Down-regulation of the Fetal Stem Cell Factor SOX17 by H33342

A MECHANISM RESPONSIBLE FOR DIFFERENTIAL GENE EXPRESSION IN BREAST CANCER SIDE POPULATION CELLS

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Human solid tumors contain rare cancer side population (SP) cells, which expel the fluorescent dye Hoechst 33342 (H33342) and display cancer stem cell characteristics. Transcriptional profiling of cancer SP cells isolated by H33342 fluorescence analysis is a newly emerging approach to discover cancer stem cell markers and aberrant differentiation pathways. Using Affymetrix expression microarrays and quantitative reverse transcription-PCR, we investigated differential gene expression between SP and non-SP (NSP) cells isolated from human mammary carcinoma cell lines. A total of 136 genes were up-regulated in breast cancer SP relative to NSP cells, one of which was the fetal stem cell factor and Wnt/β-catenin signaling pathway target SOX17. Strikingly, we discovered that SOX17 was down-regulated by H33342 in a dose-dependent manner. In SP cells, which expel H33342, down-regulation of SOX17 was less pronounced than in NSP cells, which retain H33342. As a result of this, SOX17 displayed a 10–20-fold overexpression in cancer SP relative to NSP cells. Similar results were obtained for further stemness-related genes, namely EPC1 and SPRY1. These findings establish a previously unidentified gene-regulatory impact of H33342 as a novel mechanism responsible for differential gene expression in cancer SP cells. This has significant implications for the future interpretation of cancer SP cells.

One view of cancer is that it arises from and is maintained by a small number of cancer stem cells (1). According to this concept, only cancer stem cells self-renew infinitely, whereas most of their progenies undergo aberrant differentiation and consecutively cease proliferation. Several lines of evidence suggest that tumor recurrence, chemoresistance, and metastasis are driven by a yet poorly defined subset of cancer stem cells (2, 3). There is an enormous clinical interest in cancer stem cell assessment as a future tool in the diagnosis and treatment of cancer (4). However, with respect to human solid tumors, the development and evaluation of robust techniques for the detection of putative cancer stem cells are still awaited. Hoechst 33342 (H33342)2 dual wavelength fluorescence analysis has been proven to be a remarkably powerful technology for the identification and isolation of adult tissue stem cells (5–7). This technique identifies so-called side population (SP) cells, which pump out the fluorescent dye H33342 and thus form a tail of dimly stained cells in red versus blue bivariate fluorescence-activated cell sorting dot plots (7, 8). Based on this dye efflux phenomenon, SP cells have been isolated from a wide variety of mammalian tissues and have been shown to contain multipotent stem cells (7). Great excitement has been generated by the finding that cultured human cancer cells, established cancer cell lines, and xenograft tumors possess a small fraction of cancer SP cells, which apparently represent the tumorigenic subset within a clonal cell population (9–11). Enhanced in vitro growth and in vivo tumorigenicity have been demonstrated for SP cells isolated from human gastric cancer (12), hepatocellular carcinomas (13), breast cancer (14), lung cancer (15), nasopharyngeal cancer (16), prostate cancer (11), and sarcomas (17). Contrary to cancer SP cells, freshly isolated and recultivated cancer non-SP (NSP) cells initially form very small colonies but later on fail to sustain proliferation in vitro or regress after a latency period of 1 or 2 weeks (18, 19). This has been interpreted as a deficiency of prolonged self-renewal ability of cancer NSP cells (13, 18) rather than an effect of the H33342 dye, which, although initially introduced as a nontoxic vital dye, may diminish cell vitality at higher concentrations (20). Remarkably, however, cancer SP cells generate heterologous descendent cells that no longer maintain the SP phenotype (15, 19, 21). Cancer SP cells isolated from genetically engineered mouse tumor models also display these stem cell-like properties (18, 22, 23). Thus, cancer SP cells are well established as bona fide cancer stem cells (24). Preliminary expression analyses suggested an up-regulation of stemness-related genes, such as NOTCH1, in cancer SP cells relative to NSP cells (11). Recently, global transcriptional profiling of cancer SP versus NSP cells has emerged as a novel approach to discover surrogate markers of cancer stem cells and to dissect aberrant intratumoral differentiation pathways (14, 22, 25, 26). Using Affymetrix gene expression microarrays and quantitative RT-PCR analyses, the

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2 The on-line version of this article (available at http://www.jbc.org) contains supplemental Tables 1 and 2 and Fig. 1.

