Tumor and Stem Cell Biology

Tumorigenic and Metastatic Activity of Human Thyroid Cancer Stem Cells

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Abstract

Thyroid carcinoma is the most common endocrine malignancy and the first cause of death among endocrine cancers. We show that the tumorigenic capacity in thyroid cancer is confined in a small subpopulation of stem-like cells with high aldehyde dehydrogenase (ALDH) activity and unlimited replication potential. ALDH cells can be expanded indefinitely in vitro as tumor spheres, which retain the tumorigenic potential upon delivery in immunocompromised mice. Orthotopic injection of minute numbers of thyroid cancer stem cells recapitulates the behavior of the parental tumor, including the aggressive metastatic features of undifferentiated thyroid carcinomas, which are sustained by constitutive activation of CMet and Akt in thyroid cancer stem cells. The identification of tumorigenic and metastagic thyroid cancer cells may provide unprecedented preclinical tools for development and preclinical validation of novel targeted therapies.

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Introduction

Thyroid cancer is the most frequent endocrine malignancy with a global increasing incidence. Papillary (PTC), follicular (FTC), and anaplastic (UTC) thyroid carcinomas arise from endodermal-derived follicular cells, which represent the most abundant cellular population of the thyroid gland. PTC comprises 80% to 85% of all thyroid neoplasms, whereas FTC is the second most common thyroid cancer, accounting for approximately 10% to 15% of cases (1). The least common (1–2%) histotype is UTC, which has a fast progression and a very poor prognosis (2, 3).

Tyrosine kinase receptors play a major role in the regulation of tumor initiation and progression (4, 5). In response to hepatocyte growth factor/scatter factor, the Met tyrosine kinase receptor triggers intracellular signals that positively regulate cell survival differentiation and invasion (6, 7). Met receptor activates phosphatidylinositol-3-kinase and downstream kinases critically involved in cell survival, such as GSK3β and Akt (8–10). Phosphatidylinositol-3-kinase/AKT signal transduction pathway is also involved in the epithelial-mesenchymal transition, a process that confers motility and invasiveness to epithelial tumor cells through the loss of epithelial proteins and the upregulation of the transcription factors Twist and Snail (11). Strong evidence depicts aberrant activation of β-catenin and Met in a wide variety of human tumors, including thyroid cancer (12, 13). Overexpression of activated β-catenin plays critical roles in both cell adhesion and transcriptional regulation in the Wnt signaling pathway, which has been implicated in the maintenance of self-renewal capacity of the stem cell compartment (14).

The recent discovery of cancer stem cells (CSC) in a variety of tumors has changed the view of carcinogenesis and therapeutic strategies (15, 16). Therefore, the identification and characterization of such tumorigenic population represents a crucial step to develop effective therapies.

Until a few years ago, thyroid carcinoma was believed to originate from well-differentiated normal thyroid follicular cells as a consequence of multiple mutations accumulated throughout the entire life span (17). More recently, the existence of several degrees of differentiation has lead to the assumption that a pool of stem cells at different stages of differentiation are responsible for thyroid cancer initiation and progression. A novel hypothesis of thyroid carcinogenesis posits that thyroid cancer cells are derived from the remnants of fetal cells (18). According to such a theory, thyroid cancer cells would be generated from the transformation of three types of fetal thyroid cells—thyroid stem cells, thyroblasts, and prothyrocytes, which would result in UTC, PTC and FTC, respectively. Although the phenotype

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of stem and/or progenitor cells of the thyroid gland has not yet been identified, several markers including CD133 (19–23), CD44 (24–27), and ALDH1 (28–32) have been associated with undifferentiated cells in several tissues. Increased aldehyde dehydrogenase (ALDH) activity has been described in primitive cells from multiple myeloma (30), acute myeloid leukemia (31), pancreatic (29), breast (28), and lung (32) carcinomas.

