UM-Chor1: establishment and characterization of the first validated clival chordoma cell line

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OBJECTIVE Chordomas are rare malignant tumors thought to arise from remnants of the notochord. They can be located anywhere along the axial skeleton but are most commonly found in the clival and sacrococcygeal regions, where the notochord regresses during fetal development. Chordomas are resistant to many current therapies, leaving surgery as the primary method of treatment. Cancer cell lines have been useful for developing new cancer treatments in a laboratory setting that can then be transferred to the clinic, but there are only 4 validated chordoma cell lines available. The objective of this work was to establish chordoma cell lines from surgical tissue in order to expand the library of lines available for laboratory research.

METHODS Chordoma tissue from the clivus was processed and sorted by flow cytometry to obtain an isolated population of chordoma cells. These cells were grown in culture and expanded until enough doublings to consider the line established. Identification of a chordoma cell line was made with known markers for chordoma, and the line was observed for ALDH (aldehyde dehydrogenase) subpopulations and tested in serum-free growth conditions as well as in vivo.

RESULTS A fifth chordoma cell line, UM-Chor1, was successfully established. This is the first chordoma cell line originating from the clivus. Validation was confirmed by phenotype and positivity for the chordoma markers CD24 and brachyury. The authors also attempted to identify an ALDH<sup>high</sup> cell population in UM-Chor1, UCH1, and UCH2 but did not detect a distinct population. UM-Chor1 cells were able to form spheroids in serum-free culture, were successfully transduced with luciferase, and could be injected parasacrally and grown in NOD/SCID mice.

CONCLUSIONS The availability of this novel clival chordoma cell line for in vitro and in vivo research provides an opportunity for developments in treatment against the disease.

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Brachyury gene confers chordoma susceptibility.21,40 Animals was followed. Guide for the Care and Use of Laboratory Institutional for use of the animal model were obtained through the many chordomas is also a property of cancer stem cells.1,39 Furthermore, the resistance to chemotherapy exhibited by cells, including the ability to self-renew and differentiate. Whether chordomas contain a subpopulation of stem-like cells has yet to be thoroughly investigated. A 2012 study showed a self-renewal potential in chordoma cells isolated for stem-cell markers CD15 and CD133.2 A type of aldehyde dehydrogenase (ALDH), ALDH1, has been shown to be a marker for cancer stem cells in many cancer types, and the isolation of a subpopulation of ALDH\textsuperscript{high} cells in chordomas is currently being attempted by other groups.10,16,24

Despite an increase in chordoma awareness and research after the creation of the Chordoma Foundation in 2007, understanding the molecular mechanisms of tumorigenesis and potential therapeutic targets remains a constant goal. Creation of new chordoma cell lines is a first step to achieving rigorous in vitro testing and characterization of the disease. At the time of creation of UM-Chor1, only 4 chordoma cell lines were available to researchers worldwide.17,20,33 All 4 were isolated from sacral tumors, which occurred roughly after 25 passages or 78 weeks.

### Genomic DNA Purification for Genotyping
Cells were harvested and washed in phosphate-buffered saline (PBS), then frozen at \(-80\)°C. The thawed cell pellets were resuspended in 600 μl of Promega nuclei lysis solution for 1 hour at 55°C and then allowed to cool to room temperature; 200 μl of Promega protein precipitation solution was added to each sample on ice for 5 minutes before being centrifuged at 13,000 RPM for 2 minutes. Supernatant was transferred to a tube containing 600 μl of isopropanol and centrifuged at 13,000 RPM for one minute. Supernatant was aspirated and the DNA pellet washed in 200 μl of 70% ethanol and resuspended in 50 μl of nuclease-free water.

### Analysis of Genetic Loci
DNA samples were diluted to 0.10 ng/L and were analyzed at the University of Michigan DNA Sequencing Core using the Profiler Plus PCR Amplification Kit (Invitrogen) in accord with the manufacturer’s protocol. The 10 loci D3S1358, D5S818, D7S820, D8S1179, D13S317, D18S51, D21S11, FGA, VWA, and AMEL were analyzed and compared with ladder control samples.

