Targeting Signal Transducer and Activator of Transcription 3 Pathway by Cucurbitacin I Diminishes Self-Renewing and Radiochemoresistant Abilities in Thyroid Cancer-Derived CD133\(^+\) Cells

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ABSTRACT

Anaplastic thyroid cancer (ATC) is a lethal solid tumor with poor prognosis because of its invasiveness and its resistance to current therapies. Recently, ATC-CD133\(^+\) cells were found to have cancer stem cell (CSC) properties and were suggested to be important contributors to tumorigenesis and cancer metastasis. However, the molecular pathways and therapeutic targets in thyroid cancer-related CSCs remain undetermined. In this study, ATC-CD133\(^+\) cells were isolated and found to have increased tumorigenicity, radioresistance, and higher expression of both embryonic stem cell-related and drug resistance-related genes compared with ATC-CD133\(^-\) cells. Microarray bioinformatics analysis suggested that the signal transducer and activator of transcription 3 (STAT3) pathway could be important in regulating the stemness signature in ATC-CD133\(^+\) cells; therefore, the effect of the potent STAT3 inhibitor cucurbitacin I in ATC-CD133\(^+\) cells was evaluated in this study. Treatment of ATC-CD133\(^+\) cells with cucurbitacin I diminished their CSC-like abilities, inhibited their stemness gene signature, and facilitated their differentiation into ATC-CD133\(^-\) cells. Of note, treatment of ATC-CD133\(^+\) cells with cucurbitacin I up-regulated the expression of thyroid-specific genes and significantly enhanced radiiodine uptake. Furthermore, cucurbitacin I treatment increased the sensitivity of ATC-CD133\(^+\) cells to radiation and chemotherapeutic drugs through apoptosis. Finally, xenotransplantation experiments revealed that cucurbitacin I plus radiochemotherapy significantly suppressed tumorigenesis and improved survival in immunocompromised mice into which ATC-CD133\(^+\) cells were transplanted. In summary, these results show that the STAT3 pathway plays a key role in mediating CSC properties in ATC-CD133\(^+\) cells. Targeting STAT3 with cucurbitacin I in ATC may provide a new approach for therapeutic treatment in the future.

INTRODUCTION

Thyroid cancer is a common endocrine tumor. Approximately 85 to 90% of patients with thyroid cancer are diagnosed with papillary thyroid cancer (PTC), which originates from thyroid follicular cells (Sipos and Mazzaferris, 2010). Patients with PTC are expected to survive for 10 years or more after diagnosis (Sipos and Mazzaferris, 2010). However,
patients with anaplastic thyroid cancer (ATC), an undifferentiated thyroid cancer, have a poor prognosis with a median survival of less than 6 months after traditional treatments such as surgery, radiotherapy, and chemotherapy (Sugino et al., 2002; Sipos and Mazzaferrri, 2010). Cancer stem cells (CSCs), a crucial subset within the bulk of the tumor, exhibit self-renewal and differentiation capabilities. Some studies have demonstrated that CSCs exist in many cancer types and are responsible for tumor development, relapse, and metastasis (Jordan et al., 2006). Zito et al. (2008) identified CD133⁺ cells from four human ATC cell lines that presented stem cell-like features in vitro. In addition, Friedman et al. (2009) evaluated the tumor initiation potential of CD133⁺ cells in an animal model. Together, their results suggest that the CD133 subsets within ATC may account for tumor progression, resistance to current modes of chemotherapy, and the aggressiveness of ATC.

Signal transducer and activator of transcription 3 (STAT3), a transcription factor that responds to cytokine and growth factor signaling, regulates a variety of cellular functions via its downstream target genes such as p21, c-Myc, and cyclin D1 (cell cycle progression genes); matrix metalloproteinase 9 (MMP-9) (mediating cellular invasion); and vascular endothelial growth factor (regulating angiogenesis) (Yu and Jove, 2004). STAT3 was found to be constitutively activated in numerous cancer types (Bowman et al., 2000; Bromberg, 2001, 2002; Epling-Burnette et al., 2001), and it was also reported that STAT3 mutations induce cellular transformation and tumor formation in vivo and that activation of STAT3 signaling further inhibits p53 transcriptional activity, fulfilling the definition of an oncogene (Bromberg et al., 1999; Niu et al., 2005). Previous reports have shown that STAT3 can be directly activated by rearranged in transformation/papillary thyroid carcinoma (RET/PTC) tyrosine kinase, the most frequently identified genetic rearrangement in PTC (Hwang et al., 2003). Several proteins such as phospholipase D and tumor suppressor LKB1 have been suggested to be modulators of RET/PTC-induced STAT3 activation (Kim et al., 2007, 2008). Another study demonstrated that p53 mutations in ATC play an important role in tumor progression via the STAT3 pathway (Kim et al., 2009). Abolishment of STAT3 phosphorylation by exogenous expression of suppressors of cytokine signaling or administration of a RET/PTC kinase inhibitor inhibits the growth of thyroid carcinoma cells, suggesting that targeting the STAT3 pathway could be useful in thyroid cancer therapy (Kim et al., 2006; Francipane et al., 2009). Although recent studies have shown that STAT3 activation is strongly associated with thyroid tumorigenesis, the mechanisms of STAT3 signaling pathways need to be investigated further to identify possible therapeutic targets for thyroid CSCs.

