A Novel Orthotopic Mouse Model of Human Anaplastic Thyroid Carcinoma

Carmelo Nucera,¹ Matthew A. Nehs,¹ Michal Mekel,¹ Xuefeng Zhang,² Richard Hodin,¹ Jack Lawler,² Vânia Nose,³ and Sareh Parangi¹

Background: Orthotopic mouse models of human cancer represent an important *in vivo* tool for drug testing and validation. Most of the human thyroid carcinoma cell lines used in orthotopic or subcutaneous models are likely of melanoma and colon cancer. Here, we report and characterize a novel orthotopic model of human thyroid carcinoma using a unique thyroid cancer cell line.

Methods: We used the cell line 8505c, originated from a thyroid tumor histologically characterized by anaplastic carcinoma cell features. We injected 8505c cells engineered using a green fluorescent protein–positive lentiviral vector orthotopically into the thyroid of severe combined immunodeficient mice.

Results: Orthotopic implantation with the 8505c cells produced thyroid tumors after 5 weeks, showing large neck masses, with histopathologic features of a high-grade neoplasm (anaplasia, necrosis, high mitotic and proliferative indexes, p53 positivity, extrathyroidal invasion, lymph node and distant metastases) and immunoprofile of follicular thyroid cell origin with positivity for thyroid transcription factor-1 and PAX8, and for cytokeratins.

Conclusions: Here we describe a novel orthotopic thyroid carcinoma model using 8505c cells. This model can prove to be a reliable and useful tool to investigate *in vivo* biological mechanisms determining thyroid cancer aggressiveness, and to test novel therapeutics for the treatment of refractory or advanced thyroid cancers.

Introduction

THYROID CANCERS THAT DERIVE FROM thyroid follicular cells are classified as well-differentiated (papillary and follicular thyroid carcinomas [PTC and FTC]), poorly differentiated, and undifferentiated (anaplastic) thyroid carcinomas (ATC). PTC is the most common thyroid malignancy and has excellent long-term survival with standard therapy (1). However, poorly differentiated and undifferentiated thyroid cancers more readily metastasize, frequently resist uptake of radioiodine treatments, and therefore lead to the largest number of endocrine-related deaths (2–4). ATCs have a very high proliferation rate and marked aneuploidy, are thought to develop from an existing PTC or FTC, and are responsible for more than half of thyroid carcinoma deaths (5). The diagnosis of ATC is almost uniformly fatal, with a mean survival of 6 months from diagnosis (6). Neither chemotherapy nor radiation therapy are effective at prolonging the survival of an ATC patient (7). Therefore, alternative systemic treatments for this aggressive cancer are urgently needed.

In recent years, considerable effort has been made to develop more clinically relevant models by orthotopic transplantation of human tumor cells in rodents. It is now possible to transplant tumor material from a variety of tumor histotypes into the appropriate anatomical site (orthotopic model), and often these tumors will metastasize to similar locations as the same tumor type in human cancer (8). In addition, the development and establishment of stable green fluorescent protein (GFP)-expressing cell lines that permit detection and visualization of growth of the tumor and metastases in live tissue has had a major impact on research with orthotopic models (8). The development of a reliable and reproducible orthotopic model of advanced thyroid cancer is an important step in developing and evaluating new treatments, especially for those with aggressive forms of thyroid cancer.

Several types of animal models of human thyroid cancers (HTC) have been created, including xenograft, orthotopic, and transgenic or genetically engineered mice (9). Subcutaneous implantation of HTC cells into immunocompromised mice has been most common (10). Subcutaneous xenograft models are

¹Thyroid Cancer Research Laboratory, Unit of Endocrine Surgery, Massachusetts General Hospital; ²Division of Cancer Biology and Angiogenesis, Department of Pathology, Beth Israel Deaconess Medical Center; ³Endocrine Pathology, Department of Pathology, Brigham and Women's Hospital; Harvard Medical School, Boston, Massachusetts.

