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# Phenotypic Plasticity in Uveal Melanoma Is Not Restricted to a Tumor Subpopulation and Is Unrelated to Cancer Stem Cell Characteristics

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**PURPOSE.** Uveal melanoma (UM) is the most common primary intraocular malignancy in adults and approximately half of those diagnosed will die of metastasis. This study investigates whether UM progression is driven by a subpopulation of stem-like cells, termed “cancer stem cells” (CSCs).

**METHODS.** Expression of postulated stem cell markers aldehyde dehydrogenase (ALDH), CD44, and CD133 was analyzed in UM cell lines and primary UM short-term cultures (STCs) established from tumor samples. Additionally, the notion of a “cellular hierarchy” within UM was investigated. Finally, the phenomenon of phenotypic plasticity in response to environmental factors was explored.

**RESULTS.** We demonstrate that expression of ALDH, CD44, and CD133 does not select for a subpopulation of stem-like cells in either UM cell lines or UM STCs. Furthermore, there is an absence of a cellular hierarchy in cell lines and all cells in culture are able to drive tumor progression. Last, we show that established UM cell lines and UM STCs are plastic in nature and switch their phenotype in response to environmental stimuli.

**CONCLUSIONS.** We hypothesize that this capacity to undergo phenotypic plasticity may be a consequence of neural crest lineage and renders the exploration of the CSC hypothesis extremely challenging in UM.

**Keywords:** uveal melanoma, cancer stem cells, cellular hierarchy, phenotypic plasticity

Uveal melanoma (UM) is the most common primary intraocular malignancy in adults, accounting for 80% of all noncutaneous melanoma.<sup>1</sup> These tumors are often highly aggressive and frequently metastasize to the liver, after which point survival is rarely more than 6 months. Long-term monitoring of UM patients has shown that liver metastasis can develop decades after first diagnosis, suggesting that UM cells remain dormant within the body for long periods.<sup>2</sup> One explanation for this apparent dormancy has been suggested by the idea of cancer stem cells (CSCs) within tumors. The CSC hypothesis of tumor progression states that all tumor cell types arise from a rare subpopulation of multipotent CSCs that are thought to be responsible for tumor initiation, progression, and relapse posttreatment, and therefore need to be destroyed for treatment to be successful long-term.<sup>3,4</sup>

Since the notion of the “cancer stem cell hypothesis” was first presented in leukemic studies in the 1990s,<sup>5,6</sup> there have been considerable efforts to trace this rare cell of origin in solid tumors.<sup>7–11</sup> CSCs are characterized as tumor cells with similar characteristics to normal stem cells, such as continual self-renewal and the capacity to differentiate. Normal tissue is organized in a cellular hierarchy whereby cells exhibit different capacities to proliferate and differentiate.<sup>12</sup> This was first demonstrated in in vitro clonogenic studies in keratinocytes whereby three distinct clone types were observed in culture,

termed “holoclones,” “meroclones,” and “paraclones.” Holoclones were described as large, highly proliferative clones made up of undifferentiated cells that, when isolated from culture and reseeded, were able to reestablish holoclones, meroclones, and paraclones. Meroclones were described as partially differentiated, intermediate clones that gave rise to further meroclones and paraclones, but were unable to give rise to holoclones. Paraclones were seen to be small colonies made up of cells with irregular morphologies. Paraclones were unable to give rise to further colony types when isolated from culture and therefore thought to represent terminally differentiated cells. It has since been shown that several cancers are organized in a similar fashion to normal tissue, in that cancer cells also have varying capacities to proliferate and differentiate and CSCs represent the apex of the cell hierarchy. A number of established cancer cell lines have been shown to retain a stem cell-like hierarchical structure reflective of the heterogeneity observed in tumors.<sup>11,13–16</sup>

A number of studies in cutaneous melanoma (CM) support the CSC hypothesis in this tumor type and have proposed potential biomarkers for stem cells in cancers arising from a melanocytic lineage, including cell surface markers, such as CD133 and CD44, and markers associated with normal stem cell function, such as aldehyde dehydrogenase (ALDH).<sup>17–20</sup> Expression of the cell surface antigen CD44 has been shown to



be present in tumor cells with enhanced self-renewal and tumorigenic properties in a number of cancer types.<sup>9,21–23</sup> In an established CM cell line, CD44<sup>+</sup> cells were seen to be more metastatic in *in vivo* models compared with CD44<sup>-</sup> cells.<sup>24</sup> Furthermore, ALDH expression has previously been shown to select for tumorigenic subpopulations in CM cells isolated from patient material.<sup>17</sup> However, there are studies that negate these findings and suggest that all CM cells are tumor-initiating and exhibit stem-like characteristics, not just a subpopulation of CSCs.<sup>25–27</sup> Prasmickaite and colleagues<sup>27</sup> found that low-passage cell cultures established from metastatic CM exhibited no difference in proliferative or clonogenic capacity between ALDH<sup>+</sup> or ALDH<sup>-</sup> subpopulations.