### Flow Cytometry
Neural cell adhesion molecule (NCAM) and fibroblast surface protein (FSP) expression were detected using primary antibodies (BD Pharmingen 559043; Abcam 11333) and fluorophore-conjugated secondary antibodies (BD Pharmingen 550874; eBioscience 12–5790–81). CD24 expression was detected using a fluorophore-conjugated primary antibody (BD Biosciences 555428). Cells were suspended in Hank’s balanced salt solution (HBSS; Gibco) with 2% heat-inactivated calf serum (HICS) added to a concentration of 1 million cells per ml. Five μl of primary antibody was added per ml of cell suspension, and the solution was left to incubate on ice for 20 minutes. The suspension was then centrifuged for 5 minutes at 1000 RPM, and the cell pellet was resuspended in HBSS to a concentration of 1 million cells per ml. Flow cytometry analysis was performed using a BD FACSCalibur flow cytometer (BD Biosciences).
control population in the allophycocyanin (APC) channel with excitation and emission wavelengths of approximately 650 nm/660 nm. For FSP and CD24 expression, cell-sorting gates were established using an unstained control population in the phycoerythrin (PE) channel with excitation and emission wavelengths of approximately 565 and 578 nm, respectively. Brachyury expression was detected using an antibody conjugated to FITC (fluorescein isothiocyanate; Millipore FCMAB302F). A cell suspension was fixed in 4% paraformaldehyde on ice for 10 minutes then permeabilized in methanol for 10 minutes at −20°C. The suspension was centrifuged for 5 minutes at 1000 RPM, and the cell pellet was resuspended in PBS and incubated with the antibody on ice for 30 minutes. The suspension was centrifuged for 5 minutes at 1000 RPM and the cell pellet was resuspended in PBS with DAPI as a cell viability indicator. Cell-sorting gates were established using an unstained control population in the FITC channel with excitation and emission wavelengths of approximately 495 and 521 nm, respectively. ALDH activity was detected using the Aldefluor kit (StemCell Technologies). A single cell suspension in Aldefluor assay buffer (AAB) was incubated with ALDH substrate (BAAA, 5 mol/L per 1×10^6 cells) for 45 minutes at 37°C. Concurrently, diethylaminobenzaldehyde (DEAB, 50 mmol/L) was added to a separate sample also containing BAAA for an ALDH-inhibited control. Samples were washed and resuspended in AAB. Fluorescence-activated cell-sorting gates were established using the inhibited control (DEAB) along the FITC channel.

**Immunofluorescence Staining**

Chordoma cells were plated onto collagen I–treated chamber slides (BD Biosciences). Cells were fixed with 4% paraformaldehyde on ice for 10 minutes, permeabilized with methanol at −20°C for 10 minutes, and incubated with either a brachyury-FITC antibody (Millipore FCMAB302F) or CD24-PE antibody (BD Biosciences 555428) for 30 minutes on ice. Samples were then coated with a DAPI-containing mountant (Molecular Probes) and left overnight. Immunofluorescence images were taken with an Olympus BX-51 upright light microscope at the University of Michigan Microscopy & Image Analysis Laboratory.

**Spheroid Growth**

Chordoma cells were suspended in medium prepared as above but absent of FBS and plated on ultra–low attachment plates (Corning) to promote spheroid growth as previously described. Once clusters of cells, dubbed chordospheres, were observed for 4 days they were processed and prepared for flow cytometry to determine ALDH activity as described above.

**Luciferase Transduction**

UM-Chor1 was transduced at the University of Michigan Vector Core with recombinant human immunodeficiency virus (rHIV) with a luciferase reporter. A lentiviral vector containing a pLentiLox backbone and a cytomegalovirus promoter was used to provide stable integration in permanent cell lines rather than the transient transfection typically provided by adenovirus. Polybrene was added to increase efficiency of the transduction. Successful gene delivery was confirmed via green fluorescent protein (GFP) visualization in a side-by-side transduction of the rHIV-GFP vector under identical conditions.

**Parasacral Injections**

One million UM-Chor1 cells were suspended in 100 μl of media and 100 μl of Matrigel matrix (Corning). The suspensions were injected parasacrally into 3 NOD/SCID mice and imaged at 1-week intervals.

**Bioluminescence Imaging**

All animals treated with UM-Chor1-Luc injections were imaged with the Xenogen IVIS-200 imaging system at the University of Michigan Center for Molecular Imaging. Treated mice were given intraperitoneal injections of 100 μl luciferin at a concentration of 40 mg/ml and allowed to sit for 10 minutes before being anesthetized with isoflurane and imaged.