Cucurbitacin I (also known as JSI-124), a natural cell-permeable triterpenoid compound, belongs to the cucurbitacin family of drugs isolated from various plant families (Lee et al., 2000). Cucurbitacins have been used as folk medicine as a STAT3 inhibitor in signaling research. We found that thyroid cancer stem cell-like cells were confined in a CD133⁺ subpopulation. Microarray analysis revealed that the level of activated STAT3 was significantly higher in these cells compared with that in ATC-CD133⁻ cells. In view of these findings, we further investigated whether blocking STAT3 signaling with cucurbitacin I could influence molecular biological functions in ATC-CD133⁺ cells, particularly by modulating the expression of thyroid-specific genes. Finally, we provide evidence that cucurbitacin I can improve the efficacy of treatments targeting ATC-CD133⁺ cells by reducing their resistance to radiation and chemotherapeutic drugs.

**Materials and Methods**

**Isolation of CD133⁺ Cells from Thyroid Cancer Patients and Cell Lines.** This research followed the tenets of the Declaration of Helsinki, and all samples were obtained after patients provided informed consent. Three cell lines (BHT-101, CAL-62, and 8505c) and cells dissociated from a sample from a patient with ATC were labeled with 1 ml of CD133/1 micromagnetic beads per 1 million cells using a CD133 cell isolation kit (Miltenyi Biotech, Auburn, CA). CD133⁺ cells were cultured in medium consisting of serum-free DMEM/F12 (Invitrogen, Carlsbad, CA) with N2 supplement (R& D Systems Inc., Minneapolis, MN). 10 ng/ml human recombinant bFGF (R&D Systems Inc.), and 10 ng/ml EGF (R&D Systems Inc.) (Chiou et al., 2008).

**Irradiation and Clonogenic Assay.** Ionizing radiation (IR) was delivered by a cobalt unit (Theratron International, Inc., Ottawa, ON, Canada) at a dose rate of 1.1 Gy/min (source-to-surface distance = 57.5 cm). For a clonogenic assay, cells were exposed to different radiation doses (0, 2, 4, 6, 8, and 10 Gy). After incubation for 10 days, colonies (>50 cells/colony) were fixed and stained for 20 min with a solution containing crystal violet and methanol. Cell survival was determined by a colony formation assay. The plating efficiency (PE) and survival fraction (SF) were calculated as follows: PE = (colony number/number of inoculated cells) × 100%. SF = colonies counted/(cells seeded × (PE/100)).

**Quantitative Real-Time RT-PCR.** Real-time RT-PCR was performed as described previously (Kao et al., 2009). In brief, total RNA (1 µg) from each sample was reverse-transcribed in a 20 µl reaction using 0.5 µg of oligo(dT) and 200 U of SuperScript II RT (Invitrogen). The primer sequences used for real-time RT-PCR are shown in Table 1. DNA amplification was performed in a total volume of 20 µl, and cDNA was transcribed with SuperScript II RNase H-reverse transcriptase purification kit (Qiagen, Valencia, CA). Total RNA was reverse-transcribed with SuperScript II RNase H-reverse transcriptase (Gibco-BRL) to generate Cy3- and Cy5-labeled cDNA. Duplicate PCRs were heated to 95°C for 10 min, followed by 40 cycles of the following: denaturation at 95°C for 5 s, annealing at 55°C for 5 s, and extension at 72°C for 20 s. Standard curves of cycle threshold values versus template concentrations were prepared for each target gene and for the endogenous reference (glyceraldehyde-3-phosphate dehydrogenase) in each sample. Unknown samples were quantitated using LightCycler Relative Quantification Software (version 3.3; Roche Molecular Systems, Inc.).