The highly heterogeneous nature of all types of melanomas and their propensity to metastasize are thought to be attributed to their derivation from multipotent transitory neural crest cells.<sup>28</sup> Furthermore, there is evidence suggesting that these cancer types are able to switch phenotypes, and depending on conditions, are in fact fluid in their expression of stem cell markers.<sup>25,29–32</sup> Previous studies have demonstrated that CM cells exhibit a phenomenon known as “phenotype switching,” whereby cells readily change between a differentiated state to a dedifferentiated state dependent on their surroundings.<sup>33–36</sup> This characteristic is thought to arise because melanocytes originate from highly plastic, transitory neural crest cells. To date, this phenomenon has not been shown in UM; however, UM cells do exhibit various cell lineage markers, and are capable of vasculogenic mimicry, suggesting a capacity to differentiate into several cell types.<sup>37,38</sup> The phenomenon of vasculogenic mimicry, whereby tumor cells are able to recapitulate vascular channels in the absence of endothelial cells or fibroblasts, has been previously reported in CM and UM.<sup>39–41</sup> Gene expression profiling of both CM and UM cells capable of vasculogenic mimicry showed that these cells express genes associated with an undifferentiated phenotype.<sup>39,42</sup> Furthermore CM cells have been shown to be able to undergo dedifferentiation, which, along with evidence of phenotype switching from vasculogenic mimicry studies, renders the identification of true CSCs a challenging prospect in melanoma.<sup>43</sup>

There have been limited studies investigating the CSC hypothesis in UM. For example, Thill et al.<sup>44</sup> reported the presence of a subpopulation of CD133<sup>+</sup> cells in UM cell lines; however, their functional properties were not explored. Furthermore, Kalirai et al.<sup>45</sup> demonstrated the existence of a hierarchical organization in two UM cell lines whereby holoclones demonstrated indefinite self-renewal capacity and increased chemoresistance. Studies of UM cell lines investigating the effect of growing cells as three-dimensional spheres in comparison with two-dimensional monolayers showed that expression of the CSC marker CD271 was dependent on culture conditions and associated with the phenomenon of vasculogenic mimicry.<sup>32</sup>

In this study, we initially sought to investigate the expression of putative melanoma CSC markers ALDH, CD44, and CD133 in UM cell lines and STCs, as well as in CM cell lines. We subsequently analyzed both UM cell lines and tumor cells at low passage number for their plasticity in response to environmental stimulus and whether the phenomenon of phenotype switching could be observed in UM, particularly in STCs.

## MATERIALS AND METHODS

### Clinical Information

UM primary tumor samples were obtained from patients undergoing enucleation at the Royal Hallamshire Hospital,

Sheffield, UK. Ethical approval and patient consent was sought before surgery (09/ H1008/141) and the procedures adhered to the tenets of the Declaration of Helsinki.

### Cell Line Culture

UM cell lines SOM 157d and SOM 196b were previously established from UM tissue cultured in the Academic Unit of Ophthalmology and Orthoptics at the University of Sheffield.<sup>37</sup> UM cell lines were cultured in ocular melanoma (Oc. Mel.) medium consisting of RPMI medium (Invitrogen, Carlsbad, California, USA) supplemented with 20% fetal calf serum (FCS), 100 U/mL penicillin, 100 µg/mL streptomycin, and 250 µg/mL amphotericin B (Sigma Aldrich, St. Louis, MO, USA). Two CM cell lines, A375 and WM793, were purchased from the American Type Culture Collection (ATCC) (Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium supplemented with 10% FCS, 100 U/mL penicillin, 100 µg/mL streptomycin, and 250 µg/mL amphotericin B (Sigma Aldrich).

### Establishment of UM Short-Term Cultures (STCs)

UM tumor pieces were collected immediately after surgery and processed for culture by finely mincing as described previously.<sup>46</sup> Once minced, the cell suspension was divided into two T25 flasks and cultured independently in either Oc. Mel. medium or in stem cell supportive medium termed “cancer stem cell medium” (CSCM), a basal medium supplemented with transferrin, insulin, and BSA (Provitro, Berlin, Germany).

### Clonogenic Assay

Established cell lines were seeded out at  $2 \times 10^3$  cells per 60-mm dish and allowed to give rise to colonies over a period of 14 days. Classification of each colony was determined depending on their morphology and capacity to give rise to further colonies. Colonies were isolated from culture using 3-mm clonal discs (Sigma Aldrich). Medium was removed and plates washed with  $1 \times$  PBS. Clonal discs were dipped in trypsin and placed directly over isogenic colonies for 3 minutes. Discs were reseeded into individual wells of a six-well plate containing fresh medium. Wells were checked for the presence of colonies following 14 days in culture. The percentage of successful colonies observed in comparison with cells seeded (termed the “clonal efficiency”) was calculated by the following: (number of colonies observed/number of colonies seeded)  $\times$  100. Successful colonies were considered to be those  $>0.5$  mm in diameter after 14 days of culture.

### Proliferation Assay

Cells were seeded into 96-well plates at either  $1 \times 10^3$  cells (established cell lines) or  $2 \times 10^3$  cells (UM STCs) per well. Cells were allowed to grow for 10 days at which point viable cells were determined using trypan blue staining and the number of cells per well was counted using a Vi-Cell XR cell counter (Beckman Coulter, Brea, CA, USA). Experiments were set up in six-well replicates and repeated three times.

### Expression of ALDH and Isolation of ALDH<sup>+</sup> Cells Using Fluorescent-Activated Cell Sorting (FACS)

Cells were analyzed for ALDH activity using the Aldefluor assay (Stem Cell Technologies, Vancouver, BC, Canada). The Aldefluor assay has been optimized to detect the ALDH1 isoform, which is most related to stem cell function.<sup>47</sup> Briefly, two tubes containing  $5 \times 10^5$  cells each were resuspended in Aldefluor buffer and control cells incubated with 30 µM N,N-diethylami-

nobenzaldehyde (DEAB) (a specific ALDH inhibitor) for 10 minutes before treatment with the Aldefluor substrate. Test cells did not receive DEAB treatment. FACS for ALDH expression was carried out following incubation with the Aldefluor substrate using a FACSARIA (Becton Dickinson, Oxford, UK). ALDH<sup>+</sup> cells were identified as those cells emitting fluorescence clearly greater than control inhibitor-treated cells. ALDH<sup>+</sup> and ALDH<sup>-</sup> cell populations were seeded into proliferation and clonogenic assays immediately after sorting. To determine whether ALDH expression is a fixed phenotype in CM, sorted ALDH<sup>+</sup> cells and ALDH<sup>-</sup> cells were cultured for 14 days and subsequently reassessed for ALDH activity using a FACSARIA (Becton Dickinson).