The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo accession no. GSE18773.

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present study investigated differential gene expression between cancer SP and NSP cells isolated from the CAL-51 and UACC-893 human mammary carcinoma cell lines. Here, we establish a previously unidentified gene-regulatory impact of H33342 as a novel mechanism responsible for differential expression of stemness-related genes in cancer SP cells. This is critical for the future understanding and evaluation of cancer SP cells as putative cancer stem cells.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—The human breast cancer cell lines CAL-51 and UACC-893 were obtained by the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany) and the American Type Culture Collection (ATCC, Manassas, VA), respectively. Culture conditions and routine maintenance have been described previously (19). Proliferation capacity of cancer SP cells was determined using an assay previously described by Patrawala et al. (11). Briefly, sorted cells were plated on 96-well plates at a density of 1 cell/well using a MoFlo cell sorter. Colony sizes (cells/colony) of n = 50 colonies (defined as a cluster of at least 4 cells) were assessed manually for several time points as described under “Experimental Procedures.”

**Gene Expression Microarray Analysis**—For global transcriptional profiling, cells were lysed in RLT buffer (Qiagen, Hilden, Germany) and subjected to extraction of total RNA. Quality and integrity of the RNA preparations were controlled by running all samples on a 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). RNA amplification and labeling were done according to the manufacturer’s protocol (Small Sample Target Labeling Assay Version II; Affymetrix, Santa Clara, CA), and 10 μg of each biotinylated cRNA preparation was fragmented and placed in a hybridization mixture containing four biotinylated hybridization controls (BioB, BioC, BioD, and Cre) as recommended by the manufacturer. Samples were hybridized to an identical lot of Affymetrix HG U133 Plus 2.0 GeneChips for 16 h at 46 °C. Subsequently, GeneChips were washed, stained with SA-PE, and read using an Affymetrix GeneChip fluidic station 400 and an GCS 3000 scanner. Extracted probe set signal intensities were normalized using the MASS normalization algorithm (Affymetrix). Data from all samples and probe sets were uploaded into the statistical package BRB-ArrayTools version 3.5.0-Patch_2 for filtering and statistical analyses (27). Signal intensities of probe sets with low signal intensity (<70) were raised to the threshold of 70. All subsequent analyses were done on data from log2-transformed expression values. Probe sets showing minimal variation across the set of samples were excluded from the analyses. Probe sets whose expression differed by at least 1.5-fold from the median in at least 20% of the samples were retained (2101 probe sets). Cell fraction (SP versus NSP) was introduced as a new variable into the BRB-ArrayTools experiment descriptor for examination of the expression data by scatter plot analysis (27). Then, two-class comparison was performed to identify genes differentially expressed between SP and NSP cells using parametric t tests. Genes were considered statistically signifi-
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FIGURE 3. Detection and characterization of cancer SP cells in the UACC-893 breast cancer cell line. A, H33342 dual wavelength fluorescence analysis following labeling of UACC-893 cells with $5 \, \mu g/ml$ H33342 for 90 min according to international standard procedures (upper left). Verification of proper labeling was performed by SP inhibition with the broad range ABC transporter inhibitor verapamil (50 \, \mu m) and the ABCG2-specific inhibitor fumitremorgin c (10 \, \mu m). Percentages given in the upper left corner of the dot plots refer to the relative abundance of SP cells. Dot plots in the upper right panel present H33342 fluorescence profiles of sorted SP cells and NSP cells. B, Quantitative RT-PCR analyses in SP and NSP cells isolated from the UACC-893 breast cancer cell line. Two housekeeping genes (TBP and \( \beta \)-GUS) served as reference. Data are presented as relative expression with NSP cells adjusted to a value of 1 for each gene individually. Error bars represent S.E.; n.s., not significant.