Here, we analyzed the tumorigenic activity of thyroid cancer cells. We found that FTC, PTC, and UTC contain a small fraction of tumorigenic cells that can be prospectively identified using the ALDH activity. Thyroid cells with high ALDH expression (ALDH<sup>high</sup>) possess the ability to self-renew and reinitiate serial transplantable tumors that recapitulate the phenotype and metastatic behavior of parental tumors promoted by the activation of Met and Akt.

**Materials and Methods**

**Tissues, cell culture, clonogenic and invasion assays**

Thyroid cancer tissues were obtained at the time of thyroidecomy from patients affected by PTC (n = 18; age range, 28–70 y), FTC (n = 10; age range, 21–72 y), and UTC (n = 6; age range, 51–73 y). Normal and tumor thyroid cells were purified from fresh tissues as described in ref. (33). To obtain thyroid spheres, cells were resuspended in medium containing basic fibroblast growth factor and epidermal growth factor (20, 22) and plated on ultra-low-adhesion 96-well plates at a concentration of a single cell per well. Wells containing either none or more than one cell were excluded for the analysis. For sphere-derived adherent culture, tumor spheres were cultured in adherence conditions in DMEM supplemented with 10% fetal bovine serum or with human recombinant basic fibroblast growth factor and epidermal growth factor, either in 1× or none were excluded for the analysis. For sphere-derived adherent culture, tumor spheres were cultured in adherence conditions in DMEM supplemented with 10% fetal bovine serum or with human recombinant α thyroid-stimulating hormone (400 ng/mL; Genzyme).

Cell migration was measured using growth factor–depleted Matrigel-coated (BD Biosciences) transwell inserts. Dissociated sphere cells (1.5 × 10<sup>3</sup>) were plated onto Matrigel-coated transwells with 8 μm pore size. DMEM supplemented with 5% of human serum was plated in the lower compartment of the transwell. After plating, migrated cells were counted up to 72 hours.

**Immunohistochemistry and immunofluorescence**

For immunohistochemical analysis, slides were heated for antigen retrieval in 10 mmol/L of sodium citrate (pH 6.0). Sections were subsequently exposed to specific antibodies for thyroglobulin (Tg, DAK-Tg6; Dako), TTF1 (SPT24; Novocastra), cytokeratin 19 (CK19, RCK108; Dako), CD133/1 (AC133; Miltenyi), CD44 (DF1485; Biogenex), ALDH1 (ALDH1a1, TaqMan Gene Expression Assays; Applied Biosystems) and 1× probes and primer sets Hs0016745_m1 (ALDH1a1, TaqMan Gene Expression Assays; Applied Biosystems) or 1× human glyceraldehyde-3-phosphate dehydrogenase (Pre-Developed TaqMan Assay Reagents; Applied Biosystems). Data processing and statistical analysis were performed using the ABI PRISM SDS, software version 2.1 (Applied Biosystems).

**Quantitative and reverse transcription-PCR analysis**

Total RNA was obtained using the RNeasy Mini kit (Qiagen GmbH) and reverse transcribed using the High-Capacity cDNA Archive kit (Applied Biosystems). Quantitative TaqMan PCR analysis was performed with the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) in a reaction volume of 25 μL containing 1× TaqMan Universal Master Mix (Applied Biosystems) and 1× probes and primer sets. Quantitative and reverse transcription-PCR analysis was performed with the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) in a reaction volume of 25 μL containing 1× TaqMan Universal Master Mix (Applied Biosystems) and 1× probes and primer sets. Quantitative reverse transcription-PCR analysis was performed with the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) in a reaction volume of 25 μL containing 1× TaqMan Universal Master Mix (Applied Biosystems) and 1× probes and primer sets. Quantitative reverse transcription-PCR analysis was performed with the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) in a reaction volume of 25 μL containing 1× TaqMan Universal Master Mix (Applied Biosystems) and 1× probes and primer sets.