### Expression of Stem Cell Surface Markers CD44 and CD133

To determine expression of the stem cell surface markers CD44 and CD133, cells were stained with anti-CD44 (PE-conjugated) and anti-CD133 (APC-conjugated) antibodies (Miltenyi, Begisch Gladbach, Germany). The anti-CD44 antibody detected all isoforms of CD44. An antibody against the AC133 epitope of the CD133 antigen was used because this specific epitope is expressed exclusively on stem and progenitor cells and represents the glycosylation-dependent epitope of CD133. Fluorophore-conjugated isotype control antibodies were used in all experiments.

Briefly, two tubes containing  $5 \times 10^5$  cells were centrifuged and cell pellets were resuspended in blocking buffer before antibody incubation. After incubation, cells were washed in blocking buffer and fluorescent staining was visualized via flow cytometry on a FACSCALIBUR (Becton Dickinson).

## RESULTS

### ALDH Activity in CM Cell Lines Does Not Select for Increased Proliferative or Clonogenic Capacity

In the present study, both CM cell lines A375 and WM793 were assessed for ALDH activity and the clonogenic and proliferative capacity of subsequent ALDH<sup>+</sup> and ALDH<sup>-</sup> populations tested. Both CM cell lines were seen to express ALDH in a high percentage of cells in culture (Figs. 1A, 1B). The mean percentage of ALDH<sup>+</sup> A375 cells was  $45.9\% \pm 13.4\%$  ( $n = 3 \pm SD$ ) and  $21.8\% \pm 9.7\%$  ( $n = 3 \pm SD$ ) in WM793 cells. Following identification of ALDH<sup>+</sup> cells in both CM cell lines, cells were sorted into ALDH<sup>+</sup> and ALDH<sup>-</sup> subpopulations and subsequently seeded into proliferation and clonogenic assays. In both CM cell lines, ALDH<sup>+</sup> cells and ALDH<sup>-</sup> sorted cells were found to proliferate at an equivalent rate (Fig. 1C). When seeded out at clonal density, ALDH<sup>+</sup> and ALDH<sup>-</sup> cells both readily formed colonies. There was no significant difference in proliferative or clonogenic capacity between ALDH<sup>+</sup> and ALDH<sup>-</sup> cells and no difference in colony morphology observed (Fig. 1D).

ALDH<sup>+</sup> and ALDH<sup>-</sup> populations were reassessed for ALDH expression 14 days post sort. Both A375 cells and WM793 cells demonstrated that ALDH activity is a fluid process and mixed populations of ALDH<sup>+</sup> and ALDH<sup>-</sup> cells were present in both populations of previously sorted cells, summarized in the Table. In the A375 cell line, the number of ALDH<sup>+</sup> cells arising from previously sorted ALDH<sup>+</sup> cells was comparable to the number of ALDH<sup>+</sup> cells in unsorted populations ( $44.6\% \pm 1.7\%$ ). ALDH<sup>-</sup> sorted populations were able to give rise to daughter cells that expressed ALDH, although these ALDH<sup>+</sup> cells represented only  $2.0\% \pm 0.1\%$  of the population ( $n = 3 \pm SD$ ). In the WM793 cell line, previously sorted ALDH<sup>+</sup> cells largely retained their ALDH<sup>+</sup> phenotype when grown in

culture, containing  $85.8\% \pm 2.9\%$  ALDH<sup>+</sup> cells following 14 days in culture. Sorted ALDH<sup>-</sup> WM793 cells also tended to retain their original phenotype in culture, giving rise to only  $2.6\% \pm 0.2\%$  ALDH<sup>+</sup> daughter cells.

### ALDH Activity in UM Cell Lines and STCs Is Not Associated With a Stem Cell Phenotype

Two UM cell lines and seven UM STCs were tested for expression of ALDH. Interestingly, ALDH expression was absent in both UM cell lines ( $n = 3$ ; data not shown). In contrast to observations in UM cell lines, all UM STCs were found to contain a subpopulation of ALDH<sup>+</sup> cells ranging from 0.8% to 16.0% (mean = 6%) (Fig. 1E). Where possible, repeats were performed; however, due to limited material, this was not possible in all cases. To determine whether expression of ALDH was related to proliferation capacity, sorted populations were seeded into proliferation assays. Four of seven UM STCs tested for ALDH expression were taken forward for growth assays. We show that there was no difference in the proliferative capacity of ALDH<sup>+</sup> cells versus ALDH<sup>-</sup> cells in any of the four UM cases studied (Fig. 1F; data represent SOM 577).

### CD133 Is Present in a Small Subpopulation of CM Cells and Select UM STCs

In the present study, the two CM cell lines were seen to contain small subpopulations of CD133<sup>+</sup> cells (A375: mean of 0.6% [Fig. 2B]). WM793: mean of 0.3% (Fig. 2D;  $n = 3$ ).

UM cell lines were found not to contain a subpopulation of CD133<sup>+</sup> cells ( $n = 3$ ; Figs. 2E, 2H). Of the six UM primary cultures tested, only one (SOM 588) contained a subpopulation of CD133<sup>+</sup> cells (0.3% of cells) (Fig. 2J). The remaining five cultures were negative for CD133 expression (data not shown). Therefore, it was concluded that CD133 is not likely to represent a CSC population in UM and was not studied further.