technically simple, and it is easy to palpate and measure the tumor growth after implantation. However, these models differ in the tumor microenvironment of the skin implantation site and that of the organ of origin, such as the thyroid (9,11). In xenografted tumors, naturally occurring inhibitors of angiogenesis present in the skin theoretically can limit not only the growth of the primary tumor but also patterns of metastases (12–15). These differences in the tumor microenvironment may be particularly important in studying tumor invasion and metastasis, since critical aspects of aggressive tumor behavior may depend not only on the tumor cells themselves but also on the stromal, endothelial, and lymphatic elements in the implantation site. Orthotopic tumor models are more cumbersome to develop, but they place the tumor cells into the organ of origin, thyroid in this case. They may better reproduce the stromal response and the pattern of spread of the tumor to regional lymph nodes and other common metastatic sites such as lung and bone.

Over the past 15 years, several sophisticated genetically engineered mouse models of thyroid cancer have been created to further our understanding of the genetic events leading to thyroid carcinogenesis *in vivo* (16). In most cases, engineered mice have been produced using the highly active bovine thyroglobulin (Tg) promoter to specifically target transgene expression to thyroid follicular cells, or using the human or rat calcitonin/calcitonin gene-related peptide promoter to target transgene expression to C cells. The most common models express oncogenic alterations such as B-Raf^{V600E} mutation (tumor type: PTC aggressive tall cell variant), RET/PTC-1 (tumor type: PTC) and RET/PTC-3 translocations (tumor type: PTC), c-Ha-RAS mutation (tumor type: PTC), v-Ha-RAS mutation (tumor type: C-cell hyperplasia, medullary thyroid carcinoma [MTC]), TRK-T1 translocation (tumor type: follicular cell hyperplasia and PTC), RET mutation (tumor type: MTC), and thyroid receptor beta by introduction of a dominant-negative mutation named PV (TR $\beta^{PV/PV}$ or TR $\beta^{PV/-}$) (tumor type: follicular cell hyperplasia, FTC) (16). In addition, engineered mouse models with heterozygous deletional mutation of Rb and p53 develop C-cell hyperplasia and MTC. Importantly, engineered mouse models RET/PTC-1 or RET/PTC-3 with p53 loss are histologically characterized by anaplasia, local invasion, and lymph node metastasis (16).

Advantages of these models include analysis of the role of specific genetic mutations in thyroid tumorigenesis, offering examples of positive genotype-phenotype correlation, but they also have some major downsides. Often these tumors are driven by single specific alterations such as RET/PTC, RAS, or B-Raf at least in their first phase of development, and thus they either do not reflect the complexity of human tumor biology or do so to a very limited extent. Another downside of these mice is that thyroid tumors often form either very fast (B-Raf^{V600E} transgenic mice) or very slowly (RET/PTC-1 transgenic mice) (17,18). This makes testing of therapeutics in a cohort of similar-sized tumors challenging. Therefore, genetically engineered models of thyroid cancer do have an important role in studying thyroid cancer *in vivo* but have some significant limitations, especially in testing therapeutics.

Reports of orthotopic mouse models of ATC or PTC have been published previously (1,19–26). However, some of the tumor cell lines used in these studies were subsequently determined to be of melanoma and/or colon cancer due to contamination, rather than HTC cell lines as originally re-

ported (27). Three orthotopic models of human FTC have been also generated (28–30). Given the difficulty in interpreting the literature with the recent confusion about cross-contamination and misidentification of known historical thyroid cancer cell lines, we felt it was important to establish a new orthotopic model of human ATC with a well-established, confirmed HTC cell line. Having a reliable and reproducible animal model of thyroid cancer is an important step in the testing and development of novel therapeutics. To produce our orthotopic mouse model of advanced HTC, we used the unique thyroid cancer cell line 8505c (27). This cell line harbors a homozygous mutation in B-Raf (V600E, homozygous T > A transversion at nucleotide 1799) and p53 (C:G to G:C transversion at the first base of p53 gene codon 248) and no RET/PTC-1 translocation (27,31,32).