**Results**

**Cell Line Establishment**

The primary disease showed phenotypic characteristics of a classical chordoma—physaliferous cells with small, round nuclei (Fig. 1A). Small pieces of chordoma tumor tissue were grown in flasks treated with collagen I to promote adhesion (Fig. 1B). When fibroblast overgrowth became a concern, cells were sorted by flow cytometry to separate the 2 cell types. Conventional methods of fibroblast removal involving partial trypsinizations...
were ineffective due to the detachment of chordoma cells at the same time as fibroblasts at low concentrations of trypsin. FSP and NCAM were used to separate and sort the fibroblast and chordoma populations, respectively (Fig. 2). Other chordoma markers were tested, but epithelial membrane antigen (EMA), epithelial cell adhesion molecule (EpCAM), and CD24 were found not to be optimum due to weaker staining or marker presence on the fibroblasts (data not shown). After 10 passages of purified chordoma cells, adhesion in typical tissue-culture flasks was confirmed and cells were grown until enough doublings occurred to suggest immortality (Fig. 1C). Genotyping results of the cells were unique when compared with all previously established chordoma cell lines and head and neck cell lines catalogued in our laboratory, as tested by a short tandem repeat (STR) analysis, confirming UM-Chor1 as a novel cell line.

**Chordoma Characterization**

UM-Chor1 was evaluated for brachyury and CD24 expression by immunofluorescence staining (Fig. 3A). The line was compared with previously established chordoma lines UCH1 and UCH2. Nuclear localization of the transcription factor brachyury was seen, and transcription of brachyury and CD24 was confirmed. Intracellular brachyury presence was also confirmed by flow cytometry (Fig. 3B).
Identification of an ALDH<sup>high</sup> chordoma population was attempted by measurement of a cell population with increased ALDH activity via the Aldefluor assay. UCH1, UCH2, and UM-Chor1 all had negligible populations of cells that had higher ALDH activity than the rest of the population (Fig. 4). Spheroid growth in low-attachment plates of UM-Chor1 resulted in small aggregates of cell clusters that, when analyzed for ALDH activity, showed no increase in a population of cells with higher ALDH activity (Fig. 5).

**In Vivo Model**

UM-Chor1 cells were successfully transduced with luciferase, and 1.0 × 10<sup>6</sup> cells were injected parasacrally into 3 NOD/SCID mice and measured over a 5-week period, showing slow growth of the xenograft via bioluminescence (Fig. 6). The resulting xenografts exhibited classical chordoma characteristics similar to the primary tumor when stained for intranuclear brachyury.

**Discussion**

We discuss in detail the in vitro establishment and initiation of an in vivo model for the first chordoma cell line derived from the clivus. Prior treatment may impact genetic expression in cell lines established from surgery, but we deemed it important to attempt cell line establishment due to the absence of a clival chordoma cell line available for research. We were able to process primary tissue, grow cells derived from the tissue in culture, and then purify the resulting population using a 2-antibody sorting method that our laboratory had previously developed to establish squamous cell carcinomas of the head and neck. NCAM was selected as a surface chordoma marker present on UM-Chor1 cells and absent on fibroblasts, but it is not a definitive marker for chordoma. A 2001 study revealed NCAM expression in 14 of 16 chordoma samples, with high preservation of NCAM expression in the 2 skull base chordomas tested. In this particular case, NCAM was a good marker for the skull base–derived UM-Chor1 cell line. Removal of fibroblasts from the initial cultures is vital for successful chordoma cell propagation due to the stark difference in division rates. The doubling time of fibroblasts can be less than 24 hours, in contrast to that of chordoma cells, which can be as high as 7 days. UM-Chor1 has an in vitro doubling time of approximately 5 days, making immediate removal of the more quickly dividing fibroblasts important. Thorough establishment of slow-growing chordoma cell lines does require an increased initial investment of time compared with establishment of cell lines derived from other solid tumors. The division rate of the sacral chordoma cell line UCH1 was decreased by isolation of a faster-growing subpopulation, and we suspect this may be possible for UM-Chor1 in the future.

