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Identification of a cancer stem cell-specific function for the histone deacetylases, HDAC1 and HDAC7, in breast and ovarian cancer

AE Witt1, C-W Lee2, TI Lee3, DJ Azzam4, B Wang1, C Caslini1, F Petrocca5, J Grosso1, M Jones1, EB Cohick2,3, AB Gropper3, C Wahlestedt4, AL Richardson2, R Shiekhattar6, RA Young3 and TA Ince1

INTRODUCTION
The cancer stem cell (CSC) model posits that each tumour is composed of a hierarchy of cells, of which only a small subset are CSCs that possess the ability to regenerate tumours in vivo. The remaining tumour bulk is primarily comprised of non-stem-tumour-cells (nsTCs) that descend from CSCs but are themselves incapable of initiating or propagating tumours. Multiple signalling pathways involved in the regulation of CSCs have been identified.1 However, the regulation of CSCs is unlike the reversible short-term changes in cellular phenotype induced by various extracellular factors, or the permanent changes induced by mutations. The hierarchical differentiation of CSCs to nsTCs is long-lasting over many cell generations but it is also reversible, that is more akin to tissue differentiation, which suggests that epigenomic factors such as histone modifications may also be involved in the regulation of the CSC phenotype.2

HDACs are chromatin-modifying enzymes that are involved in regulation of many aspects of cell biology including tissue differentiation, autophagy, apoptosis, migration, mitosis and angiogenesis.3 There are 11 different HDAC genes with distinct tissue-specific expression. The HDAC proteins are grouped by their homology to their orthologues in yeast; class I (HDAC 1-3, 8); class IIa (HDAC 4, 5, 7, 9); class II b (6 and 10), and class V (HDAC 11).3 While the total pan-HDAC activity, including all family members, have been implicated in differentiation, pluripotent and embryonic stem cell regulation,4–10 and the role of specific HDACs in the regulation of CSCs has not been fully explored.

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The exploration of the epigenetic factors that may be involved in the regulation of CSCs has been hampered by experimental and technical challenges, which we partly address in this study. The CSCs can be isolated with fluorescence-activated cell sorting (FACS), using antibodies that recognize putative CSC cell surface markers.1,11–22 However, the rare nature of CSCs in solid tumours, coupled with the methodological challenges of isolating and expanding them in vitro, has impeded the identification of the precise epigenetic regulators responsible for maintaining CSC phenotype.

It is worth pointing out that FACS enrichment of CSCs from primary tumours is not an innocuous process, because it exposes the tumour cells to many changes in temperature, CO2 and pH levels. Since isolation of CSCs by FACS requires single cell suspensions as starting material, the solid tumour fragments are first digested with proteases (such as collagenase and trypsin) at 37 °C for several hours depending on the tissue type (1–8 h). Next, the resulting single cell suspension is incubated with CSC-specific antibodies at 4 °C for 1–2 h, followed by FACS enrichment at 20 °C for another 1–2 h. At the end of this process, in our experience 102–3 fold enrichment of CSCs relative to nsTCs can be achieved by combining two to three CSC surface markers. However, if one is working with a cell line or tumour type with a low 10−5 to 6 CSC
frequency, this would represent at best only one in a hundred cells in the enriched population are indeed CSCs. Therefore, one can isolate relatively CSC-enriched populations with FACS; however, these are not CSC-pure populations by any means. Lastly, even when CSC populations are successfully isolated with FACS, their phenotypic instability limits their utility, because CSCs rapidly differentiate into nSTCs in standard culture.14

In summary, while FACS enrichment is used as a benchmark method to study CSCs, the lack of absolute purity, rapid dilution and potential alterations in CSC phenotype are some of the drawbacks of this approach. In this manuscript we describe a model system that addresses some of these methodological challenges, leading to identification of several HDACs involved in the regulation of CSC phenotype in several model systems.

RESULTS

BPLER cell lines as a model of CSCs

The technical challenges associated with isolation and study of CSCs can be partly circumvented by utilizing a set of genetically engineered cell lines that functionally ‘phenocopy’ CSC behaviours. Previously we derived three independent pairs of genetically matched breast cancer cell lines, BPLER2–4 and HMLER2–4. The BPLER lines were derived from normal human breast precursor epithelial cells that have a mixed luminal-myoepithelial phenotype and maintain telomerase activity in vitro.23,24 In contrast, HMLERs are derived from normal human mammary epithelial cells that exhibit a more differentiated myoepithelial phenotype and do not have telomerase activity.23,24 Both high levels of hTERT activity and lack of lineage commitment have been associated with stem cells in normal tissues. Consistent with the difference in their cell-of-origin, we now demonstrate that BPLER cells recapitulate the mRNA expression profile and the biological properties of CSCs. In contrast, HMLER cells as a whole resemble nSTCs (Supplementary Figures 1–7).