**Microarray Analysis and Bioinformatics.** Total RNA was extracted from cells using TRIzol reagent (Invitrogen) and the RNAeasy purification kit (Qiagen, Valencia, CA). Total RNA was reverse-transcribed with SuperScript II RNase H-reverse transcriptase (Gibco-BRL) to generate Cy3- and Cy5-labeled (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) cDNA probes for control and treated samples, respectively. The labeled probes were hybridized to a cDNA microarray containing immobilized cDNA fragments corresponding to 10,000 gene clones. Fluorescence intensities of Cy3 and Cy5 targets were measured and scanned separately using a GenePix 4000 B Array Scanner (Molecular Devices, Sunnyvale, CA). Data analysis was performed using GenePix Pro 3.0.5.56 (Molecular
Healthcare) by a wet transfer system. The following primary anti-
separated in a 10% SDS-polyacrylamide gel electrophoresis gel. The
2009). Fifteen microliters of sample were boiled at 95°C for 5 min and
blot analysis were performed as described previously (Kao et al.,
human NIS (Abcam Inc., Cambridge, MA); mouse anti-human
anti-human STAT3, rabbit anti-human MMP-9, and mouse anti-
ice-cold Hanks’ balanced salt solution and detached with trypsin and

**TABLE 1**
Sequences for the primers of quantitative real-time RT-PCR

<table>
<thead>
<tr>
<th>Gene (Accession No.)</th>
<th>Primers</th>
<th>Direction</th>
<th>Sequences (5’ to 3’)</th>
<th>Product Size</th>
<th>$T_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oct4A (NM_002701)</td>
<td>Forward</td>
<td>GGTTGGCAAGTCGTGAAC</td>
<td>86</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>SOX2 (NM_003106)</td>
<td>Reverse</td>
<td>TCTCTTCTCTCTTCTCTTC</td>
<td>74</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>Nanog (NM_024865)</td>
<td>Forward</td>
<td>ATCTGAGAGACGCTCTTC</td>
<td>76</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>MDR1 (NM_000927)</td>
<td>Reverse</td>
<td>TTCTTTGCTGACCTCTCCTGC</td>
<td>76</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>ABCG2 (NM_004827)</td>
<td>Forward</td>
<td>CGGAGGCGCTCTTCCTGGA</td>
<td>164</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>MRP (NM_004996)</td>
<td>Reverse</td>
<td>CTTGAGCTGCGCCCACTGA</td>
<td>122</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>NIS (NM_000453)</td>
<td>Forward</td>
<td>CCGGCTAGAGCACAAGCCG</td>
<td>174</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>Thyroglobulin (NM_003235)</td>
<td>Reverse</td>
<td>TGACCTAGAGCCGCTGCCTGT</td>
<td>152</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>Thyroperoxidase (NM_000547)</td>
<td>Forward</td>
<td>GTGCAAGATGAAGAGCCTCCTGG</td>
<td>178</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>GAPDH (NM_002446)</td>
<td>Reverse</td>
<td>GCCGCTTCAGCACCACCCTT</td>
<td>180</td>
<td>60</td>
<td></td>
</tr>
</tbody>
</table>

F, forward; R, reverse; bp, base pairs.

Devices) and GeneSpring GX 7.3.1 software (Agilent Technologies, Santa Clara, CA). The average-linkage distance was used to assess the similarity between the two groups of gene expression profiles as described below. The difference in distance from the expression profiles of the two sample groups to the expression profile of a third sample was assessed by comparing the corresponding average linkage distances (the mean of all pairwise distances of linkages between the members of the two groups). The error of this comparison was estimated by combining the S.E.s (the S.D. of pairwise linkages divided by the square root of the number of linkages) of the average linkage distances involved. Classical multidimensional scaling was performed using the standard function of the program R to provide a visual impression of how the various sample groups were related (Chiu et al., 2011).

**125I Uptake Assay.** An iodide uptake assay was performed as described previously (Furuya et al., 2004). In brief, cells grown in six-well plates were incubated with 1 ml of Hank’s balanced salt solution containing 0.2 μC of carrier-free Na$^{125}$I (PerkinElmer Life and Analytical Sciences, Waltham, MA) and 10 μM NaI with or without 300 μM NaClO₄, a sodium/iodide symporter (NIS) inhibitor. After incubation for 1 h at 37°C, cells were washed twice with ice-cold Hank’s balanced salt solution and detached with trypsin and their radioactivity was measured with a gamma counter. The counts were normalized to the number of viable cells.