**Immunoblotting**

Lysates were fractioned on SDS-polyacrylamide gels and blotted to nitrocellulose. Membranes were blocked for 1 hour with nonfat dry milk and successively incubated with antibodies specific for Met (sc-161, C20, rabbit polyclonal; Santa Cruz Biotechnology), pMet (D26, Tyr1234/1235, rabbit IgG; CST), AKT (9272, rabbit polyclonal; CST), pAKT (9271, Ser473, rabbit polyclonal; CST), E-cadherin (4065, rabbit polyclonal; CST), and β-actin (Ab-1 mouse IgM; Calbiochem). Membranes were then washed, incubated for 1 hour with horseradish peroxidase–conjugated anti-mouse or anti-rabbit immunoglobulins (Amersham), and developed with a chemiluminescence detection system (Pierce Chemical, Co.).

**Production of lentiviral particles and infection**

Gene transfer was performed using a TWEEN lentiviral vector containing luciferase (LUC) and green fluorescent protein (GFP) as reporter genes (34). Transfection of packaging human embryonic kidney cell line HEK-293T was assessed using FuGENE 6 Reagent (Roche) and following the instructions of the manufacturer.

Stable short hairpin RNA (shRNA) expressing UTC cells were obtained by infection with pLKO1 lentiviral plasmid containing the interfering sequence of puromycin-resistant Akt (Sigma).
UTC cells were selected with puromycin drug (2 μg/mL, Sigma) for 15 days to enrich for positive transfectants. ShMet (pCCLSsin.PPT.JhPGK.GFP.Wpre) was obtained as described in ref. (33).

Animal model

Five-week-old nude mice from Charles River Laboratories were maintained in accordance with the institutional guidelines of the University of Palermo animal care committee.

After s.c. injection, tumor size was measured using the formula: (π/6) × larger diameter × (smaller diameter)². For orthotopic xenografts, 6-week-old nonobese diabetic/severe combined immunodeficiency mice were injected into the right thyroid gland using a 25 μL Hamilton syringe and a 32-gauge needle with the support of a dissecting microscope. All mice were analyzed on a weekly basis by in vivo imaging (Biospace Lab) upon i.p. injection (100 μL) of d-luciferin (40 mg/mL; Sigma-Aldrich; ref. 35).

Statistical analysis

Data were expressed as mean ± SD. Statistical significance was determined by ANOVA (one-way or two-way) with Bonferroni post-test. Results were considered significant when P values were less than 0.05 (*, P < 0.05; **, P < 0.01; and ***, P < 0.001).

Results

A small population of thyroid carcinoma cells retains clonogenic capacity

We measured the clonogenic activity of tumor cells freshly isolated from thyroid tumors. Limiting dilution analysis showed that the mean number of clonogenic cells was 2% in PTC, 1.2% in FTC, and 3.5% in UTC. This small cell subset could be expanded unlimitedly as thyrospheres after serial passages, whereas the remaining cells displayed a limited growth that did not last more than 2 weeks (Fig. 1A and B; data not shown). Although we found some CD133 transcripts in normal thyroid and UTC cells, clonogenic thyrosphere cultures expressed ALDH1 and CD44 at the protein level, whereas CD133 and ALDH1 were almost abolished after 5 days of culture in PTC and FTC, and maintained in the majority of UTC spheres–derived adherent cells (Supplementary Fig. S1E). Nestin was highly expressed in PTC and FTC spheres and gradually lost in their respective spheres–derived adherent cells, whereas UTC cells were consistently negative in both conditions.

Collectively, these data suggest that epithelial thyroid cancers are hierarchically composed by a small cell population with clonogenic potential that gives rise to a more differentiated progeny of the respective tumor.