Importantly, the frequency of CSCs in unsorted bulk BPLER cultures is equivalent to the CSC population obtained after FACS enrichment of standard cell lines and is stably maintained in routine 2D culture. Hence, while as few as five unsorted BPLER cells are capable of forming tumours in nude mice, the genetically matched HMLERs require injection of 103–5 cells to form tumours in vivo (Figure 1a). The CSC-like BPLERs also form significantly higher number of tumour spheres compared to matched HMLER lines (Figure 1b), which is a measure of CSC frequency in vitro.25–29

In addition, the BPLER CSC phenotype is stable, maintaining high endogenous levels of the CSC-associated markers CD166,1,11–16 CD326 (EpCAM, ESA),18–20 CD44v22 and BMI-12 throughout extended periods of culture (Figure 1c), and the CSC attributes of BPLER can be even further enhanced by FACS enrichment using individual CSC markers, in particular, CD44 and

Figure 1. BPLER and HMLER cells differ in their in vivo and in vitro tumor initiating cell (TIC) frequency, and in CSC surface marker expression. (a) In serial dilution xenograft assays BPLER lines display 2–4 orders-of-magnitude (102–104) greater in vivo TIC frequency than paired HMLER cell lines. BPLER2/HMLER2, BPLER3/HMLER3, BPLER4/HMLER4 isogenic cell lines were derived from normal human breast primary cells isolated from three different donors as previously described23 (See Supplementary Figure 2a for further details). (b) BPLER lines (blue bars) demonstrate greater capacity for sphere formation than HMLER cells (red bars) in 3D sphere formation assays used as an in vitro measurement of CSC self-renewal. Data presented as a mean +/− s.d. of sphere counts from triplicate wells (P < 0.05). Results are representative of at least three independent experiments. (c) BPLER lines express higher levels of the CSC markers CD264 (EpCAM/ESA), CD166 (ALCAM), and BMI-12 than HMLER. Additionally, BPLER lines express the CSC-specific CD44 isoform (CD44v-250 kDa), while HMLER express the standard CD44 isoform (CD44s-80 kDa). Western blot of whole-cell lysates. β-Actin represents loading control. *BMI-1-matched β-actin in Supplementary Figure 3f. (d) FACS-enriched BPLER CSCs, sorted for high expression of individual CSC markers (CD44, CD166, CD326), or a combination of all three markers, demonstrate enhanced mammosphere formation when compared to BPLER with low CSC-marker expression (black bars, CSC marker high; white bars, CSC marker low). Data presented as a mean +/− s.d. of sphere counts performed in triplicate (P < 0.05). Results are representative of at least three independent experiments.
CD166 (Figure 1d). The unique stability of the CSC phenotype in the BPLER model enabled us to carry out the CSC-specific experiments described below, which would not have been possible with conventional cell lines.

Lastly, we found that the BPLER cells co-cluster with the triple-negative breast cancer (TNBC) cell lines (Supplementary Figure 4), TNBC patient tumours (Supplementary Figures 5 and 6), express a CSC-like mRNA expression signature identified in other cell lines (Supplementary Table 1), and recapitulate the accurate human breast adenocarcinoma morphology in xenograft tumours (Supplementary Figures 7 and 8). These results cumulatively indicate that the BPLER model at least partially phenocopies naturally occurring CSCs.

Discovery of CSC over-expression of HDAC1 and HDAC7 in BPLER CSCs

We hypothesized that specific members of the HDAC family, chromatin-modifying enzymes involved in epigenetic regulation of differentiation, might be involved in the regulation of the CSC phenotype in BPLERs. Since the CSC-like BPLER and nsTC-like HMLER cell line pair are derived from the same patient and transformed with identical oncogenes, they are ostensibly isogenic, which makes them a good model system to survey epigenetic differences between CSC and nsTC. In order to initially examine these potential epigenetic differences, the paired BPLER/HMLER lines were treated with Trichostatin A (TSA), a pan-HDACi that inhibits all the HDAC family members. These dose-response studies reveal that BPLER proliferation is significantly more sensitive to TSA inhibition as compared to matched HMLERs in 2D culture (Figure 2a).

Next, we tested the effect of short-term (24 h) TSA treatment on subsequent 2D proliferation in routine culture vs 3D sphere formation, a surrogate assay for measuring CSC frequencies. Interestingly, we found that TSA pretreatment preferentially inhibits BPLER sphere formation (3D growth). In contrast, standard chemotherapeutics preferentially inhibit HMLERs and 2D proliferation (Figure 2b, Supplementary Figures 9a and b). Furthermore, these conventional chemotherapeutics (Taxol, 5-fluorouracil and Doxorubicin) preferentially induce apoptosis in HMLERs, but not BPLERs (Supplementary Figure 9c), consistent with the chemotherapeutic resistance typically associated with CSCs. Finally, TSA pretreatment preferentially inhibits 3D growth of standard breast cancer cell lines as compared to 2D proliferation (Supplementary Figure 9d). These findings demonstrate that short-term treatment with a pan-HDACi has lasting effects on sphere-initiating capacity in multiple models, and this effect is not simply due to a non-specific inhibition of cell proliferation.

These results prompted us to evaluate whether specific HDAC family members are expressed at higher levels in CSC-like BPLERs compared tonsTC-like HMLERs. We found that only two of the 11 HDAC family members, HDAC1 and HDAC7, were consistently expressed at significantly higher levels in BPLERs at the protein level (Figure 2c). Interestingly, there is no consistent difference at the mRNA level between BPLER and HMLER lines for any of the HDACs 1–11 (Figure 2d), suggesting that these differences are maintained at the protein level in CSCs. The regulation of HDACs at the protein level may be one reason why the association between CSCs and HDAC1/7 has not been identified in previous mRNA profiling experiments. Furthermore, we found that TSA treatment of BPLER lines downregulates HDAC1 and HDAC7 protein expression, as well as CD44 and CD166, suggesting that HDAC-dependent mechanisms may regulate these CSC markers (Figure 2e). Consistent with this, immunofluorescent (IF) staining of unsorted BPLER cells revealed that CD44high/CD166high-CSC subpopulations express higher levels of HDAC7 (and HDAC1, data not shown) compared to CD44low/CD166low cells (Figure 2f).