Gene Ontology Enrichment Analysis—Functional annotation and gene ontology (GO) term enrichment analysis were performed with DAVID software (28). The GO chart analytical module was run with an EASE score of 0.1 and a count threshold of 9. Affymetrix probe sets with a signal intensity of $\geq 70$ in CAL-51 ($n = 14.151$) served as population background. Term enrichment $\geq 2$-fold was considered relevant (28).

Quantitative RT-PCR—Extraction of total RNA and cDNA synthesis were performed as described previously (29). Quantitative assessment of gene expression normalized to two housekeeping genes (\( \beta \)-GUS and TBP) was performed with Platinum Taq DNA polymerase (Invitrogen), Sybr Green I (Invitrogen), and QuantiTect\textsuperscript{\textregistered} primer assays (Qiagen) on an ABI Prism 7700 system (Applied Biosystems, Foster City, CA).

Statistical significance was determined by parametric \( t \) tests using GraphPad Prism software.

Western Blotting—Cells were lysed in radioimmune precipitation assay buffer, and 40 \, \mu g of total cellular protein was separated by 12% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were probed with anti-SOX17 (clone 2F9; OriGen Technologies, Inc., Rockville, MD) and anti-\( \beta \)-actin antibodies (clone AC15; Acris, Hiddenhausen, Germany). 293T cells transiently transfected with a pCMV6 expression vector encoding for full-length SOX17 (OriGen Technologies) served as positive control. Digital band densitometry was performed with AIDA software.

RESULTS

The CAL-51 human breast cancer cell line is composed of $\sim 99\%$ NSP cells and a small fraction of about 1% classical SP cells, which expel H33342 in a verapamil- and fumitremorgin c-sensitive manner (Fig. 1A) (19). Consistent with the findings in other cancers and our previously reported data (16, 18, 19), freshly isolated and recultivated CAL-51 NSP cells initially formed very small colonies but later on failed to sustain proliferation, whereas purified CAL-51 SP cells maintained neoplastic cell growth (Fig. 1B). Thus, CAL-51 represents a suitable model to study cancer SP cells, which are thought to function as cancer stem cells (24). To investigate differential gene expression between cancer SP and NSP cells, three different, consecutive cell culture passages of CAL-51 were each subjected independently to H33342 dual wavelength fluorescence analysis and were each sorted into SP and NSP cell fractions (Fig. 2A). Subsequently, these biological replicates of CAL-51 SP and NSP cells were all subjected to global transcriptional profiling using Affymetrix expression microarrays.