ALDH expression is restricted to a small population of thyroid cells and its relative abundance correlates with malignancy

We analyzed 18 PTC, 10 FTC, and 6 UTC (Table 1) for the presence of putative cancer stem/progenitor cells. Immunohistochemistry and flow cytometry analyses of differentiation/stemness markers revealed distinct protein expression profiles among the different histologic thyroid cancer types (Fig. 2A and B). The terminal thyroid differentiation marker Tg and the transcription factor TTF1 were detected in all the PTC and FTC. In contrast, their expression was reduced or missing in UTC, confirming the absence of thyroid-specific functions in the latter (Fig. 2A and B). CK19 was preferentially expressed in PTC with respect to FTC, and was barely detectable in cells purified from the UTC aggressive histologic variant (Fig. 2A and B). Analysis of the potential stem cell markers showed that CD133 was not detectable (Fig. 2A), whereas CD44 was constitutively expressed in all the thyroid tissues tested, both in tumors and their uninvolved pairs (Fig. 2A). In contrast, we observed the presence of rare ALDH1+ cells in normal tissues, which increased progressively in the more aggressive thyroid cancer histotypes (Fig. 2A). ALDH1+ cells were more abundant in UTC (16 ± 4%, n = 5) than in PTC (7 ± 1.8%, n = 12) and in FTC (3 ± 1.2%, n = 9; Fig. 2B–D). A small percentage (2%) of ALDH1+ cells was found in the UTC primary adherent cultures (Supplementary Fig. S1B). Thus, ALDH1 expression is restricted to a small population of cells of the thyroid gland whose relative abundance in UTC may correlate with higher malignancy.

Cells with high ALDH expression are tumorigenic and reproduce the phenotypic characteristics of the original tumor

To determine whether ALDH expression could be used as a prospective marker of thyroid tumorigenic cells, we isolated ALDHhigh cells from all three histologic variants. As shown in Fig. 3A, cells with ALDHhigh expression were detected in all the tumors examined, with the highest percentage in freshly dissociated UTC tissues (on average, 14 ± 3% of positive cells). The analysis of cells from PTC and FTC revealed a smaller ALDHhigh fraction, accounting for 5 ± 2% and 2 ± 1.2% of the whole-cell pool, respectively.

A prerequisite of putative CSCs is the ability to initiate tumor development in recipient animals and reproduce the phenotype of the human parental tumor. Therefore, we injected ALDHhigh, ALDHlow, and unsorted cells isolated from
human thyroid cancers into the flanks of nude mice. Although not all the tumors contained cells able to initiate thyroid cancer in nude mice, ALDH\textsuperscript{high} and unseparated cells efficiently engrafted under the subcutis of the majority of the animals (ALDH\textsuperscript{high} efficiency: 75\% PTC, 70\% FTC, and 80\% UTC; Fig. 3B; Table 1). In all such animals, 5,000 ALDH\textsuperscript{high} cells were more tumorigenic than 25,000 bulk cells, whereas 5,000 ALDH\textsuperscript{low} cells isolated from the same tumors were unable to form a tumor xenograft (Fig. 3B). The injection of a higher number (25,000) of ALDH\textsuperscript{low} FTC and PTC cells did not improve the tumor incidence. However, a limited and delayed tumor growth was observed 8 to 12 weeks from the delivery of 25,000 ALDH\textsuperscript{low} cells (Fig. 3B), as a possible result of the presence of contaminating ALDH\textsuperscript{high} cells (in the order of 0.2–0.3\%) in the sorted ALDH\textsuperscript{low} fraction. The failure of the ALDH\textsuperscript{low} population to generate tumors

Figure 1. A small population of thyroid cancer cells is clonogenic and forms spheres. A, percentage of clonogenic cells in freshly dissociated tumors. B, representative phase contrast microscopy analysis of UTC single cell–derived spheres cultured in serum-free medium at the indicated days. C, confocal microscopy analysis of DIC (left) and immunofluorescence for the indicated antigens on thyroid cancer spheres. D, confocal microscopy analysis of CK19 in PTC, FTC, and UTC spheres–derived adherent cells cultured for 1 and 5 d. E, immunofluorescence analysis of thyroglobulin (Tg), CD44, and ALDH1 in UTC single cell–derived spheres cultured for 5 d in fetal bovine serum (FBS). F, ALDH1 mRNA expression in UTC single cell–derived spheres cultured for 5 d in fetal bovine serum (FBS).
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NOTE: The symbol (/) indicates that the experiments was not performed.
following xenotransplantation was not due to a total depletion of cancer cells during the cell sorting procedure because this nontumorigenic cell population was mutated for BRAF in 5 out of 12 PTC and in 2 out of 6 UTC (Supplementary Fig. S2A; Table 1; data not shown). Interestingly, the resultant tumor xenografts reproduced the genotypic and phenotypic characteristics of their respective parental tumors, as shown by histologic and immunohistochemical analyses (Fig. 3C; Supplementary Table S1). The presence of \( \text{BRAF} \) mutation was associated with a lower ALDH activity than \( \text{RET/PTC1} \) rearrangement and \( \text{p53} \) mutations, whereas the combination of \( \text{BRAF} \) and \( \text{p53} \) mutations was associated with the highest ALDH activity in the xenografts (Supplementary Table S1).