### CD44 Is Highly Expressed in All Melanoma Cells and Not Just a Minor Subpopulation

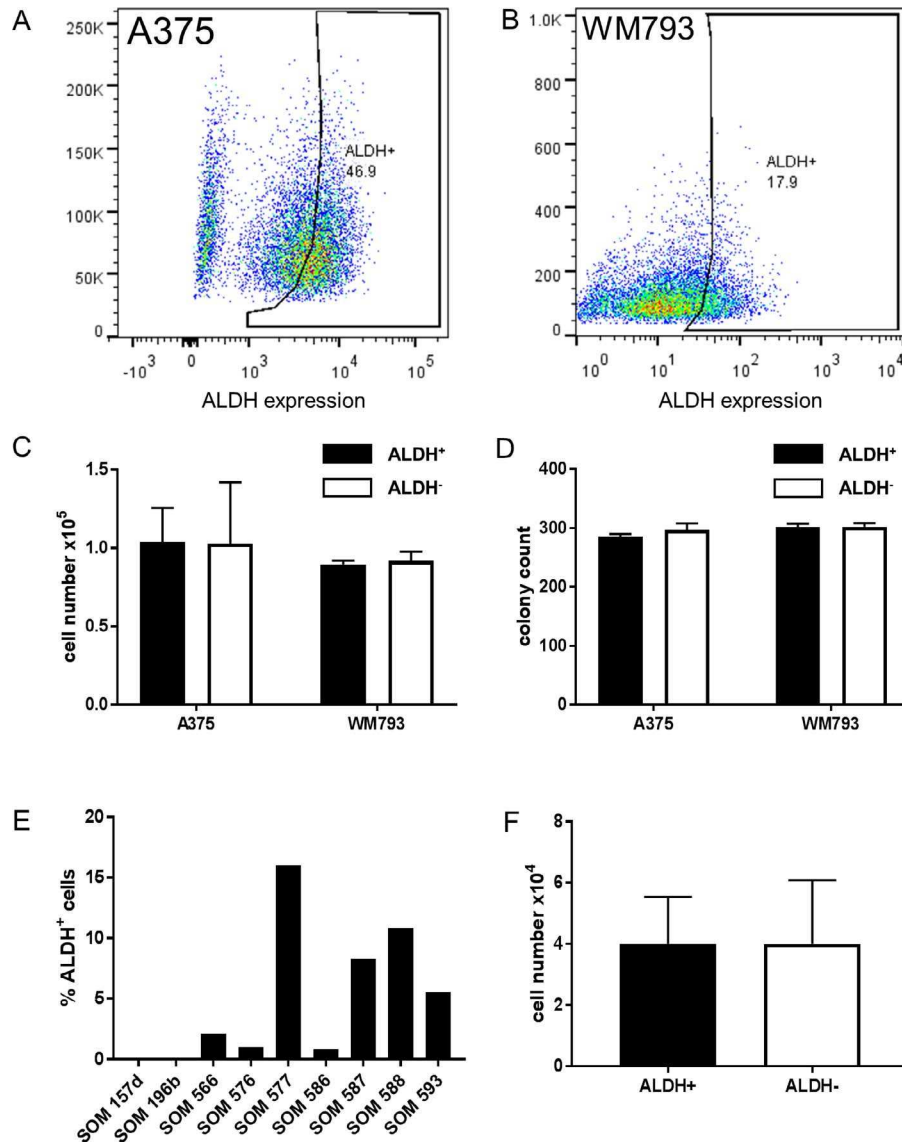
The present study found that in the two CM cell lines (>95% CD44<sup>+</sup>), and also the two UM cell lines (>95% CD44<sup>+</sup>), CD44 was expressed in most cells in culture and not just a subset of cells (Figs. 2A-G). Furthermore, CD44 was found to be expressed in most cells in the six UM cultures tested, ranging from 77% to 92% CD44<sup>+</sup> cells (Fig. 2I; data represent SOM 588).

### A Cellular Hierarchy Is Absent in Established UM and CM Cell Lines

To determine whether UM cell lines SOM 157d and SOM 196b were organized in a hierarchical manner, cells were seeded out into clonogenic assays. Colonies were observed after 14 days. The clonal efficiency for SOM 157d cells was  $16.0\% \pm 0.4\%$  and  $13.0\% \pm 1.0\%$  for SOM 196b (data represent the average colony count and clonal efficiency of three repeat triplicate experiments  $\pm SEM$ ). In both UM cell lines, colony morphologies remained consistent with no variation in cell type or proliferative capacity (Fig. 3). When isolated from culture using clonal discs, all colonies picked and reseeded were able to reestablish further colonies which resembled the parent colony. This observation could be seen in all subsequent generations of colonies picked, with 100% efficiency. There appears to be only one clone type present in UM cell lines whereby every cell within the population has the capacity to drive tumor progression.