FIGURE 2. Global transcriptional profiling of CAL-51 SP cells using Affymetrix gene expression microarrays. A, replicate isolations of SP and NSP cells from CAL-51. Three different, consecutive cell culture passages of CAL-51 were each subjected independently to H33342 dual wavelength fluorescence analysis and were each sorted into SP and NSP cell fractions. Dot plots in the upper panel present H33342 fluorescence profiles prior to the sorts. Percentages given in the upper left corner of the dot plots refer to the relative abundance of SP cells. Dot plots in the lower panel present H33342 fluorescence profiles of sorted SP cells and NSP cells. B, examination of the expression data by scatter plot analysis. Affymetrix signal log values of NSP cells from sort 1 are plotted on the abscissa, and signal log values of SP cells isolated from the same culture dish (also from sort 1) are plotted on the ordinate. Each dot represents a single probe set. Thin lines indicate 2-fold expression difference. Outliers are highlighted in red. C, scatter plot analysis of SP versus NSP cells obtained from different, consecutive cell culture passages by sorts 1 and 2, respectively. D, scatter plot analysis of SP versus NSP cells obtained from different, consecutive cell culture passages by sorts 1 and 2, respectively. E, two-dimensional presentation of gene expression pattern for 144 genes identified as differentially expressed in CAL-51 SP relative to NSP cells at the $p \leq 0.001$ significance level. For visualization, cluster analysis was performed for 175 probe sets corresponding to the 144 differentially expressed genes. Each column represents a sample, and each row represents a single probe set. Expression levels above the mean of individual genes/probe sets are shown in red, and expression levels below the mean of individual genes/probe sets are shown in green. F, functional annotation and GO term enrichment analysis of 136 genes up-regulated in CAL-51 SP relative to NSP cells. Each section of the pie chart represents a GO term enriched $\geq 2$-fold in the genes up-regulated in CAL-51 SP cells compared with a population background consisting of genes generally expressed in CAL-51. The size of the pie chart sections reflects the number of genes associated with a GO term. Fold enrichment and number of genes are given in parentheses. Examples of genes associated with a GO term are highlighted in red. Genes previously implicated in SP cells are underlined. G, validation of selected genes identified as up-regulated in CAL-51 SP relative to NSP cells by quantitative RT-PCR. Two housekeeping genes (TBP and \( \beta \)-GUS) served as reference. Data are presented as relative expression with NSP cells adjusted to a value of 1 for each gene individually. Error bars represent S.E.
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Examination of the expression data by scatter plot analysis indicated that CAL-51 SP and NSP cells from the same culture passage had clearly dissimilar gene expression profiles (Fig. 2B). Noteworthy, NSP cells from different culture passages showed virtually identical gene expression patterns (Fig. 2C). SP cells from different culture passages showed almost identical gene expression profiles as well (Fig. 2D). Hence, a two-class comparison was performed, in which the cell fraction represented the class-defining label. At the $p = 0.001$ significance level, parametric $t$ tests identified 165 probe sets corresponding to 136 genes as up-regulated and 10 probe sets corresponding to 8 genes as down-regulated in CAL-51 SP cells (Fig. 2E and supplemental Table 1). Functional annotation and gene ontology term enrichment analyses revealed that the genes up-regulated

SOX17. Strikingly, brief H33342 exposure indeed induced a dose-dependent down-regulation of SOX17 in both CAL-51 and UACC-893 cells (Fig. 4, A–C). Next, SOX17 mRNA levels were monitored during the entire process of SP cell detection and isolation performed according to international standard procedures. In unsorted CAL-51 cells, SOX17 mRNA expression declined 13-fold in response to the labeling with H33342 (Fig. 4D). In isolated CAL-51 SP cells, SOX17 mRNA expression had declined 60-fold, whereas in CAL-51 SP cells, SOX17 mRNA expression had declined only 3-fold and slowly recovered during long term recultivation (Fig. 4D). As a result of the differential suppression following H33342 labeling, SOX17 mRNA expression was 20-fold higher in CAL-51 SP cells compared with CAL-51 NSP cells (Fig. 4D). Similar obser-
vations were made for additional genes. The enhancer of polycomb EPC1 and the regulator of branching morphogenesis SPRY1 were both up-regulated in SP relative to NSP cells isolated from CAL-51 and UACC-893 (Figs. 2G and 3B). Like SOX17, both genes were down-regulated by H33342 in a dose-dependent manner, indicating that this was the underlying mechanism responsible for the differential expression of those genes between cancer SP and NSP cells (Fig. 4, A and B). However, H33342 did not influence the expression of the basal mammary epithelium marker JAG1, which was up-regulated in CAL-51 SP cells (Fig. 2G and supplemental Fig. 1). Moreover, H33342 did not affect the expression of the ABC transporter ABCG2, the mediator of H33342 efflux, which is preferentially expressed in cancer SP cells (supplemental Fig. 1).