Consistently, the highly tumorigenic ALDH\textsuperscript{high} UTC cells generated CK19 and Tg-negative undifferentiated tumors, reproducing the same phenotypic characteristics of the parental tumor (Fig. 3C1). PTC, FTC, and UTC primary xenografts retained a similar amount of ALDH\textsubscript{1} expression of the parental tumors (Fig. 3C; Supplementary Fig. S3A). Thus, a high ALDH expression characterizes the tumorigenic thyroid cancer population.

**ALDH\textsuperscript{high} thyroid cancer spheres retain tumor-initiating capacity**

We first evaluated the self-renewal capacity of the total, ALDH\textsuperscript{high}, and ALDH\textsuperscript{low} thyroid cancer cell populations by seeding freshly isolated tumor cells at clonal density under nondifferentiating conditions. In such culture conditions, ALDH\textsuperscript{high} cells generated viable spheres and showed significant sphere enrichment as compared with an equal number of unseparated cells (Fig. 4A). Although at 21 days, \( 2 \times 10^5 \) ALDH\textsuperscript{high} cells from PTC, FTC, and UTC primary tumors yielded \( 490 \pm 100 \), \( 330 \pm 74 \), and \( 550 \pm 80 \) thyrospheres, respectively, very few of the ALDH\textsuperscript{low} cells generated spheres (Fig. 4A; Supplementary Fig. S2B). The analysis of the clones generated from cells undergoing flow cytometry sorting...
indicated that the sorting procedure reduced the number of clonogenic cells from PTC and FTC by 50% without affecting the clonogenic performance of UTC cells. Thus, the number of clonogenic cells within the ALDH\textsuperscript{high} population was in the order of 40%, 60%, and 25% for PTC, FTC, and UTC, respectively. Clonogenic analysis showed that thyroid cancer sphere cells display a considerably higher degree of self-renewal than normal thyroid sphere cells (Fig. 4B), which were ALDH\textsuperscript{+} and CK19\textsuperscript{−} as the tumor counterparts (Supplementary Fig. S2C). To determine whether ALDH\textsuperscript{high} cells retained their tumorigenic potential after growing in vitro as thyrospheres, we injected ALDH\textsuperscript{high} spheres into nude mice kept for three passages under nondifferentiating conditions or grown in monolayer under adherent conditions for 20 days. PTC and FTC adherent cells did not engraft whereas UTC cells formed very small tumors, which could be derived from the small number of ALDH\textsuperscript{high} cells that persisted under adherent conditions (Fig. 4C; data not shown). In contrast, all thyroid cancer spheres were highly tumorigenic, as tumors invariably formed following the injection of as few as 5 × 10\textsuperscript{3} cells (Fig. 4C).

Repeated in vitro passages of thyrospheres for up to 1 year did not affect cell growth or the ability to generate tumors in immunocompromised mice (Supplementary Fig. S2D). Furthermore, serial transplantation experiments were conducted using repurified ALDH\textsuperscript{high} spheres expanded in vitro from primary, secondary, and tertiary PTC, FTC, and UTC xenografts (Supplementary Fig. S2E and F). Analysis of the clonogenic activity and ALDH1 expression indicated that the number of tumorigenic cells did not change significantly in primary thyroid cancer xenografts as compared with the corresponding parental tumors (Fig. 2A; Supplementary Fig. S2G), suggesting