To determine whether the CM cell lines, A375 and WM793, reflect the clonal behavior of UM cell lines, cells were seeded





**FIGURE 1.** ALDH activity does not select for increased proliferative or clonogenic capacity in melanoma. Background fluorescence was determined by treating a control population with the specific ALDH inhibitor DEAB in the presence of ALDH substrate. Gating for positive staining was subsequently measured against inhibitor-treated control cells. Scatter plots of A375 (A) and WM793 (B) CM cell lines showing ALDH<sup>+</sup> populations. Mean percentage of ALDH<sup>+</sup> population in A375 cells was 45.91% ± 13.35% (*n* = 3 ± SD) and 21.84% ± 9.69% (*n* = 3 ± SD) in WM793 cells. No difference in proliferative (C) or clonogenic (D) capacity was observed between sorted ALDH<sup>+</sup> or ALDH<sup>-</sup> populations in either cell line. Of seven UM STCs tested, ALDH expression was seen in all seven, ranging from 0.8% to 16% (E). No difference in proliferative capacity was observed following sorting of ALDH<sup>+</sup> and ALDH<sup>-</sup> cells (data represent SOM 577) (F).

out at clonal density in the same manner. A375 had an average clonal efficiency of 16.0% ± 0.2%. WM793 had an average clonal efficiency of 15.0% ± 0.6%. All colonies were seen to be of a similar morphology and when isolated from culture all colonies picked were able to reestablish subsequent colonies resembling the parent colony for both A375 cells and WM793 cells, reflecting a similar colony-forming pattern as the UM cell lines (data not shown).

### UM Cells in Culture Exhibit a Highly Plastic Phenotype and Switch Phenotypes Dependent on Conditions

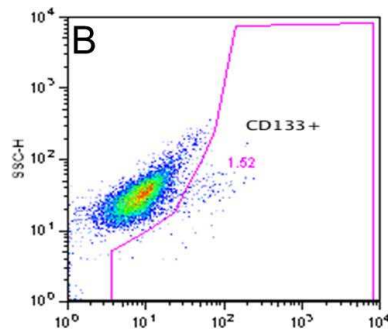
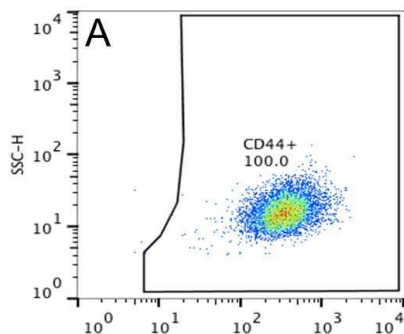
To investigate the effects of varying culture conditions on UM cell behavior, we selected CSCM, as it is specifically optimized to support a stem-like phenotype as a comparison to Oc. Mel.

**TABLE.** Plasticity of ALDH Expression in CM

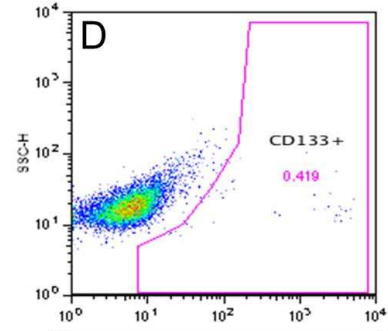
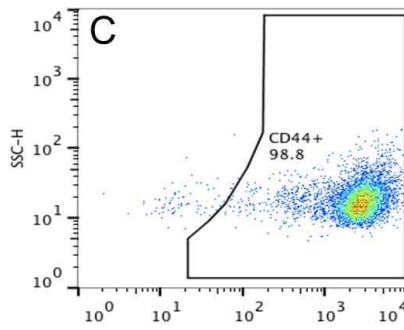
Cell Line	% ALDH <sup>+</sup> in Unsorted Population	% ALDH <sup>+</sup> Arising From ALDH <sup>+</sup> Population	% ALDH <sup>+</sup> Arising From ALDH <sup>-</sup> Population
A375	45.9	44.6	2.0
WM793	21.8	85.8	2.6

The number of ALDH<sup>+</sup> A375 cells arising from sorted ALDH<sup>+</sup> populations was comparable to the number of ALDH<sup>+</sup> cells in unsorted populations (44.6% ± 1.7%). ALDH<sup>-</sup> sorted populations were able to give rise to ALDH<sup>+</sup> daughter cells, although representing only 2.0% ± 0.1% of the population (*n* = 3 ± SD). In WM793 cells, ALDH<sup>+</sup> cells largely retained their ALDH<sup>+</sup> phenotype, containing 85.8% ± 2.87% ALDH<sup>+</sup> cells following 14 days in culture. Sorted ALDH<sup>-</sup> WM793 cells also tended to retain their original phenotype in culture, giving rise to 2.6% ± 0.2% ALDH<sup>+</sup> daughter cells 14 days post sort.

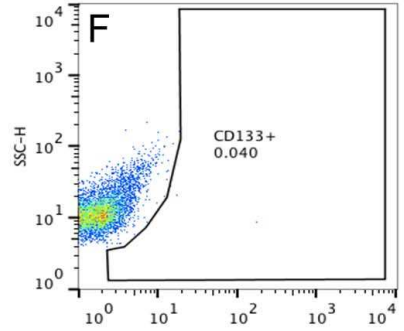
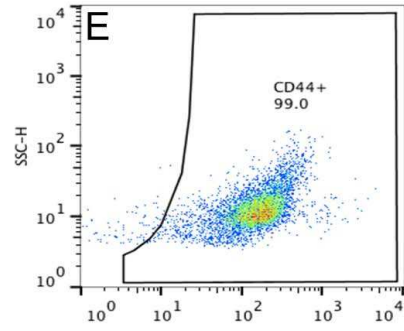
A375



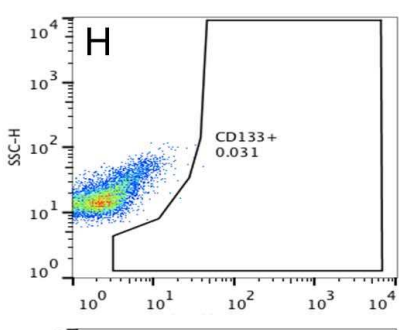
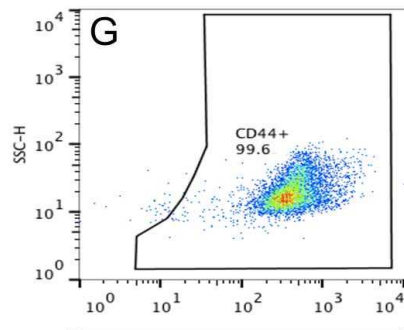
WM793