**Western Blot Assay.** Protein extraction from cells and Western blot analysis were performed as described previously (Kao et al., 2009). Fifteen microliters of sample were boiled at 95°C for 5 min and separated in a 10% SDS-polyacrylamide gel electrophoresis gel. The proteins were transferred to Hybond-ECL nitrocellulose paper (GE Healthcare) by a wet transfer system. The following primary antibodies were used: rabbit anti-human phospho-STAT3, rabbit anti-human STAT3, rabbit anti-human MMP-9, and mouse anti-human (Cell Signaling Technology, Danvers, MA); mouse anti-human NIS (Abcam Inc., Cambridge, MA); mouse anti-human Bel-2 (Millipore Corporation, Billerica, MA); and mouse anti-β-actin (Millibore Bioscience Research Reagents, Temecula, CA). The reactive protein bands were detected with the ECL detection system (GE Healthcare).

**In Vitro Cell Migratory Analysis and Soft Agar Assay.** A 24-well plate Transwell system with a polycarbonate filter membrane was used (8-μm pore size; Corning, Essex, UK). Cell suspensions were seeded in the upper compartment of the Transwell chamber coated with Matrigel at a density of 1 × 10⁴ cells in 100 μl of serum-free medium. The surface of the filter membrane facing the lower chamber was stained with Hoechst 33342 for 3 min, and the migrating cells were visualized under an inverted microscope. For the soft agar assay, the bottom of each well (35 mm) of a six-well culture dish was coated with 0.2 ml of an agar mixture [DMEM, 10% (v/v) fetal calf serum, and 0.6% (w/v) agar]. After the bottom layer solidified, 2 ml of a top agar-medium mixture [DMEM, 10% (v/v) fetal calf serum, and 0.3% (w/v) agar] containing 2 × 10⁴ cells was added and incubated at 37°C for 4 weeks. The plates were stained with 0.5 ml of 0.005% crystal violet, and the number of colonies was counted using a dissecting microscope.

**In Vivo Analysis of Tumor Growth and Metastasis.** All procedures involving animals were conducted in accordance with the institutional animal welfare guidelines of Taipei Veterans General Hospital. In total, 10⁵ CD133(+/-) cells were injected into the tail vein of nude mice (BALB/c strain) at 8 weeks of age. Cucurbitacin I (1 mg/kg i.p. for 5 days) was administered in xenotransplanted nude mice by intraperitoneal injection. In vivo GFP imaging was performed using an illuminating device [LT-9500 Illumatool TLS equipped with an excitation illuminating source (470 nm) and a filter plate (515 nm)]. Tumor size was measured using calipers, and the volume was calculated according to the following formula: (length × width²)/2. Tumors were subsequently analyzed using Image Pro Plus software (Kao et al., 2009).

**Statistical Analysis.** The Statistical Package of Social Sciences software (SPSS, Inc., Chicago, IL) was used for statistical analysis. An independent Student’s $t$ test was used to compare the continuous variables between groups. The Kaplan-Meier procedure was used to calculate survival probability estimates. A log-rank test was used to compare the cumulative survival durations in different patient groups. The statistical significance level was set at 0.05 for all tests.