Verification of HDAC1 and HDAC7 over-expression in primary tumour and cell line CSCs

In order to verify these results in other models, we used FACS to isolate CSCs. Consistent with previous reports, we observed that CD44high/CD166high populations have greater sphere-forming capacity compared to CD44low/CD166low counterparts (Figure 3a)(1,11–14). CD44high/CD166high CSCs from multiple breast (SUM159, MDA-MB-231, MCF7), ovarian (SKOV3, OV90) and colon (HT29) tumour cell lines. These FACS-enriched CSC subpopulations demonstrate increased HDAC enzymatic activity (Figure 3b), and in agreement with the BPLER/HMLER system, consistently expressed significantly higher HDAC1 and HDAC7 levels compared to paired nsTC populations (Figure 3c, Supplementary Figure 10a). Consistent with these findings, IF staining confirmed that CD44high/CD166high cells express higher levels of HDAC7 compared to CD44low/CD166low cells (Figure 3d, Supplementary Figures 10b and c). While several other HDAC family members have variable expression patterns in sorted CSCs, among these HDAC1 and HDAC7 are most consistently associated with CSCs in all cell lines examined.

In addition to CD44 and CD166, we verified the association between HDAC7 overexpression and CSC phenotype using six additional CSC markers (ALDH1a1, ALDH1a3, CD29, CD44v, CD44f and CD326) in a panel of seven additional breast and ovarian cancer lines. These experiments identified a broad correlation between high HDAC7 protein expression with a variety of well-established CSC markers (Figure 3e, Supplementary Figure 10d). These results were also confirmed in tumour cells freshly isolated from seven primary human ovarian and breast cancers, and in formalin-fixed paraffin-embedded sections of primary human breast cancer tissues (Supplementary Figure 11). In sum, we observed a correlation between eight CSC markers and high HDAC7 protein expression in seven cell lines and seven primary tumours using IF staining. These results establish an association between high HDAC1/7 protein and CSCs in multiple cell line models and primary tumour cells.

Re-identification of HDAC inhibitors through a BPLER siRNA lethality screen

Reassuringly, HDAC sensitivity of the BPLER lines was confirmed in an independent genome-wide high-throughput unbiased siRNA lethality screen.31 A genome-wide siRNA screen was used to identify 154 genes that are specifically lethal when inhibited in BPLER cells but not in HMLER cells.31 This signature correlates with poor survival and early onset of metastasis in breast cancer patients independent of tumour subtype.31 We interrogated the mRNA expression of these 154 genes in the Broad Institute connectivity map (CMAP), which contains mRNA expression data from cancer cell lines exposed to 1309 compounds in over 7000 experiments.31 This analysis identified 37 compounds that preferentially reduced the expression of 154 BPLER-lethality genes in other cell lines (Supplementary Table 2). Interestingly, the HDACi, TSA, was the top candidate that emerged out of this in silico screen, and Vorinostat (SAHA) was the fourth most significant (Figure 4a). Next, we evaluated the dose-response of BPLER vs HMLER cells to the top 10 compounds, and found that only the pan-HDACis, TSA and SAHA have differential efficacy towards BPLER (Figure 4b). Interestingly, the other eight in silico candidate compounds did not exhibit any differential effects on BPLER vs HMLER cells (Figure 4b, Supplementary Figure 12). Hence, we found the same differential HDAC sensitivity between BPLER and HMLER cells, both by a hypothesis-driven candidate-based approach and a high-throughput unbiased approach. Remarkably, the two HDAC inhibitors were the only two candidate hits that were confirmed from the high-throughput screen.
Re-identification of HDAC inhibitors in a small molecule CSC lethality screen

In order to confirm the HDAC sensitivity of CSC-like BLERs in other CSC models, we used FACs-isolated CD44\(^{\text{high}}\)/CD24\(^{\text{low+}}\) MDA-MB-231 CSCs. This subpopulation of cells have been previously shown to possess greater sphere-forming potential, increased capacity for tumour-initiation and metastasis in xenograft models, and higher expression of stem-like and metastatic mRNA signatures compared to CD44\(^{\text{high}}\)/CD24\(^{\text{neg}}\) cells.\(^{17,32}\) Consistent with the BPLER results, we found that the CD44\(^{\text{high}}\)/CD24\(^{\text{low+}}\) CSCs express significantly higher HDAC1, HDAC7 and CD166 proteins compared to their CD44\(^{\text{high}}\)/CD24\(^{\text{neg}}\) counterparts (Figure 4c). Also