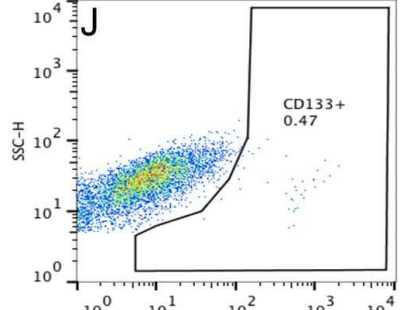
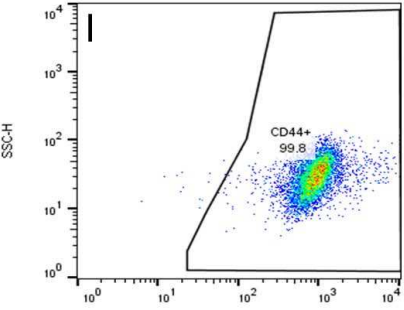
SOM 157d



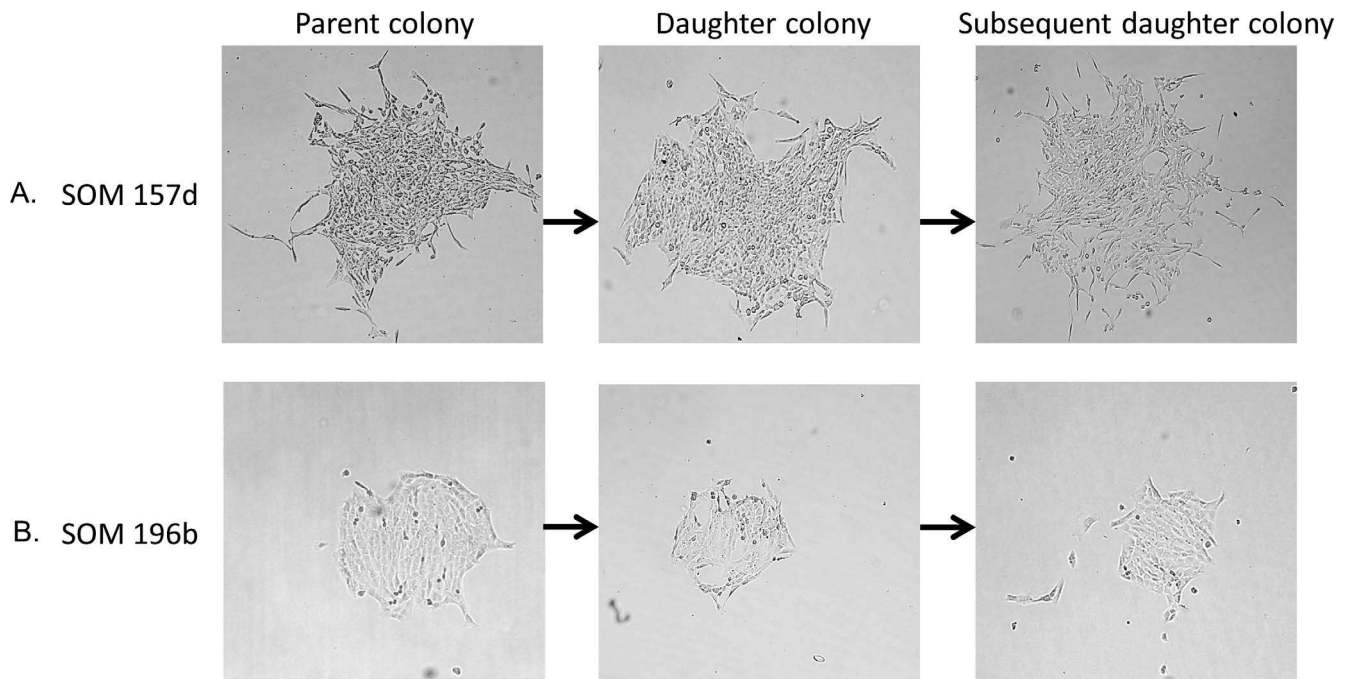
SOM 196b



SOM 588



**FIGURE 2.** Expression of stem cell markers CD44 and CD133. Control cells were stained with IgG antibodies to determine positive gating strategy. CD44 was seen to be expressed in most cells in culture (A) A375 (>95% CD44<sup>+</sup> cells), (C) WM793 (>95% CD44<sup>+</sup> cells), (E) SOM 157d (>95% CD44<sup>+</sup> cells), (G) SOM 196b (>95% CD44<sup>+</sup> cells), and (I) SOM 588 (>95% CD44<sup>+</sup> cells). CD133<sup>+</sup> cells represented a small subpopulation of cells in CM cell lines. (B) A375 (0.62% CD133<sup>+</sup> cells) and (D) WM793 (0.32% CD133<sup>+</sup> cells). CD133<sup>+</sup> cells were absent in both UM cell lines (F) and (H); however, a small subpopulation was detected in one of six UM STCS (J) SOM 588 (0.3% CD133<sup>+</sup> cells). Data represent mean % positive cells, *n* = 3.