**Results**

**Isolation and Characterization of ATC-Derived CD133⁺ Cells.** Recent studies have shown that the expression of CD133 in thyroid cancer results in high tumorigenicity and resistance to conventional therapy (Zito et al., 2008; Friedman et al., 2009). To look for additional CSC-like characteristics in CD133⁺ cells from ATC cells and tissues, we isolated...
CD133+ cells (Fig. 1A, left) from tissue sample of 17 patients with ATC using the magnetic bead method. ATC-CD133+ cells formed floating spheroid-like bodies in DF-12 serum-free medium with bFGF and EGF (Fig. 1A, right). Quantitative real-time RT-PCR results showed that the transcript levels of "stemness" genes (Oct4, Sox2, and Nanog) and drug resistance genes (ABCG2, MDR1, and MRP) were higher in ATC-CD133+ cells than in ATC-CD133- cells (Fig. 1B). Furthermore, ATC-CD133+ cells displayed greater ability for sphere formation (Fig. 1C), foci formation (Fig. 1D), and invasive activity (Fig. 1E), as well as enhanced resistance to conventional chemotherapeutic drugs than ATC-CD133- cells of the same origin (\( p < 0.05 \)) (Fig. 1, F–H). To evaluate the tumor-restoration capability of ATC-CD133+ cells, 3 \( \times \) 10^3 and 3 \( \times \) 10^4 cells were subcutaneously injected into SCID mice. The results showed that 3 \( \times \) 10^4 ATC-CD133+ cells did not develop tumor formation, but 3 \( \times \) 10^3 CD133+ cells from 12 patients, as well as 3 \( \times \) 10^4 CD133+ cells from all 17 patients, developed into visible tumors 6 weeks after injection into xenotransplanted mice (Table 2). To further explore the key regulators governing stemness and tumorigenicity in ATC-CD133+ cells, we performed microarray studies to compare the transcriptional profile of CD133- and CD133+ cells (Fig. 2A). To gain additional insights into the functional consequences of differential gene expression patterns and to provide quantitative evidence, signature genes were submitted to the gene ontology database to identify statistically represented functional groups. The predominant processes up-regulated in ATC-CD133+ cells include transcription, activation of transmembrane receptor, protein tyrosine kinase activity, DNA repair, and stress response (Fig. 2A; Supplemental Table 1).
ties of ATC-CD133 and JNK signaling pathways (Blaskovich et al., 2003). How-
acin I-treated ATC-CD133<sup>+</sup> cells was shifted toward that of ATC-CD133<sup>−</sup> cells (Fig. 4B). These results suggested that the treatment with cucurbitacin I can promote the transcriptional pattern of ATC-CD133<sup>+</sup> cells shifted into the expression profiling of ATC-CD133<sup>−</sup> cells. To further validate the microarray and bioinformatic findings, we examined whether the proportion of CD133<sup>+</sup> cells was suppressed by STAT3 inhibition. First, CD133<sup>+</sup> cells were incubated with 100 or 150 nM cucurbitacin I for 24 h. Fluorescence-activated cell sorter analysis showed that cucurbitacin I decreased the number of ATC-CD133<sup>+</sup> cells in a dose-dependent manner (<i>p</i> < 0.05) (Fig. 4, C and D). Taken together, these results suggest that CD133<sup>+</sup> cells share stem cell-related gene signatures, and cucurbitacin I can further promote ATC-CD133<sup>+</sup> cells differentiated into ATC-CD133<sup>−</sup> cells.

**Cucurbitacin I Restores the Sensitivity of ATC-CD133<sup>+</sup> Cells to Radiochemotherapy.** Lack of NIS, thyroperoxidase (TPO), and thyroglobulin (Tg) expression in ATC cells makes them resistant to conventional radioiodide therapy (Spitzweg, 2009). Re-expression of these genes in ATC cells has proven to be a promising gene therapy approach. To evaluate the effects of STAT3 abrogation on these thyroid-specific genes, their transcripts were analyzed by quantitative real-time RT-PCR. Addition of 100 or 150 nM cucurbitacin I induced the expression of NIS, TPO, and Tg mRNA in ATC-CD133<sup>+</sup> cells (<i>p</i> < 0.05) (Fig. 5, A–C). The effects of cucurbitacin I on STAT3 downstream genes and cell survival-related genes such as survivin and Bcl-2, as well as on the expression of NIS, were examined by Western blot. Protein levels of Bcl-2 and survivin were down-regulated by cucurbitacin I treatment, whereas NIS production was enhanced (Fig. 5D). In addition, the protein level of MMP-9, which is a key regulator in the metastatic process, declined when STAT3 signaling was abrogated (Fig. 5D). To assess the effects of STAT3 inhibition on iodide uptake, untreated and cucurbitacin I-treated cells were incubated with Na<sup>125</sup>I for 1 h. Nonspecific iodide accumulation in the presence of NaClO<sub>4</sub> did not significantly change in untreated cells; however, when CD133<sup>+</sup> cells were treated with cucurbitacin I for 24 h, a marked increase in <sup>125</sup>I uptake was observed (Fig. 5, E and F), which was abolished by NaClO<sub>4</sub> (a specific inhibitor of NIS-mediated iodide uptake). In agreement with the microarray and quantitative real-time RT-PCR findings, the <sup>125</sup>I uptake assay showed that cucurbitacin I-induced iodide uptake might be facilitated by the differentiation of CD133<sup>+</sup> cells and the subsequent re-expression of functional NIS.
Cucurbitacin I Sensitizes ATC-CD133+ Cells to Radiochemotherapy. To examine the influence of cucurbitacin I on the chemoresistance of ATC-CD133+ cells, cytotoxicity analysis was performed. Cucurbitacin I treatment of ATC-CD133+ cells rendered them more sensitive to the chemotherapeutic agents cisplatin (Fig. 6A), 5-fluorouracil (Fig. 6B), and doxorubicin (Fig. 6C), all of which are currently used for thyroid cancer chemotherapy. In addition, sphere formation (Fig. 6D), colony formation (Fig. 6E), and invasiveness (Fig. 6F) were dramatically reduced in the cells treated with chemotherapeutic agents plus cucurbitacin I compared with reduction in cells treated with either agent alone.