![Image of ALDH activity analysis](image-url)

**Figure 3.** ALDH\textsuperscript{high} cells retain tumorigenic capacity. A, representative FACS analysis of 7 PTC, 7 FTC, and 4 UTC cells using ALDEFLUOR assay. Thyroid cancer cells exposed to ALDEFLUOR substrate (BAAA) and specific inhibitor of ALDH (DEAB) were used to define the population with high ALDH activity (R2, green region) and population with low ALDH activity (R1, red region). B, size of tumors developed following s.c. injection of different thyroid cancer subpopulations. Data are mean tumor size ± SD of five tumors per group derived from four separated patients. C, histologic and immunohistochemical analysis of paraffin-embedded sections of xenografts obtained 12 wk after injection of 5 × 10\textsuperscript{3} ALDH\textsuperscript{high} cells. One representative experiment of four performed with cells from different donors is shown. c1, representative s.c. tumor growth in nude mice 12 wk after injection of 5 × 10\textsuperscript{3} UTC ALDH\textsuperscript{high} cells. No tumor growth was found at the UTC ALDH\textsuperscript{low} cells’ (5 × 10\textsuperscript{3} cells) injection site.
that thyroid sphere–derived xenografts maintain the same hierarchy as thyroid cancer. However, the number of tumorigenic cells together with the tumor growth rate tended to increase with serial xenografting, as the possible result of a selection process (Supplementary Fig. S2F and G and S3B).

**ALDH**\(^\text{high}\)**–thyroid cancer spheres form tumors and metastasis when orthotopically injected into mouse thyroid gland**

The development of experimental models of thyroid cancer able to reproduce the human condition would be a powerful tool for preclinical validation of new therapies, particularly for the most aggressive histotypes. We injected 100 cells from PTC, FTC, and UTC spheres into the thyroid gland of immunocompromised mice. This procedure resulted in 100% tumor formation efficiency within a few weeks from injection.

*In vivo* imaging and microscopic examinations of thyroid tumors generated after 4 weeks by ALDH\(^\text{high}\) PTC spheres revealed local tumor growth, whereas FTC spheres determined thyroid gland infiltration and compression of adjacent structures such as larynx and trachea (Fig. 5A). Similarly, the injection of ALDH\(^\text{high}\) UTC spheres resulted in tumor development with a rapid invasive growth causing tracheal and esophageal compression (Fig. 5A), thus recapitulating the clinical features of this tumor. Mice injected with UTC cells became moribund and cachetic within 4 weeks after injection due to the impediment of the upper aerodigestive tract. Histologic examination of tumor specimens confirmed compression and infiltration of mouse thyroid gland, trachea, and esophagus (Fig. 5A and B). Tumorigenicity of orthotopically injected thyroid cancer spheres was significantly faster than after s.c. xenografting, even following serial *in vivo* passages at the ectopic location (Supplementary Fig. S3B).

Moreover, mice injected with ALDH\(^\text{high}\) UTC spheres exhibited cervical lymph node metastasis and a high rate of lung metastasis, whereas animals inoculated with ALDH\(^\text{high}\) FTC spheres generated a moderate local invasion but not regional or distant metastases (Fig. 5A and B). Immunohistochemistry and flow cytometry analyses of tumors and metastases of mice orthotopically injected with ALDH\(^\text{high}\) UTC spheres showed a significant enrichment of the putative tumorigenic ALDH\(^1\)+ cells in lung metastases as compared with the thyroid tumor (Fig. 5B; Supplementary Fig. S3A). Similar to parental tumors, Tg and CK19 differentiation markers were expressed in PTC and FTC orthotopic xenografts (Fig. 5B). These findings show that the orthotopic...
injection of ALDH\textsuperscript{high} thyroid cancer spheres reproduces the clinical and pathologic behavior, including tracheal and esophageal invasion, and high incidence of local and distant metastases, which are peculiar characteristics of poorly differentiated human thyroid tumors.