**DISCUSSION**

The present study investigated gene expression in putative cancer stem cells isolated as SP cells. Using the example of SOX17, a fetal hematopoietic stem cell factor and Wnt/β-catenin signaling pathway target (30, 31), we herein establish a previously unidentified gene-regulatory impact of H33342 as a novel mechanism responsible for differential gene expression in cancer SP cells. Up-regulation of SOX17 in breast cancer SP relative to NSP cells was identified by global transcriptional profiling and was validated by quantitative RT-PCR. Subsequently, we demonstrated that SOX17 is transcriptionally down-regulated by H33342 in a dose-dependent manner. In SP cells, which expel H33342, down-regulation of SOX17 was less pronounced than in NSP cells, which retain H33342. This differential suppression of SOX17 was shown to be the underlying mechanism responsible for a 10–20-fold overexpression of SOX17 in breast cancer SP relative to NSP cells. Similar observations were made for further stemness-related genes, namely EPC1 and SPRY1. Interestingly, Zheng et al. (33) have previously speculated that H33342 labeling may selectively deprive cancer NSP cells of stem cell characteristics or factors. The present study provides the first direct evidence that H33342 labeling indeed induces differential expression of well-established stem cell genes, such as SOX17, in cancer SP versus NSP cells. Moreover, a brief survey of recently published profiling data revealed that SOX17 and EPC1 were among the candidate genes differentially expressed between SP and NSP cells from markedly different cellular contexts (25, 34, 35). This suggests that the transcriptional deregulation of SOX17 and EPC1 by H33342 during cancer SP cell isolation is a common phenomenon. Further stemness-related genes, such as NOTCH1 and CTNNB1, whose preferential expression in cancer SP cells has been reported before and reasoned to reflect the cancer stem cell nature of cancer SP cells (11), may in fact be deregulated in an H33342-dependent manner as well. Taken together, this has significant implications for the understanding of cancer SP cells: (i) due to the gene-regulatory impact of H33342, neither cancer SP nor NSP cells maintain their original gene expression profile after the cell isolation, highlighting that comparative transcriptional profiling of isolated SP cells versus unsorted, H33342-unlabeled or NSP cells does not allow the distinction between true differential expression in SP cells and unwanted side effects of H33342; (ii) candidate genes or surrogate markers proposed to characterize cancer SP cells in their original state require a refined validation by demonstrating independence of their expression from H33342 exposure; (iii) due to the differential deregulation of multiple genes by H33342 in cancer SP relative to NSP cells, neither NSP cells nor H33342-unlabeled cells represent appropriate controls for the functional analysis of cancer SP cells. With these prerequisites in mind, one will refrain from further comparing and interpreting growth characteristics of cancer SP and NSP cells because of the overlapping effects of truly different cellular phenotypes and H33342-induced cellular changes. These conclusions necessitate a much more careful interpretation of the cancer SP cell phenotype in future studies. However, they should not give cause generally to reject the notion that cancer SP cells represent a sort of stem cell-like cancer cells. There is no doubt that SP cells from a wide variety of nonmalignant mammalian tissues are multipotent stem cells (7). Currently, however, there is no compelling explanation for the presence of SP cells in cancer except that these cancer SP cells are either derived from or mimic nonmalignant adult tissue stem cells (24). It is interesting to note that this study successfully identified JAG1, a basal mammary epithelium marker, as up-regulated in CAL-51 breast cancer SP cells. Importantly, up-regulation of JAG1 was not related to H33342-dependent gene perturbation, and JAG1 has previously been implicated in the regulation of putative breast cancer stem cells (36–38). Moreover, expression of JAG1 is associated with poor outcome in breast cancer patients in the clinics (39–41). Thus, cancer SP cells may prove to be a valuable resource for the discovery of cancer stem cell surrogate markers or aberrant differentiation pathways if future analyses account for the gene-regulatory impact of H33342.

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