We next examined whether the radioresistance of CD133+ cells was due to modulation of STAT3 signaling. Vehicle- or cucurbitacin I-treated ATC-CD133+ cells and CD133− cells were exposed to different doses of ionizing radiation. Cellular survival fraction analysis indicated that CD133+ cells were more radioresistant than CD133− cells (Fig. 7A). Of note, silencing of STAT3 signaling with cucurbitacin I sensitized CD133+ cells to radiation. Cucurbitacin I in combination with 4 Gy IR dramatically diminished sphere formation (Fig. 7B) and colony formation and invasion (data not shown) in both CD133+ cells and CD133− cells. Based on clinical observations, the propensity to metastasize is one of the major causes of the poor prognosis associated with ATC. To investigate whether cucurbitacin I treatment could suppress ATC cell migration, cell mobility assays were performed with both CD133+ cells and CD133− cells treated with cucurbitacin I and/or IR. Compared with the control group, a single treatment with cucurbitacin I or IR reduced cell mobility; a more prominent reduction in cell mobility was observed after the cells were treated with cucurbitacin I plus IR (Fig. 7C).
Moreover, caspase 3 activity analysis revealed that the cytotoxic effect of IR on ATC-CD133\textsuperscript{+} cells was significantly enhanced with the addition of cucurbitacin I ($p < 0.05$) (Fig. 7D). In addition, terminal deoxynucleotidyl transferase dUTP nick-end labeling-positive cells concomitantly increased among ATC-CD133\textsuperscript{+} cells treated with cucurbitacin I and IR compared with cells treated with either method alone (Fig. 7E). These results suggest that cucurbitacin I can greatly increase the effectiveness of radiotherapy against ATC-CD133\textsuperscript{+} cells by driving apoptosis.

**Effects of Cucurbitacin I on the In Vivo Tumorigenicity and Survival Time of ATC-CD133\textsuperscript{+} Cells in a Xenotransplantation Animal Model.** We next investigated the role of STAT3 signaling and the effects of cucurbitacin I in ATC-CD133\textsuperscript{+} cells in vivo. ATC-CD133\textsuperscript{+/-} cells were transfected with a lentiviral vector containing the GFP gene. We injected $2 \times 10^5$ CD133\textsuperscript{+}-GFP cells and $2 \times 10^5$ CD133\textsuperscript{-}-GFP cells into the subcutaneous regions of nude mice that had received different treatment protocols (Fig. 8). We found that the subset of nude mice receiving CD133\textsuperscript{-}-GFP cells had formed no tumors in the subcutaneous region by 10 weeks after transplantation. CD133\textsuperscript{+}-GFP cells presented the greatest ability to form tumors in transplanted mice (Fig. 8A). Mice bearing subcutaneous tumors received cucurbitacin I (1 mg/kg i.p. for 5 days) or cisplatin plus 4 Gy IR. Both treatment strategies suppressed the proliferation of CD133\textsuperscript{-}-GFP cells in the transplanted mice (Fig. 8A). Of note, the combination triple treatment (cucurbitacin I + cisplatin + IR) caused a significant amount of tumor shrinkage in CD133\textsuperscript{+}-GFP cell-transplanted mice ($p < 0.05$) (Fig. 8A).
To confirm that STAT3 was crucial for metastasis in vivo, mice were orthotopically injected with either CD133<sup>+</sup>/H11001-GFP or CD133<sup>+</sup>/H11002-GFP cells. After transplantation, CD133<sup>+</sup> cells showed significantly higher levels of local invasion and distant metastasis to the lungs than CD133<sup>+</sup> cells, and they formed larger tumors (Fig. 8B). Either strategy (cucurbitacin I alone or cisplatin plus IR) modestly decreased local invasion and distant metastasis in a statistically significant manner. Most importantly, the triple treatment (cucurbitacin I, cisplatin, and IR) of CD133<sup>+</sup> cells effectively reduced the number of lung metastases and volumes in vivo (Fig. 8B). Moreover, CD133<sup>+</sup> cell-transplanted mice treated with the triple treatment had a mean survival rate that was significantly prolonged compared with that of CD133<sup>+</sup> cell-transplanted mice that had received other treatments (Fig. 8C). Overall, these in vivo results show that the effectiveness of conventional chemoradiotherapy in mice bearing ATC-CD133<sup>+</sup> tumors can be significantly improved with the addition of cucurbitacin I treatment.