**FIGURE 3.** UM cell lines are not organized in a hierarchical manner. When seeded at clonal density, UM cell lines SOM 157d (A) and SOM 196b (B) readily give rise to colonies. When isolated from culture, all colonies were able to give rise to subsequent colonies, which continued to proliferate. In both cell lines, all subsequent daughter colonies resembled the parent colony. There was no difference in morphology or proliferative capacity in daughter colonies.

medium. Tumor pieces were split, and half seeded into CSCM culture conditions and the other half seeded into Oc. Mel. medium. Thirteen STCs (see [Supplementary Table S1](#) for clinical details) were observed over a period of 2 years and their morphology, melanin production, and proliferative rate monitored. Figure 4 demonstrates primary UM cells seeded directly into either Oc. Mel. medium (Fig. 4A) or CSCM (Fig. 4B) at approximately 1 week after seeding. UM cells seeded into Oc. Mel. medium adhered and grew as an adherent monolayer in all 13 UM cases monitored. In Figure 4A, amelanotic spindle cells can be seen around the melanotic tumor piece (white arrows). Over time, all cells in culture appear to be amelanotic spindle cells (Fig. 4C). In contrast, UM cells seeded into CSCM grew as nonadherent spheres and remained highly melanotic over long-term culture. These spheres were termed “melanospheres” and cycled at a much slower rate in comparison to their amelanotic Oc. Mel. counterparts.

To determine whether UM cells were able to switch their phenotype in response to environmental variations, as represented by different media, we reseeded UM cells, cultured exclusively in Oc. Mel. medium for between 3 and 12 months directly from the tumor, into CSCM. All cells in culture exhibited an adherent amelanotic phenotype (Fig. 4C) at the point before reseeding into CSCM. Cells were pelleted, washed with PBS, and subsequently reseeded into fresh CSCM. Following introduction into CSCM, UM-adherent cells detached and formed spheres (Fig. 4D), and produced melanin within 1 month in CSCM culture. By approximately 3 months, very few adherent cells could be seen and all cells continued to grow as melanospheres. To determine whether this phenomenon of phenotypic switching was reversible, we reseeded these melanospheres back into Oc. Mel. medium. We observed that melanospheres that had been exclusively cultured in CSCM from surgery readily attached and downregulated melanin production within 1 week of exposure to Oc. Mel. medium (Fig. 4F). This capacity to switch melanin on and off and

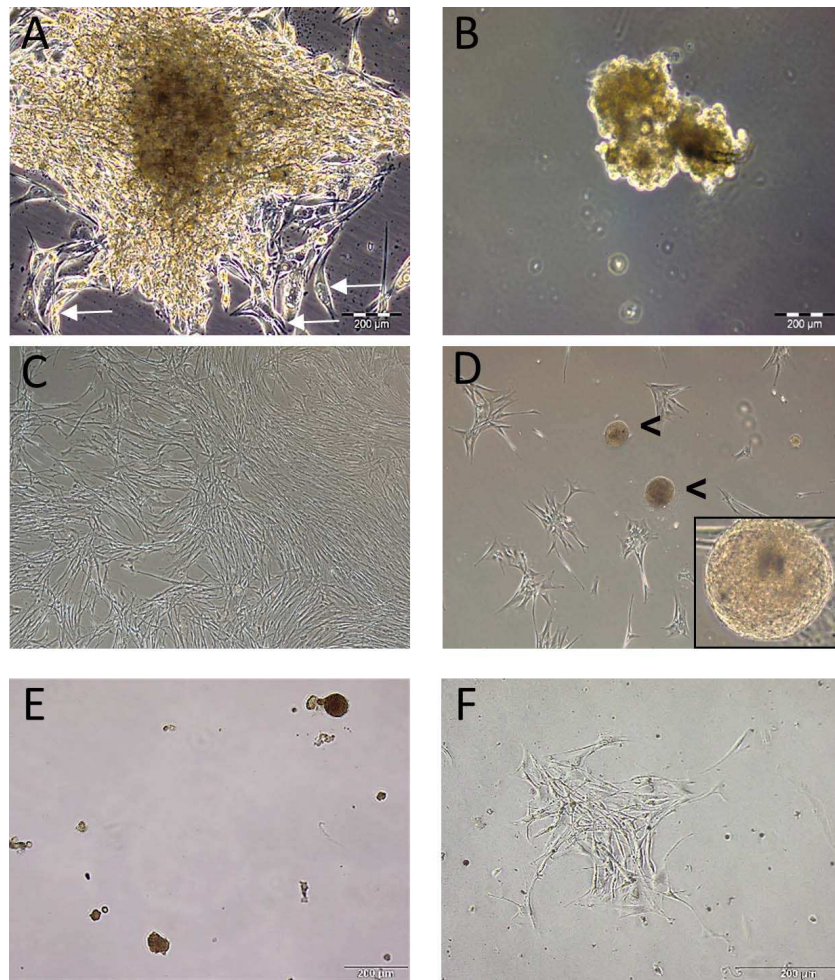
change between adherent and nonadherent was observed in all UM STCs tested.

To determine whether this plastic phenotype is exclusive to UM STCs or whether established cell lines also retain a degree of phenotypic plasticity, we seeded UM cell lines SOM157d and SOM196b into CSCM and observed their behavior. In standard Oc. Mel. Medium, these cells grow as adherent amelanotic spindle-like cells (Fig. 5A); however, when reseeded into CSCM, melanospheres were observed as early as 2 to 3 days after seeding. To determine whether these UM cell lines cultured as melanospheres for an extended period could readily reverse back to an adherent amelanotic phenotype as observed in Oc. Mel. medium, cells were cultured in CSCM for 2 months (Fig. 5B). No adherent cells were observed in culture at 2 months after seeding. Melanospheres were harvested at 2 months, washed in PBS to remove any residual CSCM, and subsequently seeded into Oc. Mel. medium. Within 3 days, melanospheres could be seen to adhere down and amelanotic adherent cells were observed (Fig. 5C). After 1 week, these adherent amelanotic cells had overgrown in culture and melanospheres could no longer be observed. Both SOM157d and SOM196b cells behaved in this way, indicating that established UM cell lines, like UM STCs, retain a degree of phenotypic plasticity in culture.