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**Figure 2.** CSC-Like BPLER cells are associated with high HDAC1 and HDAC7 expression and sensitivity to pan-HDAC inhibitors. (a) The pan-HDAC inhibitor TSA preferentially inhibits BPLER proliferation (blue line), compared to HMLER (red line). The results are representative of at least three independent experiments, presented as a percentage of vehicle treated control, the error bars represent standard deviation of the mean (\(P < 0.005\)). (b) Short-term (24 h) pretreatment with TSA (0.35 μM), preferentially inhibits BPLER sphere formation (3D) in drug-free medium with no effect on 2D proliferation in either BPLER or HMLER. In contrast, pretreatment with Taxol (50 nM) and 5-Fluorouracil (1.0 μM), preferentially inhibit 2D proliferation as compared to 3D sphere formation. The number of viable colonies from triplicate wells were determined by 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride (INT) staining. Results are representative of at least three independent experiments presented as percentage of vehicle treated control (\(P < 0.05\)). BPLER: 2D proliferation (white bars with blue outline) vs 3D sphere formation (blue bars), HMLER: 2D proliferation (white bars with red outline) vs 3D sphere formation (red bars). The error bars represent standard deviation of the mean. (c) BPLER cell lines express higher levels of HDAC1 and HDAC7 proteins compared to matched HMLER lines. Western blot of whole-cell lysates. \(\beta\)-Actin represents loading control. (d) Heatmap of the mRNA expression profile of HDAC1-11 does not reveal any consistent differences between BPLER and HMLER lines (red, increased expression; green, decreased expression). (e) Treatment of BPLER cells for 48 h with TSA (0.35 μM) leads to downregulation of HDAC1, HDAC7, CD44 and CD166 protein expression in BPLER cells. Western blot of whole-cell lysates. \(\beta\)-Actin represents loading control. (f) Double immunofluorescence staining of BPLER cells simultaneously with HDAC7 and CD44 antibodies demonstrate that HDAC7 and CD44 are co-expressed. DAPI (blue), HDAC7 (red) CD44 (green). Scale bar 25 μM.
with BPLER/HMLER, GSK126, which speciﬁes HDACis, including TSA and Vorinostat (Figure 4e). Also, consistent assays. Interestingly, 11 of the 12 positive hits turned out to be tumorigenic, CD44high/CD24neg MDA-MB-231 population in this CMV-driven constructs, we knocked down or overexpressed each were necessary to maintain the CSC phenotype. Using shRNA or subpopulations, we sought to determine whether these HDACs were necessary to maintain the CSC phenotype. Given the high levels of HDAC1 and HDAC7 expression in CSCs isolated from standard breast and ovarian cancer cell lines and examined the effects on the CSC phenotype. Experimental lines were selected based on endogenous HDAC1/7 expression levels (Supplementary Figures 13 and 14).

Consistent with a role for HDACs in CSC regulation, HDAC1 depletion decreases CSC marker expression and inhibits sphere formation (3D) and 2D cell proliferation (2D) (Figures 5a and b). These results are conﬁrmed with additional shRNA constructs in multiple cell lines (Supplementary Figure 13). Furthermore, HDAC1-shRNA expression results in signiﬁcant reduction in xenograft tumour size and ~ 4-fold reduction in tumour-initiating cell (TIC) frequency compared to control cells expressing a scramble-shRNA construct (Figures 5c and d).

Short-term HDAC7 depletion also decreases CSC marker expression, and inhibits 3D proliferation to a signiﬁcantly greater extent than 2D in multiple cell lines, conﬁrmed with several shRNA constructs (Figures 5e and f, Supplementary Figure 14). The speciﬁc inhibition of sphere formation, with lesser effects on 2D cell growth, suggests that reduction of sphere formation by CSCs with HDAC7 depletion is not simply due to a non-speciﬁc effect on regular cell proliferation. We found that HDAC7 protein is upregulated in multiple breast and ovarian cell lines and examined the effects on the CSC phenotype. Experimental lines were selected based on endogenous HDAC1/7 expression levels (Supplementary Figures 13 and 14).

Figure 3. CSCs isolated from standard breast and ovarian cancer cell lines express higher protein levels of HDAC1 and HDAC7, and have increased HDAC enzymatic activity. (a) The higher sphere forming capacity of CD44high/CD166high CSCs (black bars) compared to CD44low/CD166low nsTC cells (white bars) is observed in multiple breast (SUM159, MDA-231, and MCF7) and ovarian (SKOV3 and OV90) cell lines. The data are presented as the mean of sphere counts from triplicate wells, the error bars represent standard deviation of the mean (P < 0.01). The results are representative of at least three independent experiments. (b) The higher HDAC enzyme activity is observed CD44high/CD166high CSCs (black bars) compared to CD44low/CD166low nsTCs (white bars) in multiple breast (SUM159, MDA-231, and MCF7) and ovarian (SKOV3 and OV90) colon (HT29) cancer cell lines. Results are representative of at least three independent experiments. The error bars represent standard deviation of the mean (P < 0.01). (c) HDAC1 and HDAC7 protein expression is signiﬁcantly higher in CD44high/CD166high CSCs (+/+ from multiple breast (BPLER, MDA-MB-231, MCF7, SUM159) and ovarian (SKOV3) cancer cell lines compared to nTC (−/−, CD44low/CD166low). Merged image of two Western blots run with whole-cell lysates, gel1 (BPLER, MDA-MB-231, MCF7, SUM159) and gel2 (SKOV3). β-Actin represents loading control. (d) HDAC7high (red) and CD44high (green) co-expression in ovarian cancer cell line SKOV3, demonstrated with double IF staining. Scale bar 25 μm. (e) Double IF staining identiﬁes co-expression of CSC-markers (CD44, CD326, CD166, ALDH1, CD29) with HDAC7 in standard breast cell lines (MDA-MB-231, MCF7, SUM159) and primary human ovarian (OCI-P5x and OCI-E1p) and breast cancer cells (BCI-1009 and BCI-1133). (Y) represents consistent positive correlation of HDAC7 and CSC marker expression in surveyed lines. (y) represents positive, but inconsistent correlation. (N) represents lack of correlation. (n/a), not analyzed. (n/e) CSC marker not expressed in cell line. IF staining was repeated a minimum of two times for each line. See Supplementary Figure 10 for additional markers and cell lines.

reminiscent of the BPLER results, this difference was only present at the protein level, with no signiﬁcant difference in HDAC mRNA level (Figure 4d).