As recently as 2010, there were 7 chordoma lines described in the literature, but they lacked characterization. Thorough investigation eliminated all but 2 lines, UCH1 and UCH2, as valid chordoma cell lines. The discovery of brachyury as a prime diagnostic marker has improved the validation process for potential chordoma cell lines, with over 90% of chordomas expressing the transcription factor. Chromosomal aberrations resulting in copy number gain of brachyury are present in both sporadic and familial disease, and knockdown of the gene induces...
apoptosis and arrests cell growth in vitro.\textsuperscript{32,40} In other cancers, brachyury has been shown to be involved in epithelial to mesenchymal transition, and overexpression leads to downregulated expression of E-cadherin and induction of metastasis.\textsuperscript{12} UM-Chor1 exhibited intranuclear localization of brachyury as well as cytoplasmic mislocalization. These observations were independently verified by the Chordoma Foundation, which also measured it as having a nearly 6-fold increase in expression when compared with UCH2.

CD24, a heat-stable antigen, is another surface marker expressed in many tumor types.\textsuperscript{22,26,34,35} CD24 is involved in cell adhesion and metastasis, and it increases proliferation and affinity of tumor cells to collagen, fibronectin, and lamin.\textsuperscript{23} CD24 has been shown to have variable glycosylation, resulting in a diverse set of functions in different cell types.\textsuperscript{13,29} While high expression of CD24 enhances tumor progression in most cancers studied, intracellular expression in pancreatic cancer inhibited cell invasion and metastasis.\textsuperscript{36} CD24 was discovered to be a marker for nucleus pulposus cells located in the inner core of the vertebral disc and was used to distinguish chordoma from chondrosarcoma.\textsuperscript{15} The function of CD24 in chordoma is not yet understood, but it can be used as a chordoma marker to validate potential new cell lines. UM-Chor1 expressed CD24 on the cellular surface, and this was independently verified by the Chordoma Foundation, with an expression nearly 3-fold that of UCH2.

In an effort to isolate a cancer-initiating subpopulation in UM-Chor1, we looked at ALDH\textsuperscript{high} activity. Our laboratory first described ALDH\textsuperscript{high} cancer stem cells in head and neck squamous cell carcinomas in 2010 and has consistently isolated an ALDH\textsuperscript{high} population in each new carcinoma cell line established at the University of Michigan since that time.\textsuperscript{10} We tested UCH1, UCH2, and UM-Chor1 for enzymatic activity of ALDH1 but did not find a discernible population of increased activity, with 0.48\%, 0.37\%, and 0.17\%, respectively. Other groups have found similar numbers, with 0.35\% isolated from UCH1 and 0.30\% isolated from MUG-Chor1.\textsuperscript{33} In contrast, nearly 6.83\% of our control fibroblasts exhibited increased ALDH1 activity, and an evaluation of 20 head and neck cell lines stored in our laboratory yielded an average ALDH\textsuperscript{high} population of 7.19\% (data not yet published). Perhaps more importantly, the ALDH\textsuperscript{high} cell populations in non-chordoma cell lines were readily apparent in flow cytometry plots, separated by at least a 10-fold increase in bioluminescence caused by the enzymatic reaction with the Aldefluor substrate. Chordoma cell lines did not exhibit such separation, and the low percentages recorded cannot be ruled out as background luminescence. UM-Chor1 cells were successfully grown as spheroids in serum-free culture, which enhances the stem cell population in other cancer lines. Upon reanalysis, the ALDH\textsuperscript{high} population of the chordospheres rose to just 0.24\%. It is possible that ALDH is not as potent of a marker in chordomas as it is for other cancers, but with the
small number of cell lines currently available, it should not be discounted entirely.

Other groups have reported successful xenograft passaging of primary chordoma tissue in mice, which may have a higher chance of propagating chordoma cells than growing the primary in culture. While we prefer to establish cell lines in vitro, eliminating possible complications of an animal host, the benefits of developing an animal model for newly established cell lines allows for vital research to be conducted before clinical trials may be proposed. To this end, we performed flank injections of $1.0 \times 10^6$ cells from UM-Chor1 into the flanks of nude mice. No growth was observed after 6 months, so the more immunodeficient NOD/SCID strain was then used. At this point, UM-Chor1 had been successfully transduced with luciferase, allowing in vivo imaging of tumor growth. Cells were injected parasacrally as suggested by the literature, and initiation of tumors was seen at the injection sites in each of the 3 mice tested. Bioluminescent imaging revealed slow tumor growth over the duration of 5 weeks, with the UM-Chor1 xenografts resembling the patient disease histologically. The availability of a clival chordoma cell line that is capable of successful growth in an animal model will be a valuable tool to future chordoma research.