Materials and Methods

HTC cell lines

The HTC cell line used in the study was 8505c (purchased from DSMZ—German collection of microorganisms and cell culture). This cell line was established by Dr. M. Akiyama (Radiation Effects Research Foundation, Hiroshima, Japan) from the primary tumor of a 78-year-old woman with undifferentiated carcinoma, histologically a largely papillary thyroid adenocarcinoma with some spindle, polygonal, and giant cells.

Cell transfections

The 293FT cells (5×10^5) were seeded in 60 mm dishes and transfected the following day with a GFP-positive lentiviral construct named HIV-U6-GL3B-GFP, kindly provided by Dr. Y. Kawakami (Institute for Advanced Medical Research, Tokyo, Japan) (33). We cotransfected this construct with packaging plasmids (gag-pol and VSV-G) using Eugene 6 (Roche, Nutley, NJ) in OptiMEM for 48 hours to prepare lentivirus according to the manufacturer's instructions.

Cell infections

Prior to implantation, 8505c cells were infected to view the primary tumor and metastatic burden. Lentiviral infections were performed as follows: the media containing the progeny virus released from the 293FT cells were filtered by 0.45 μ m filters, collected, and used to infect the 8505c cells for 3–6 hours in the presence of 8 μ g/mL polybrene (Sigma, St. Louis, MO). The 8505c cells were infected at 50 or 100 multiplicity of infection according to Sumimoto *et al.* (33). Finally, the GFP-positive 8505c cells were sorted by flow cytometry analysis (MoFlo/FACSARIA Sorting, Beckman Coulter, Fullerton, CA).

Tumor implantation

All animal work was done in the animal facility at Beth Israel Deaconess Medical Center or Massachusetts General Hospital (Boston, MA) in accordance with federal, local, and institutional guidelines. For the orthotopic tumor implantation model, 10 severe combined immunodeficient (SCID) female mice, 4 to 6 weeks of age (Taconic, Germantown, NY), were anesthetized using 200 μ L of a mixture of 90 mg/mL ketamine and 10 mg/mL xylazine per mouse. The neck was

FIG. 1. (A) Macroscopic anatomy of normal mouse thyroid. (B) Histological sections of mouse thyroid tissue (hematoxylin and eosin) and adjacent anatomical structures (magnification, 4 $\times$). (C) Histology of the right thyroid showing normal follicular structure (10 $\times$). (D) Gross 8505c cell tumor mass 35 days posttumor implantation (10 $\times$). (E) Orthotopic human thyroid mass with tracheal impingement (10 $\times$). (F) and (G) Green fluorescent protein (GFP) excitation at 470 nm shows a large mass on the right with extension in the left side. (H) Gross of mouse lung (10 $\times$). (I, J) GFP excitation reveals miliary multifocal lung micrometastases (10 $\times$). Color images available online at www.liebertonline.com/thy.

washed with betadine, the skin and subcutaneous tissues were incised horizontally, and the salivary glands were reflected laterally. The central component of the neck was exposed and the overlying strap muscles were bluntly dissected away from the right thyroid. Normal anatomy of the mouse thyroid and perithyroid tissues is described in Figure 1. Once the right thyroid was exposed, 5×10^5 8505c cells resuspended in 10 μ L of serum-free RPMI medium were injected using a Hamilton syringe attached to a 27-gauge standard needle (Fig. 1A). Only the right thyroid was injected; the left side of the thyroid was not manipulated and used as an internal control. Following the injection, the salivary glands were repositioned over the anterior neck and the incision was closed using two or three interrupted 3-0 nylon sutures. The mice were placed under a warming lamp during recovery from anesthesia. For subcutaneous implantation, three SCID mice were injected subcutaneously in the right flank using the same concentration of 8505c cells. Mice were checked daily for signs of postoperative complications (hematoma, stridor, etc.). Tumor burden was measured by palpation and calipers every week in nonanesthetized mice.