Having conﬁrmed the association between CSCs and HDAC1/7 in this second cell line model, we used the CD44high/CD24low+ CSCs to screen a library of 60 epigenetic-targeting-compounds. This analysis identiﬁed 12 compounds that preferentially inhibit HDAC1/7 protein, HDAC activity and CSCs in an independent cell assay also preferentially inhibits HMLERs (data not shown). In conclusion, these results establish an association between high HDAC1/7 protein, HDAC activity and CSCs in an independent cell line model.

HDAC1 and HDAC7 are necessary to maintain the CSC phenotype. Given the high levels of HDAC1 and HDAC7 expression in CSC subpopulations, we sought to determine whether these HDACs were necessary to maintain the CSC phenotype. Using shRNA or CMV-driven constructs, we knocked down or overexpressed each
Therefore, long-term \textit{in vivo} tumorigenesis analysis with stable cell lines expressing HDAC7-shRNA was not possible. The short-term shRNA results were also confirmed using transient siRNA transfection (Supplementary Figures 15c and d).

HDAC7 is sufficient to augment the CSC phenotype
These knockdown experiments indicate that HDAC1 and HDAC7 are necessary for the maintenance of the CSC phenotype. We next examined whether HDAC1 or HDAC7 overexpression are also sufficient to augment the CSC phenotype. We found that HDAC7 overexpression upregulated CSC markers, increased sphere formation two- to sixfold, and significantly enhanced sphere size, without any effect on 2D proliferation, as compared to control cells expressing the empty vector (Figures 6a–c, Supplementary Figure 16). Furthermore, HDAC7 overexpression in MCF7 cells upregulates 334 pro-metastatic or CSC-associated genes, alters gene expression of CSC-associated metabolic pathways, and down-regulates expression of established HDAC7 targets and micro-RNAs associated with CSC phenotype (Supplementary Data 1, Supplementary Tables 3–5). Finally, \textit{in vivo} limiting dilution analysis demonstrates that HDAC7 over-expression increases TIC frequency approximately twofold (Figure 6d). These findings indicate that HDAC7 is necessary and sufficient for augmenting the CSC phenotype in these breast cancer cell lines.

Inhibition of HDACs with class-specific drugs inhibits CSC phenotype
Motivated by the potential clinical implications of these findings we explored the effect of isoform-specific HDACis on CSC proliferation. While pan-HDACis (SAHA and TSA) have been used...
to target CSCs, these HDACis indiscriminately target all 11 HDAC family members, resulting in toxicity that limits their clinical use. Consequently, we hypothesized that HDAC1/7-specific HDACis might prove more selective in inhibiting CSCs compared to pan-HDAC inhibitors. Although drugs that inhibit single HDAC isoforms are not currently available, we were able to obtain class selective HDACi.

Of particular interest, we discovered that MS275 (Entinostat) and MGCD0103 (Mocetinostat), members of the benzamide class of HDACi known to specifically inhibit HDAC1/2/3 enzyme...
dramatically downregulated HDAC7 protein levels, with limited effects on the protein levels of other HDAC family members (Figure 7). Based on our knockdown experiments implicating both HDAC1 and HDAC7 as necessary for the maintenance of the CSC phenotype, we found that both drugs preferentially target CSCs, as a 4-day treatment killed \( \geq 85\% \) of BPLER population (as compared to vehicle treated controls), while having no effect on HMLER proliferation (Figure 7a). This differential effect was apparent as early as 24 h post-treatment. Furthermore, 24-h pretreatment of SUM159 with MS275 decreases the number of CD44 hi/CD166hi, CD44 hi/CD133hi and CD44 hi/CD326hi cells as seen by FACS analysis (Supplementary Figure 18).

In order to rule out MS275 and MGCD0103 effects on HDAC2 and HDAC3, we treated BPLER with the HDAC2/3/8-selective Apicidin and HDAC3/6/8-selective Droxinostat, and determined that neither drug downregulates HDAC7 protein levels (Figure 7c). As anticipated from our results, neither drug preferentially inhibits BPLER proliferation (Figure 7a). These results indicate that the selective effects of benzamides on BPLER appear to be due to the combined inhibition of HDAC1 and HDAC7, and not related to HDAC3 or HDAC8.

While single isoform HDAC7-specific inhibitors are not yet available, HDACi that enzymatically inhibit HDAC4/5/7/9 have been developed (MC1568 and MC1575). Although these drugs inhibit HDAC7 enzymatically, they do not alter HDAC7 protein levels (Figure 7c). Interestingly, these drugs preferentially reduce 2D BPLER proliferation, with no effect on HMLER lines (Figure 7a), but have no effect on sphere formation (Figure 7b and data not shown), suggesting combined pharmacological inhibition of HDAC1 and downregulation of HDAC7 protein is important for CSC-specificity.