## DISCUSSION

Tumor progression in cancers such as prostate, colon, breast, and leukemia has been shown to be driven by a rare subpopulation of CSCs. Hence, considerable efforts have been made to identify and target this small subpopulation. In melanoma, however, the CSC hypothesis remains controversial, as CM cells, regardless of the expression of stem cell markers, have been shown to be capable of initiating tumors in immunocompromised mice.<sup>25,26</sup> For example, both CD133<sup>+</sup> and CD133<sup>-</sup> cells from metastatic CM cell lines are reported as





**FIGURE 4.** Phenotypic plasticity in UM STCs. (A) UM STC SOM 585 seeded directly from the tumor into Oc. Mel. medium. Image represents 2 weeks after seeding. *Arrows* indicate adherent amelanotic cells in contrast to the dense melanotic tumor piece. (B) SOM 585 cells seeded directly into CSCM from the tumor 2 weeks after seeding. Cells grew as nonadherent melanospheres. (C) Adherent amelanotic UM STC cells cultured in Oc. Mel. medium for more than 12 months before seeding into CSCM. (D) Cells 1-month post reseeding into CSCM. Melanospheres can be seen to be forming (*black arrows* and *inset*). (E) UM STC cells cultured in CSCM for 11 months before seeding into Oc. Mel. medium growing as melanospheres. (F) One week post reseeding into Oc. Mel. medium melanospheres had adhered and switched off melanin production. Within 3 weeks, no melanospheres were observed in culture and all cells grew as amelanotic adherent cells. *Scale bar*: 200  $\mu$ m.

equally able to give rise to tumors *in vivo*,<sup>48</sup> whereas in contradictory studies only CD133<sup>+</sup> CM cells exhibited tumorigenic capacity.<sup>18,19</sup> Less is known about the existence of CSCs in UM, but a previous study found evidence of CSCs in cell lines from a paired primary and metastatic UM using a clonogenic

assay to identify a cellular hierarchy.<sup>45</sup> The study presented here in UM STCs and cell lines SOM 157d and SOM 196b found no evidence to support a CSC hypothesis based on expression of the putative melanoma stem cell markers chosen and an absence of a cellular hierarchy in the cell lines studied. The marker expression



**FIGURE 5.** UM cell lines also retain a plastic phenotype reflective of UM STCs. SOM 157d cells originally maintained in Oc. Mel. medium (A) were reseeded into CSCM. All cells could be seen to grow as melanospheres within 1 month of seeding (B). After 2 months in CSCM, SOM 157d cells were subsequently reseeded into Oc. Mel. medium. (C) After 3 days, cells reverted to an adherent phenotype and amelanotic cells were seen to grow outward from melanospheres. All cells appeared amelanotic following 7 days' reintroduction to Oc. Mel. medium.



data presented here suggest that in contrast to other solid tumors, expression of ALDH and CD44 does not select for a subpopulation of putative CSCs. The data in ALDH<sup>+</sup> populations in UM STCs supports previous work in CM and suggests an alternative role for ALDH expression in these cancers.<sup>27</sup>

The changeable behavior of both established and STC UM cells demonstrated here supports the hypothesis that UM is able to undergo phenotypic plasticity. We observed that all cells in culture are able to undergo the phenomenon of phenotype switching; supporting previous data in CM and noncancerous melanocytes that these neuroendocrine derivative cell types retain a plastic phenotype and readily adapt in response to their environment.<sup>49,50</sup> Previous studies have shown that fully differentiated melanocytes produce melanin, whereas melanocyte precursors are amelanotic.<sup>51</sup> Changes in melanin production could therefore signal and correlate with dedifferentiation into a precursor state. In this study, UM cells cultured in the presence of 20% FCS grew as amelanotic, highly proliferative cells. The production of melanin by UM cells is, however, not likely to be the only indication of dedifferentiation and may just signify the plasticity of UM cells in response to specific culture conditions. In this study, UM cells cultured in serum-free conditions were found to readily produce melanin and grow as melanospheres. The development of melanospheres demonstrated here mirrors the findings of Zabierowski and colleagues<sup>52</sup> when studying neural crest stem cell precursors. Zabierowski and colleagues<sup>52</sup> determined that activation of the Notch1 pathway alone was capable of reprogramming fully differentiated melanocytes into neural crest stem cell precursors. These precursor cells, like the UM cells in this study, grew as nonadherent spheres under stem cell culture conditions. It would therefore seem that UM cells, regardless of cultural conditions, retain features reminiscent of melanocyte precursors. As further confirmation of this innate ability to be reprogrammed, other studies have found that normal cutaneous melanocytes, as well as CM cells, can dedifferentiate into multipotent self-renewing cells in response to exposure to endothelin-1, endothelin-3, and OCT4.<sup>34,50,53</sup> We therefore hypothesize that cultured cells derived from primary UM also retain the capacity to dedifferentiate, as they too are derived from multipotent, transient neural crest cells, and so their future behavior is not fixed. Ultimately, it seems likely that their inherent changeable phenotype may explain why hierarchical CSCs have not been conclusively identified in UM.

In the present study, we could not identify a CSC population in UM with the markers selected, and although some UM cell lines may have a hierarchical organization, our evidence suggests that in STCs, most cells retain a high degree of plasticity, reminiscent of a precursor state.

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