**Met and Akt activation drives tumorigenicity and metastatic activity of thyroid cancer stem cells**

To define the metastatic potential of thyroid cancer stem cell population, we quantitatively measured the posttranslational modifications of molecules commonly associated with the metastatic signaling pathways (Supplementary Fig. S4A). Reverse phase protein microarray technology and immunoblot validation revealed a considerable upregulation of cMet, β-catenin, and E-cadherin, with an increased phosphorylation status of cMet and Akt in sphere cells from UTC as compared with those from normal thyroid (Control) PTC and FTC (Fig. 6A; Supplementary Fig. S4A and B). Moreover, UTC sphere cells showed a higher percentage of β-catenin nuclear accumulation (Supplementary Fig. S4C).

**Figure 5.** Thyroid cancer sphere cells form tumors and metastasis upon orthotopic injection. A, whole body imaging analysis of primary and metastatic thyroid tumors after injection of 100 cells from dissociated PTC, FTC (top), and UTC (bottom) spheres into the mouse thyroid gland. One representative experiment of eight per group is shown. SG, salivary glands; T, thyroid tumor; L, lung, and H, heart. B, H&E staining and immunohistochemical analysis of ALDH1, Tg, or CK19 revealed by 3-amino-9-ethylcarbazole (red) on paraffin-embedded sections of mouse thyroid gland invaded by human PTC, FTC, and UTC cells, and mouse lung metastasis by human UTC cells. Experiments were performed as in A. Thy, normal mouse thyroid.
and showed a higher migration capacity than the stem cells from the other thyroid tumor histotypes (Fig. 6B). Interestingly, migrated UTC stem cells showed pMet and pAKT upregulation, nuclear accumulation of β-catenin, and complete loss of E-cadherin expression (Supplementary Fig. S4D). To understand whether Met and AKT activation is involved in the regulation of CSC migratory ability, we downregulated Met and AKT expression levels in UTC spheres. Transduction of spheres with lentiviral vectors encoding specific Met and AKT shRNA sequences resulted in decreased amount of these two proteins and in efficient knockdown of their activated forms (Fig. 6C). We observed that functional blockade of these molecules dramatically reduced the migration of UTC sphere cells (Fig. 6D). Moreover, knockdown of MET and AKT significantly reduced the mRNA levels of Snail and Twist (Supplementary Fig. S5A), two key transcription factors that characterize epithelial-mesenchymal transition.

We next determined whether Akt and Met silencing might have in vivo therapeutic benefits. UTC sphere cells transduced with ShMet or ShAkt were grown orthotopically into mouse thyroid gland. Met knockdown delayed tumor outgrowth by about 9 weeks whereas ShAkt dramatically reduced the capacity to give rise to orthotopic tumors generated by UTC stem cells (Fig. 6E, left; Supplementary Fig. S5B). Moreover, silencing of either Met or Akt resulted in the complete abrogation of the metastatic capacity (Fig. 6E, right; Supplementary Fig. S5B). These results clearly...
indicate that Met and/or Akt could be very promising therapeutic targets in the treatment of thyroid cancer because they play a role both in CSC growth and invasion.

**Discussion**

Here, we showed that thyroid cancer contains a population of tumorigenic cells that can be considerably enriched based on the expression of ALDH. Our data show that ~1.2% to 3.5% of the whole thyroid cancer population are ALDH$_{high}$ tumorigenic cells that can be clonally expanded *in vitro* in serum-free medium. A considerable percentage of ALDH$_{high}$ cells can self-renew and grow unlimitedly as thyroid spheres able to generate serial tumor xenografts in immunocompromised mice. During thyroid sphere formation, single clonogenic ALDH$_{high}$ cells undergo symmetrical and asymmetrical division, giving rise to other clonogenic cells together with a progeny of cells with limited proliferation potential.