To confirm these findings in other models we treated several standard breast and ovarian cancer cell lines with the HDACi panel using low-dose, short-term (24 h) pretreatments determined by dose-response studies to have minimal effects on subsequent 2D growth. Compellingly, these studies reiterate that, while pretreatment with MS275 or MGCD0103 significantly reduces or eliminates sphere formation, pretreatment with MC1568 or MC1575 has limited effects on sphere formation in most lines (Figure 7c and data not shown). Similarly, pretreatment with Droxinostat or Apicidin had minimal effects on sphere formation (Figure 7c and data not shown). In addition to standard cell lines, we repeated these experiments in a panel of primary human ovarian cancer cell lines recently established by our lab, and observed that MS275 pretreatment also inhibits sphere formation in all primary lines tested (Figure 7d). Consistent with our results with BPLER cells, these findings suggest that MS275 or MGCD0103 inhibition of sphere formation is not due to a generic inhibition of cell proliferation, and support the concept that these HDACis specifically inhibit CSCs. Furthermore, we found that MS275 treatment of BPLER cells reduces HDAC7 chromatin binding at stem cell associated transcription factor genes (Supplementary Figure 6).
Figure 19), alters Histone 3 (H3) and H3K27 acetylation of stem cell transcription factor genes and reduces expression of several stem cell factors such as myc (Supplementary Figures 20 and 21). Lastly, we found that treatment of a primary ovarian cell line (OCI-P5X), established from a human high-grade serous ovarian carcinoma, inhibited mouse xenograft tumour formation in vivo (Figures 8a–c), and reduced the tumour sphere formation capacity of the explanted cells from these xenograft tumours. (Figure 8d).

**DISCUSSION**

In summary, our results show that the paired isogenic BPLER (CSC) and HMLER (nsTC) cell line pairs can provide a suitable model system to uncover epigenetic differences between CSC and nsTC. From a mechanistic perspective, our results suggest that HDAC1 and HDAC7 may be important for maintaining both normal stem cells and the CSC phenotype. Interestingly, HDAC1 and HDAC7 have been implicated in the regulation of normal pluripotency-associated...
In malignant cells the class IIA HDACs are upregulated in breast and colon cancers compared to other cancers, highly expressed and associated with poor prognosis in astrocytoma, medulloblastoma and in childhood acute lymphoblastic leukemia. HDAC1/7 are shown to be repressed by the CSC-suppressor MiR-34a, which correlate with prognosis in gliomas and osteosarcomas. In addition, it was found that Vorinostat downregulates HDAC7 in bladder and prostate carcinomas.

The class I HDACs are upregulated in gastric, colorectal, esophageal, prostate and pancreatic cancer, high HDAC1 expression is associated with poor prognosis in colorectal cancer, pancreatic cancer, hepatocellular carcinoma, and lung cancer.

So far, the limited clinical studies have been completed with MS275 and MGCD0103 predominantly in leukemia and lymphoma patients. However, as the potential CSC-specific actions of these drugs were previously unknown, CSC biomarkers were not used in patient selection or for measuring patient response. As it appears that the regulation of HDAC1 and HDAC7 levels is predominantly occurring at the protein level, it would be feasible to use immunohistochemistry to stratify patients based on HDAC expression levels for future clinical trials. Furthermore, our results indicate that in addition to inhibiting HDAC1, MS275 and MGCD0103 significantly downregulate HDAC7 protein levels. Thus, it might be possible to take advantage of this observation in monitoring patient response by comparing tumour biopsies before and after treatment. The significant reduction of HDAC7 in post-treatment biopsies might retrospectively distinguish patients in whom effective doses were achieved. A similar strategy might be used to individualize optimum dosing for each patient prospectively.

In standard trial design with cytostatic and cytotoxic drugs, the reduction in tumour size has been one of the main metrics for monitoring patient response. However, as suggested previously, CSC targeting drugs may not cause a rapid reduction in tumour size. This is because CSCs are thought to comprise a small portion of the tumour bulk and the remaining nsTC can proliferate for some time even when CSCs are completely eliminated. Hence, the effects of CSC targeting drugs may take a long time to result in a clinically measurable reduction in tumour size. The lag in clinical response will depend on how many population doublings nsTC can go through without being replenished by CSCs, which may be patient and tumour type dependent. These considerations make the use of biomarkers such as HDAC1 and HDAC7 for patient selection and monitoring response particularly compelling as a supplement to tumour size measurements.

Our results also indicate that these drugs should be tested more extensively in solid tumours, perhaps in combination with conventional chemotherapeutics to target the nsTC comprising the tumour bulk. This strategy may address some of the considerations regarding the lag time in tumour response with CSC-targeting drugs outlined above. Furthermore, it is thought that CSCs might be particularly relevant in recurrence and metastasis. Hence, a reduction in long-term recurrence might be an important metric, in addition to tumour size, to assess the role of CSC targeting drugs. To our knowledge the CSC-specific strategies outlined above were not used for patient selection or assessing response in previous studies. This is expected because the CSC-specific actions of MS275 and MGCD0103 we describe...
here were previously not fully recognized. Hence, the response in ongoing and previous studies to HDAC inhibitors should be viewed with caution; it is possible that better results might be achieved with careful attention to CSC-associated activities of this class of drugs. We hope that this report will stimulate efforts for more innovative clinical trials and development of more selective HDAC1 and HDAC7 inhibitors that may be even more effective than those currently available.