There were no surgical or anesthetic complications in any of the mice. All 10 mice with orthotopic tumor implantation and the 3 subcutaneously implanted ones underwent necropsy 35 days after tumor cell implantation. Body weight (grams) was measured by an electronic digital scale, and tumor size was measured using an electronic caliper. Tumor volume was calculated as $(\pi/6) \times \text{length} \times \text{width} \times \text{height}$. Paratracheal and

regional cervical lymph nodes were removed en bloc with the tumor for histological analysis. Lungs, femoral bones, ribs, spine (vertebrae), pelvic bones, and liver were also collected and analyzed.

Microscopy, histological, and immunohistochemical analyses

Specimens from those animals with tumors labeled with GFP were subjected to fluorescence microscopy (Leica MZFL III [Leica Microsystems, Bannockburn, IL], wavelength absorption: 488 nm) immediately after necropsy. In addition, we used the multispectral fluorescence scanner (CRi Maestro 500, Woburn, MA), designed to image fluorophores between 450 and 900 nm, to view the GFP in the primary tumor in the whole mouse. Thyroid and lung tissue from control SCID mice, which were not implanted with 8505c cells, were analyzed to determine natural basic tissue fluorescence.

All tissue specimens were fixed with 4% paraformaldehyde and embedded in paraffin blocks. Bone specimens were processed by a standard mild decalcification procedure before embedding in paraffin. Histopathology evaluation was performed by an endocrine pathologist (Vânia Nose) on hematoxylin and eosin (H&E) stained tissue sections of the orthotopic thyroid tumor, regional lymph nodes, bones, lungs, and liver. This was done using an Olympus BX 41 microscope and the Olympus Q COLOR 5 photo camera. Four-micron-thick sections of formalin-fixed paraffin-embedded tissues

were used for immunohistochemical procedures. After baking overnight at 37°C, deparaffinization and rehydration were performed (100% xylene four times for 3 minutes each, 100% ethanol four times for 3 minutes each, and running water for 5 minutes). For peroxidase activity, the sections were blocked for 30 minutes with 1% hydrogen peroxide in methanol and washed under running water for 5 minutes.

Immunohistochemistry was performed using the following primary antibodies: galectin-3 (Fitzgerald, Concord, MA), HBME-1 (Dako, Carpinteria, CA), p53 (Immunotech, Glendale, CA), HMB-45 (Dako), pancytokeratin (Dako), PAX8 (Protein Tech Group, Chicago, IL), thyroid transcription factor (TTF)-1 (Dako), and MIB-1 (Ki-67) (Dako). The sections, treated in a pressure cooker for antigen retrieval (Biocare Medical, Concord, CA), were incubated at 123°C in citrate buffer (DAKO Target Retrieval Solution, S1699; Dako). The slides were cooled for 15 minutes and transferred to phosphate buffer saline. Antigen retrieval was performed using proteinase digestion for pancytokeratin and citrate buffer for galectin-3, HBME-1, TTF-1, p53, PAX8, and MIB-1. No treatment was done for HMB-45. Primary antibodies were diluted (galectin-3, 1:200; HBME-1, 1:200; pancytokeratin, 1:700; TTF-1, 1:1000; p53, 1:1200; PAX8, 1:300; MIB-1, 1:200; HMB-45, 1:400) and incubated for 60 minutes at room temperature.

Primary antibody was detected using a biotin-free secondary antibody (K4011, DAKO Envision System) and incubated for 30 minutes. All incubations were carried out in a humid chamber at room temperature. Slides were rinsed with phosphate buffer saline between incubations. The sections were developed using 3,3'-diaminobenzidine (Sigma) as a substrate and counterstained with Mayer's hematoxylin.

The immunohistochemical markers were assessed semi-quantitatively using the following scoring method: 0 = negative, 1 = 1–10% positive cells (low expression), 2 = 11–50% positive cells (moderate), and 3 = >50% positive cells (high expression).

Proliferative index

Nuclear immunostaining for MIB-1 was assessed semi-quantitatively as negative when no tumor cells stained, 1+ (<10% of tumor cells stained positive), 2+ (11–50% of tumor cells stained positive), and 3+ (>51% of cells stained positive). The MIB-1 reactivity was evaluated in tumor cells only. Reactivity with MIB-1 in more than 11% of tumor cells (2+ and 3+) was considered a high proliferative index. In addition, the mitotic index was calculated as the number of mitoses per high-power microscopic field (400×), and at least 200 fields were evaluated for each case. A mitotic index with 5–10 mitoses/field was considered high.