Upon exposure to serum or thyroid-stimulating hormone, clonogenic cells gradually lose the expression of ALDH, together with the tumorigenic potential. In such differentiating conditions, PTC and FTC ALDH$_{high}$ cells undergo aberrant differentiation, as indicated by the acquisition of CK19 and Tg upregulation, whereas UTC cells remain largely undifferentiated. Sphere-derived tumor xenografts reproduce the original tumor, both in terms of morphology and antigen expression. The pattern of ALDH1, CK19, and Tg expression and the percentage of tumorigenic cells did not significantly vary in parental tumors and respective primary xenografts, indicating that thyroid cancer cells possess a functional hierarchy and heterogeneity.

Previous studies on malignant cancer development showed that the transcriptional program awarding migration and invasion involves transcription factors belonging to Snail and Twist families (36), which play a similar role in the epithelial-mesenchymal transition displayed in embryogenesis. Met and its ligand are overexpressed in a variety of tumors. The activation of Met induces proliferation, invasion, and angiogenesis, contributing to tumor growth and spreading. In thyroid cancer, Met activation has been proposed as a negative prognostic factor (37). The current study reveals the important relationships between Met/Akt pathway and the aggressive phenotype of thyroid CSCs. Our data show that pMet and pAkt expression is greater in UTC than in PTC and FTC. We found that activation of Akt, Met, and β-catenin, together with downregulation of E-cadherin, confers motile and invasive behavior to UTC stem cells. Loss of E-cadherin at the invasive front was shown in a variety of tumors during the transition from adenoma to carcinoma (38–40). Although targeting E-cadherin or its regulatory transcription factors Snail and Twist would seem to be a good strategy, these factors are strictly essential for normal cell activity. Moreover, Snail has been shown to inhibit a great number of genes unrelated to E-cadherin downregulation, and re-establishment of E-cadherin does not restore an epithelial phenotype (41). Interestingly, Akt or Met targeting represses Twist and Snail expression, abolishing migration and metastatic activity of UTC stem cells. Our findings support a role for Akt and Met targeting for the treatment of invasive malignant thyroid cancers.

A recent study indicates that the evaluation of tumorigenic potential in melanoma is strictly dependent on the system used for the assay (42). Several studies have relied on the use of immunocompromised mouse recipients to quantify the percentage of tumorigenic cells present in the tumors, which were defined as ~0.0001% in melanoma or 0.057% in colon cancer (19, 43). At least in the case of melanoma, the system used for such calculation has considerably undervalued the percentage of tumorigenic cells, which seems to be on the order of 27% (42). In our analysis of the tumor-initiating cells in thyroid cancer, we evaluated the percentage of clonogenic cells to define the cell subset endowed with tumorigenic potential. Although our assay may have underestimated the percentage of tumor-initiating cells present in the tumors, both *in vitro* and *in vivo* analyses indicated that tumorigenic activity is confined in the ALDH$_{high}$ cell population, which may contain 25% to 60% of the clonogenic cells able to generate tumor xenografts in immunocompromised mice after *in vitro* expansion.

The absence of reliable experimental models has delayed the development of new therapies for thyroid tumors. Because thyroid cancer is particularly resistant to conventional chemotherapy, the therapeutic procedures have remained essentially the same in the last 20 years and rely on surgery followed by radioactive iodine treatment (44–46). However, in advanced thyroid cancer, surgery is unable to eradicate the tumor. Although the more differentiated forms can be cured by radioactive iodine treatment (47), poorly differentiated histotypes often do not express the iodide symporter and are resistant to this therapy (48).

The possibility of prospectively isolating and growing tumorigenic clones from thyroid cancer has considerable implications. An extensive characterization of tumor-initiating cells may allow the identification of new biomarkers for prognostic and therapeutic purposes. Moreover, these cells can be screened for drug sensitivity or used for generating novel animal models of thyroid cancer. In this context, the orthotopic delivery of thyroid cancer stem cells is able to recapitulate the aggressive behavior of undifferentiated thyroid carcinomas, including local and distant metastases. Because anaplastic thyroid tumors are invariably lethal, this orthotopic model could be exploited for experimental testing and preclinical validation of new treatments.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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Tumorigenic and Metastatic Activity of Human Thyroid Cancer Stem Cells

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