chondrogenic 3D pellets and reached values similar to conventional central CS biopsies. CH34 cells cultured in monolayer lost expression of numerous chondrogenic markers, whereas CH56 cells still expressed *COMP* and *ACAN*, two genes coding for important components of the hyaline cartilaginous matrix.²⁵ In conclusion, the chondrogenic status of all CS cell lines was affected by the culture conditions. The CH56 cell line was the least affected, whereas chondrogenesis in CH03 cells could be manipulated to recapitulate the dedifferentiated or the cartilaginous areas of the original patient tumor. Moreover, CS cell lines cultured in monolayer expressed abnormal levels of proliferation and apoptotic markers, such as *BIRC5*, *BAX*, *E2F1* or *XIAP*, thus stressing a deregulation of cell cycle mechanisms.^{39,40}

One of the main biological features of CS is their chemoresistance. Previous studies demonstrated that several CS cell lines overexpress efflux pumps or anti-apoptotic proteins, reducing their sensitivity to DOX or CIS.^{12,13} For instance, OUMS27 or SW1353 cells in monolayer had IC50 values for these drugs between 5 and 400 μ M,¹³ ie 10–100 folds higher than the IC50 values calculated here. This discrepancy could be explained by the use of different cell lines, passages, culture conditions, and/or drug formulation. Yet, our comparison with OS and ES cell lines, usually considered as chemosensitive cell lines, clearly indicates that CS cell lines are not grossly chemoresistant. Indeed in our culture condition, CS cell lines in monolayer appeared as sensitive as OS or ES cell lines to conventional chemotherapy except for OUMS27 and CH03 cells, which showed only a relative intrinsic resistance to DOX.

Most interestingly, CS cells became resistant to low doses of DOX or MAF when they were cultured in chondrogenic

3D pellets, indicating a crucial role of environmental conditions in triggering chemoresistance. Several mechanisms could be implicated; however, we believe that a major one relies on the inefficient diffusion of the drugs through the cartilaginous matrix. First, using CH03 and CH56 cells, we found a good correlation between the presence of a cartilaginous matrix in the 3D pellets and chemoresistance. Second, the intranucleic accumulation of DOX was impaired when a cartilaginous matrix was produced. Third, CS cells were not resistant to CIS even if they produce a cartilaginous matrix. This striking difference may be explained comparing the physicochemical properties of the three drugs, particularly their hydrophobicity. Indeed, CIS is the most soluble molecule and may diffuse more easily through the cartilaginous matrix, while DOX and MAF may be excluded.

Additional chemoresistance mechanisms are however still possible. *ABCB1* (encoding P-glycoprotein 1) expression is not induced in chondrogenic 3D CS pellets but the gene encoding the ATP-binding cassette transporter 5 (*ABCA5*) is significantly more expressed in biopsies and chondrogenic 3D pellets than in monolayer cells. One study described its potent role in OS tumorigenesis and chemoresistance⁴¹ and therefore *ABCA5* may be an important efflux pump involved in CS chemoresistance. Several genes involved in cell cycle and apoptosis are also poorly expressed in CS chondrogenic 3D pellets or biopsies, such as *BIRC5*, *BAX*, or *E2F1*, suggesting that CS cells may also be relatively resistant to apoptosis in these chondrogenic conditions. These observations may have important implications for CS treatment. If the main resistance mechanism is at the level of drug diffusion in the cartilaginous matrix and is not cell intrinsic, a better pharmacokinetic, distribution or cancer cell targeting of the drug may be effective to treat CS. Indeed, DOX incorporated into hydroxyapatite beads and implanted in the CS tumor of mice showed a better tissue distribution and effective anti-cancer effects compared with intravenous administration of DOX.⁴² Similarly, based on the affinity of the quaternary ammonium moiety for proteoglycans present in the cartilaginous matrix, a quaternary ammonium-melphalan conjugate was engineered and showed antitumor effects in CS-bearing rats.⁴³

In conclusion, the phenotype of CS cell lines in monolayer is significantly altered, whereas chondrogenic 3D pellets represent much more valuable models for this pathology, especially to study the link between chondrogenesis and chemoresistance. In comparison with animal models, these culture conditions are easier, more reproducible and ethical, although important factors such as angiogenesis or metastasis are still missing. They may be also adapted for high-throughput screening of new therapeutic agents, which is a major goal for this devastating disease.

Supplementary Information accompanies the paper on the Laboratory Investigation website (<http://www.laboratoryinvestigation.org>)

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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