**Discussion**

Most well differentiated thyroid cancers of follicular cell origin have good prognosis with a 10-year survival rate in the literature of greater than 85% (Sipos and Mazzaferri, 2010). However, for patients with ATC with metastasis to distant organs and for those with postoperative recurrence or lymphoid metastatic lesions without radiodine uptake, the success of therapy is limited and the cancer may be life-threatening. At present, neither surgery nor chemoradiation is an effective treatment for ATC with respect to achieving long-term survival. Rapid local recurrence and distant metastasis with resistance to chemotheraphy or radiotherapy are the major causes of treatment failure (Cornett et al., 2007). With
the increasing awareness of the parallels between stem cells and cancer cells, evidence supporting the existence of CSCs that contribute to tumorigenicity has been accumulating (Jordan et al., 2006). A current approach to enriching for CSCs is the use of specific cell surface markers combined with cell sorting. CD133, a 5-transmembrane glycoprotein, is a hematopoietic stem cell and endothelial progenitor marker that seems to be involved in angiogenesis (Corbeil et al., 2000; Hilbe et al., 2004). Recent studies have identified a subpopulation of CD133- cells in ATC cell lines that exhibit definitive properties of cancer stem cells: self-renewal, multipotency, and tumorigenicity in vitro or in vivo (Zito et al., 2008; Friedman et al., 2009). The expression of Oct4 has been found to be consistently up-regulated in CD133- cells, suggesting the Oct4, an embryonic stem cell gene, plays a critical role in thyroid CSCs. Moreover, an immunohistochemical analysis of ATC tissue specimens performed by Friedman et al. (2009) showed that tumor cells expressed CD133, whereas neighboring normal thyroid cells did not, suggesting that CD133 could be a therapeutic target in ATC.

However, the regulatory mechanisms active in ATC CSCs have not been clarified, impeding the subsequent development of potential therapies against these cells. In this study, we showed that relative to CD133- cells, CD133+ cells exhibited greater CSC-like properties such as increased tumorigenicity and expression of embryonic stemness and drug-resistant genes (Fig. 1). Microarray and bioinformatic analyses indicated that the STAT3 and p-STAT3 signaling axis is an important link between the tumorigenic and stemness gene expression profiles and the metastasis-associated pathways in ATC CSCs (Fig. 2). Exploiting this finding, we next demonstrated that 100 nM cucurbitacin I, a specific JAK-STAT inhibitor, efficiently inhibits the proliferation of ATC-CD133+ cells and induces apoptosis in them (Figs. 3 and 7). Furthermore, 100 nM cucurbitacin I was found to inhibit the stemness gene signatures of ATC-CD133+ cells and facilitate their differentiation into ATC-CD133- cells (Figs. 3 and 4). Moreover, blocking STAT3 signaling with cucurbitacin I significantly suppresses the self-renewal ability, tumorigenicity, and radiochemoresistance of ATC-

Fig. 6. Characterization of chemosensitivity in cucurbitacin I-treated (Cu) CD133+ cells. A to C, A total of 2 × 10^4 cells were plated in a 24-well plate and treated with or without cucurbitacin I plus various concentrations of cisplatin (A), 5-FU (B), or doxorubicin (C) for 24 h in 10% fetal calf serum/DMEM/F-12 medium. The survival rate was determined by the MTT assay. Data shown are the mean ± S.D. of three experiments. Sphere formation (D), colony formation (E), and invasive ability (F) of ATC-CD133+ and ATC-CD133- cells with the indicated treatment were measured. Scale bar, 100 μm. *, p < 0.05.
CD133⁺ cells, suggesting that STAT3 activation plays a role in maintaining their CSC phenotype (Figs. 6 and 7). Finally, Kaplan-Meier survival analysis indicated that the effect of cisplatin or 4 Gy IR on ATC-CD133⁺ cells is significantly enhanced by cucurbitacin I treatment (Fig. 8). To our knowledge, this is the first study to not only show that the STAT3 axis plays an important role in maintaining CSC-like properties but also demonstrate that targeting STAT3 with cucurbitacin I significantly suppresses tumorigenicity and radiochemoresistance in ATC-derived CSCs.