MATERIALS AND METHODS

Cell culture

The BPE, BPLER and OCI cells and their culture media BMI-P, BMI-T and OCMi are available from the Live Tumor Culture Core (LTCC) LTCC@med.miami.edu, at Sylvester Comprehensive Cancer Center (SCCC), Miller School of Medicine, University of Miami http://sylvester.org/shared-resources/Live-Tumor-Culture-Core. The BPE and BPLER cells were cultured in the BMI-P and BMI-T medium, respectively. The primary breast cancer cells (OCI) were cultured in OCMi medium as previously described,45 while the primary ovarian cancer cells (OCI) were cultured in OCMi medium as previously described.23,45 The standard cancer cell lines were cultured in the BMI-P and BMI-T medium, respectively. The primary ovarian cancer cells (OCI) were cultured in OCMi medium as previously described.23 The standard cancer cell lines were cultured in ATCC recommended media, at 37 °C and 5 % CO2 on standard plates (Falcon, New York, NY, USA). All standard cancer cell lines were recently short tandem repeat profiled and tested for mycoplasma contamination prior to experimentation. The BPE, BPLER, OCI and BCI cells are cultured on Primaria plates (BD Biosciences, San Jose, CA, USA) as previously described.23,45

Serial dilution in vivo (BPLER/HMLER)

Single-cell suspensions were prepared in a WIT:Matrigel (1:1) mixture and injected in 25 μl (orthotopic) or 100 μl (subcutaneous) volumes. Female athymic nude mice (Balb/c nu/nu, Taconic) were irradiated (400 rad) 12 h prior to subcutaneous injections. Sample size was determined by degree of dilution as follows: nine mice/experimental group for 105 and 104 dilution conditions, 12 mice/experimental set for 103, 102 and 101 dilution conditions. Animals were randomly assigned to each treatment group. Injections of tumorigenic cells into mammary fat pads were performed in 8-week-old female Nod/Scid mice that were anesthetized with intraperitoneal injections of pentobarbital (50 mg/kg). The primary breast cancer cells (BCI) were cultured in BCMI (unpublished). The primary ovarian cancer cells (OCI) were cultured in OCMi medium as previously described.45 The standard cancer cell lines were cultured in the BMI-P and BMI-T medium, respectively. Animals were randomly assigned to each treatment group. All treatments were done in triplicates, and repeated a minimum of three times. The error bars represent the standard error of the mean of the three replicates.

Inhibition of 2D cell proliferation with continuous drug treatment

To assess the effect of long-term drug treatment on 2D proliferation of BPLER and HMLER cells, they were plated at a density of 250 000 cells/well in six-well Primaria plates. After 24 h, drug-containing media was added to each well. Cells were treated continuously for 4 days, at which point cell counts and viability (based on Trypan blue exclusion) were quantified using a Nexcelom Cellometer, and assessed as a percentage of control cells. All treatments were done in triplicates, and repeated a minimum of three times.

Inhibition of 2D/3D cell proliferation with drug pretreatment

To assess effect of short-term drug pretreatment on 2D/3D growth, cells are plated in six-well plates and allowed to recover for 24 h. At this point, drug-containing media was added to each well. After 24 h of treatment, cell counts were then plated for mammospheres or 2D proliferation assays. 2D proliferation was determined after 3 days by viable cell counts, and assessed as a percentage of the dimethyl sulfoxide control. All treatments were carried out in triplicate, and repeated a minimum of three times. The error bars represent the standard error of the mean of the three replicates. The small molecule drugs that are used to treat the cells for various experiments are: TSA (Selleck, Boston, MA, USA) Doxirubicin (Selleck), Paclitaxel (Selleck), 5-Fluorouracil (Sigma), SAHA (Selleck), GSK126 (Cayman Chem), MG275 (Selleck), MC20103 (Selleck), MC1568 (Selleck), MC1575 (Gift of Dr. Antonello Mai), Apicidin (Sigma), Droxinostat (Selleck). All drugs are prepared in dimethyl sulfoxide, which was used as vehicle control in each assay.

mRNA expression analysis

mRNA analysis as previously described22 using Affymatrix human 133 2.0.

Apoptosis

The cells lines were treated with drugs for 24 h at the following doses: 5-FU (50 μM), Taxol (50 μM), Doxirubicin (5 μM). Cells were then assayed for apoptosis with Annexin V-FITC apoptosis kit (Abcam#ab14155) as per the manufacturer’s protocol. All experiments were carried out in triplicates, and repeated a minimum of three times.

Immunofluorescence

The cells were plated on to eight-well chamber slides, fixed with 4 % paraformaldehyde for 15 min, washed three times with PBS/100 mM Glycine, and then permeabilized for 15 min with 0.5 % Tween-20. Slides were then blocked for 1 h in 10 % Goat serum/0.1 % BSA/PBS, incubated for 1.5 h with primary antibody, followed by 45 min incubation with fluorophore-conjugated secondary antibody. Slides were mounted with Fluoro-Gel II, with DAPI (EM Sciences, Hatfield, PA, USA) for nuclear staining. Cells were imaged on an Evos fluorescence microscope (AMG & Life Tech, Millcreek, WA, USA). Antibodies: HDAC7 (Abcam#ab12174), CD44 (R&D#BB8A10), CD166 (AbD Serotec, Raleigh, NC, USA; #MC19267). All experiments were repeated a minimum of three times.