**Conclusions**

UM-Chor1 is the first established chordoma cell line derived from classical chordoma of the clivus, or skull base. The cells exhibit a physaliferous chordoma phenotype and express the diagnostic chordoma markers brachyury and CD24. The cell line was validated independently by the Chordoma Foundation and stored in the foundation’s cell line repository, and it is available for research through the foundation and ATCC. We have been able to successfully transduce UM-Chor1 with a lentiviral vector as well as grow it in a NOD/SCID mouse model. There are only a handful of validated chordoma cell lines worldwide, all derived from the sacrum, and the addition of a clival line adds a valuable second disease site to study.

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**Appendix**

**Source Case**

This 64-year-old man initially presented with headaches and progressively diminishing vision in the left half of his visual field. MRI revealed a 2 cm × 2 cm sellar and suprasellar lesion with solid, cystic, and hemorrhagic components. It extended along the clivus, preoptic cistern, and into the bilateral cavernous, sphenoid, and right posterior ethmoid sinuses. The optic chiasma and
third ventricle were upwardly displaced. The patient underwent an uncomplicated transnasal transsphenoidal resection of the lesion, and pathological examination demonstrated a pituitary adenoma that stained positively for adrenocorticotropic hormone. Clinically, he did not have signs of Cushingle disease. He recovered well from his operation, although a follow-up MRI showed a small amount of residual mass in the posterior sella and within the cavernous sinus. He recovered well until 4 months after the operation, when he presented to the emergency department with diplopia and was found to have a partial right-sided oculomotor nerve palsy. Imaging showed some enlargement of the cavernous sinus lesion, and he thus underwent fractionated radiation therapy for a total dose of 4500 cGy. Fifteen months postoperatively, he again presented to the emergency department with severe headaches and decreased visual acuity. An MRI showed significant enlargement of the suprasellar lesion, and he was taken emergently for decompensation of what was later diagnosed histologically as an acute hematoma, felt to be in part attributable to warfarin, taken for atrial fibrillation. He tolerated this operation well, and was followed with serial imaging. Approximately 2 years after the initial operation, the patient returned to the clinic with complaints of right-sided facial numbness. In addition to worsening of his right oculomotor nerve palsy, he did not have signs of Cushing disease. He recovered well from his operation, although a follow-up MRI showed a small amount of residual tumor in the V2 distribution. Imaging showed further progression of his mass, and it was recommended that he begin temozolomide therapy and undergo resection via the expanded endoscopic endonasal approach. Tissue from this operation was analyzed and, surprisingly, revealed a pituitary adenoma with superimposed lobules of vacuolated cells separated by fibrous septae. Additionally, there was invasion of the sinonasal mucosa and bone. Tissue stains were strongly positive for cytokeratin, S100, and EMA. The final pathological diagnosis revealed chordoma in a background of pituitary adenoma. The patient recovered well following this operation and was again evaluated by radiation oncologists. Intensity-modulated radiation therapy was prescribed to a total dose of 7400 cGy in 180-cGy fractions. The patient tolerated the radiation therapy well, although unfortunately, his most recent MRI showed extensive anterior extension of his lesion into the posterior ethmoids. He enrolled in a clinical trial including imatinib and panobinostat. However, he presented with the acute onset of left ophthalmo-paresis, and thus he was taken for urgent decompensation and staged resection via the expanded endoscopic endonasal approach. He regained oculomotor function and tolerated this operation without complication. Histopathological analysis again confirmed the diagnosis of chordoma.

References


Disclosures
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Conception and design: Prince, Owen, Wang, Abuzeid. Acquisition of data: Owen, Komarck, Wang, Abuzeid. Analysis and interpretation of data: Owen, Abuzeid, Fan. Drafting the article: Owen. Critically revising the article: Prince, Wang, Abuzeid, Keep, McKean, Sullivan. Reviewed submitted version of manuscript: Prince, Owen. Approved the final version of the manuscript on behalf of all authors: Prince. Statistical analysis: Owen. Administrative/technical/material support: Owen. Study supervision: Prince.

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