The NIS is a central mediator of active iodide transport into the thyroid gland (Carvalho and Ferreira, 2007). Because of its key role in thyroid physiology and pathophysiology, both re-expressing endogenous NIS and introducing exogenous NIS have become candidate anticancer strategies. In addition to gene therapy, several compounds have been found to harbor an ability to induce endogenous NIS gene expression. Retinoic acid was shown to trigger redifferentiation of thyroid cancer cell lines and up-regulate NIS expression (Riesco-Eizaguirre and Santisteban, 2006; Carvalho and Ferreira, 2007). A number of clinical trials using retinoic acid have been performed but have shown variable results. Because Pax8-PPARγ rearrangement is a common occurrence in thyroid cancers (Marques et al., 2002; Castro et al., 2006), PPARγ may be involved in thyroid carcinogenesis. Troglitazone, a PPARγ agonist, has been reported to induce differentiation and NIS mRNA expression in human thyroid cancer cell lines (Park et al., 2005). Another emerging class of pharmacological agents belongs to the group of histone deacetylase inhibitors, which are used to re-express silenced genes in...
cancer cells. Depsipeptide (FR901228), trichostatin A, and valproic acid have been shown to successfully restore the expression of NIS (Furuya et al., 2004; Riesco-Eizaguirre and Santisteban, 2006). Moreover, STAT3, a transcription factor regulated by various cytokines and growth factors (particularly interleukin-6 and EGF) is aberrantly activated in numerous cancers (Bollrath and Greten, 2009; Yu et al., 2009). Several cytokines such as interleukin-6 have proven to suppress NIS mRNA levels in the Fisher rat thyroid cell line (FRTL-5) (Spitzweg et al., 1999; Riesco-Eizaguirre and Santisteban, 2006). In a more recent study, treatment with sunitinib, a selective inhibitor of receptor tyrosine kinases, seemed to block STAT3 activation and induce the expression of NIS in papillary thyroid cancer (Fenton et al., 2010). In the present study, we found that treating ATC-CD133+ cells with cucurbitacin I up-regulates their expression of thyroid-specific genes including sodium/iode symporter, thyroperoxidase, and thyroglobulin, and it significantly enhances NIS-mediated radioiodine uptake (Fig. 5). Of importance, our data suggest that the abrogation of the STAT3 signaling pathway induces the expression of NIS protein and subsequently augments radioiodide accumulation, indicating that the survival rates of animals receiving CD133+ cells and treated with cucurbitacin I combined with IR and cisplatin were significantly prolonged compared with those receiving other treatments (each group, n = 6).

The CSC hypothesis has been bolstered by the clinical observation that malignant tumors are relatively resistant to chemoradiotherapies (O’Brien et al., 2010). The existence of cancer stem cell-like cells may explain why conventional
The resistance of ATC-CD133 may be responsible for the CSC-like properties and radio- and chemosensitivity in ATC-CD133 cells, in part through the inactivation of p-STAT3 and its downstream effectors survivin, Bcl-2, and MMP9 (Fig. 5). Furthermore, our data suggest that inhibition of the STAT3 pathway by cucurbitacin I significantly blocked the migration and lung metastasis of ATC-CD133 cells in both in vitro and in vivo xenotransplantation models (Figs. 7 and 8). Of note, in vivo xenotransplantation analysis showed that cucurbitacin I can be a sensitizer synergistically enhancing both radio- and chemosensitivity in a xenotransplantation model of human thyroid cancer stem cells (Fig. 8). Taken together, our findings indicate that the antiproliferative, proapoptotic, and radiochemosensitizing effects of cucurbitacin I on CD133+ cells could be used as a strategy to overcome the resistance of highly tumorigenic ATC-CD133 cells in patients with advanced ATC. Treating ATC by targeting JAK/STAT3 signaling together with other components of the CSC-related microenvironment that biologically affect radiochemoresistance, recurrence, and metastasis should be further investigated.

In conclusion, we showed that the STAT3 signaling axis may be responsible for the CSC-like properties and radioresistance of ATC-CD133 cells. Cucurbitacin I potently attenuated the malignancy of ATC-CD133 cells, presenting a potential clinical benefit for thyroid cancer and CSC treatment. Thus, there is a great need to determine the underlying mechanisms of the STAT3 pathway in ATC-CD133 cells and to further evaluate the therapeutic potential of STAT3 pathway inhibitors. In addition, persistent activation of STAT3 promotes tumor cell proliferation and survival, contributing to tumor progression and migration. Therefore, abrogation of STAT3 signaling may be a potent therapeutic strategy for malignant thyroid cancers as well as cancer stem cells in general.

Authorship Contributions

Participated in research design: Tseng, Y.-C. Chen, Chioi, and Lee.


Performed data analysis: Y.-C. Chen, Chang, Huang, Chi, and Yang.

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