HDAC activity assay

The cells were harvested and sorted by flow cytometry as described above. After three washes with cold HBSS buffer cells were lysed in cold lysis buffer and centrifuged at 4 °C. The protein concentration of the cell lysates were quantified by Coomassie Plus Reagent (Thermoscientific #1856210). HDAC activity assay (Cayman Chemical Co., Ann Arbor, MI, USA;...
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#10011563) was performed according to the manufacturer's protocol. The data are presented as fluorescence units. All experiments were carried out in triplicates in each of at least three independent experiments.

shRNA knockdown of HDAC1 and HDAC7

The cell lines were transduced with GIPZ lentiviral shRNA constructs purchased from Open Biosystems (Lafayette, CO, USA)/ThermoScientific (Catalog # V3LHS_351665, V2LHS_96400, V2LHS_96401). HDAC1 cell lines were transduced with MISSION shRNA purchased from Sigma (Catalog# TRCN0000195467, TRCN0000195672, TRCN0000195103, TRCN0000195672). The cell lines that stably express the shRNAs were selected for puromycin resistance. All experiments were performed in triplicate, and repeated a minimum of three times.

siRNA knockdown of HDAC7

HDAC7 siRNA (Sigma#EUHU78781) was transfected into cell lines with Mission Transfection Reagent (Sigma#S1452) as per manufacturer's protocol. Transfected cells plated in triplicate in 2D/3D assays and lysates harvested at 72 h post-transfection. All experiments were repeated a minimum of three times.

Over-expression of HDAC1 and HDAC7

HDAC1-Flag (provided by Eric Verdin through Addgene, Cambridge, MA, USA; plasmid#13820) and HDAC7 (purchased from Origene, Rockville, MD, USA; cat. #RC215233) were cloned into pENTR vectors. These constructs were recombined into pLenti CMV DEST lentiviral vectors (provided by Eric Campeau through Addgene). Lentivirus was packaged in 293T cells using the manufacturer's recommended protocols. Stable lines were selected with G418 (HDAC7) or Blast (HDAC1). All experiments were carried out in triplicate, and repeated a minimum of three times.

Serial dilution in vivo (knockdown/overexpression)

MDA-MB-231-luciferase cells were transduced with lentivirus and selected for stable expression of HDAC7-CMV, empty vector control (EV), HDAC1 shRNA (Construct #1 and Construct #2), or a scramble shRNA control. HDAC7-overexpression, or HDAC1 knockdown was confirmed by western blot. Serial dilutions of MDA-MB-231-luciferase cells were injected (1,000,000, 100,000, 10,000, 1000, or 100 cells suspended in 100 μl of HBSS/Matrigel) into the mammary fat pad of 5-week-old, female, Balb/C nude mice (Charles River, Wilmington, MA, USA) at the rate of three injections per mouse. The mice were randomly assigned to each treatment group. Tumours were followed by weekly IVIS analysis beginning at 5 weeks. The mice that died post-injection and prior to the beginning at 5 weeks. The mice that died post-injection and prior to the weekly IVIS analysis beginning at 1 week. Tumour luciferase signals were analysed by Living Image In Vivo imaging software (Perkin Elmer, Waltham, MA, USA) and plotted using GraphPad Prism 5.0. Investigators were not blinded in the animal studies. All animal studies were approved and performed in accordance with relevant institutional IACUC committee guidelines.

Serial dilution in vivo (MS-275 treatment)

The primary ovarian cancer cell line OCI-P5x that expresses luciferase-GFP (OCI-P5x Fluc-IRES-GFP) was plated on T-75 flasks and treated with either 0.5 μM MS-275 or vehicle control for 24 h. After trypsinization, serial dilutions were injected (1,000,000, 100,000, 10,000, or 1000 cells suspended in 100 μl of 1:1 HBSS: Matrigel) into the left and right flanks of 5-week-old Balb/C nude mice (Charles River). Animals were randomly assigned to each treatment group. Tumours were followed by weekly IVIS analysis beginning at 1 week. Tumour luciferase signals were analysed by Living Image In Vivo imaging software (Perkin Elmer) and plotted using GraphPad Prism 5.0. Investigators were not blinded in the animal studies. All animal studies were approved and performed in accordance with relevant institutional IACUC committee guidelines.

Statistical analysis

Cell proliferation, sphere formation and HDAC activities were compared by two-tailed Student's t-test, using GraphPad Prism 5.0, GraphPad Software with P < 0.05 considered significant. Limiting dilution analysis was done with http://bioinf.wehi.edu.au/software/elda/index.html.

CONFLICT OF INTEREST

Tan A. Ince discloses pending patent intellectual proprietary interest as the inventor of BMI and OCM media.

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AUTHOR CONTRIBUTIONS

Conception, design and study supervision (Tan A. Ince, Abigail E. Witt), development of methodology and cell culture medium (Tan A. Ince), acquisition of data (Abigail E. Witt, Chung-Wei Lee, Tong Ihn Lee, Diana J. Azzam, Fabio Petrocca, James Grosso, Evan B. Cohick, Bin Wang, Corrado Caslini, Michelle Jones and Adrienne B. Gropper), bioinformatic, computational, statistical analysis and interpretation (Tan A. Ince, Abigail Young, Fabio Petrocca, Andrea L. Richardson, Ramin Shiekhattar), writing, review and revision of the manuscript (Tan A. Ince and Abigail E. Witt).

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