Statistical analysis

Mean tumor volume and standard error of the mean (SEM) were calculated and compared in animals with subcutaneous and orthotopic implantations sites by a Student *t*-test with Microsoft Excel Software. *p*-Values less than 0.05 were considered significant (**p* < 0.05, ***p* < 0.01, ****p* < 0.001).

Results

Normal thyroid tissue in the mouse resides on each side of the thyroid cartilage and trachea, and is histologically

similar to human thyroid tissue with a follicular appearance (Fig. 1A–C). All the mice with orthotopic thyroid tumor implantation had tumor growth, which became palpable on neck examination 20 days postimplantation. Tumors steadily grew up to day 35 after tumor implantation and were easily palpable at that point. On examination, the mice demonstrated cachexia and weight loss. Mice with neck tumors had an average decrease in weight of 5.6 grams compared to age-matched controls (average weight 19.4 ± 4.1 grams in tumor-implanted mice versus 25.8 ± 1.8 grams in mice with no injection of 8505c cells). These mice were therefore euthanized and at necropsy had orthotopic thyroid tumors (Fig. 1D–G) with a mean tumor volume of 246.9 ± 68.8 mm³. Many of the orthotopic thyroid tumors were very large and grew between the trachea and the ipsilateral strap muscles displacing the trachea to the left (Fig. 1D). The orthotopic GFP-engineered 8505c cells were also detected by fluorescence microscopy, with the primary tumors showing a bright green signal in the neck (Fig. 1G) and lung metastasis appearing as multiple micrometastases, similar to the disease seen in humans (Fig. 1I, J). The 8505c cells infected with HIV-U6-GL3B-GFP and uninfected 8505c cells did not show any significant difference in cellular proliferation.

Subcutaneous implantation resulted in smaller tumors (18.4 ± 3.5 mm³) compared to orthotopic placement (246.9 ± 68.8 mm³, *p* < 0.01) of the tumor at 35 days postimplantation (Fig. 2). Mice injected subcutaneously with 8505c cells did not demonstrate any neck mass or fluorescence in the thyroid, nor were there any metastases in the lungs (data not shown). Neither orthotopic nor subcutaneous implantation resulted in metastasis to bones or liver.

Histologically, all orthotopic thyroid tumors (Fig. 3C) displayed the ATC features of a high-grade malignant neoplasm characterized by high-grade nuclear atypia, marked cellular pleomorphism, necrosis, high mitotic index (Fig. 3D), and invasion into adjacent organs (Fig. 3E–G). Some tumors also demonstrated tracheal invasion (Fig. 3C and G), skeletal muscle invasion (Fig. 3C and E), perineural invasion (Fig. 3F),

FIG. 2. The 8505c thyroid tumor volume in severe combined immunodeficient (SCID) mice injected orthotopically (*n* = 10) or subcutaneously (*n* = 3), 35 days posttumor implantation. Student *t*-test indicates *p* < 0.01 for 8505c cells injected orthotopically compared to those injected subcutaneously after 35 days of tumor implantation. These data represent mean values and standard error of the mean (SEM).

FIG. 3. Histological sections of mouse thyroid tissue stained with hematoxylin and eosin (H&E). (A) and (B) Normal mouse thyroids (asterisks) near the trachea of SCID mice (magnifications, 4 $\times$ and 10 $\times$) show a typical follicular structure by micro- and macrofollicles. (C) Orthotopic anaplastic thyroid carcinoma (ATC) within the right thyroid by 8505c human thyroid cancer cell line orthotopically injected into the right thyroid of immunocompromised SCID mice. The left thyroid shows absence of malignant features (4 $\times$). (D) Orthotopic ATC showing high-grade features such as mitoses (arrow), necrosis (asterisk), and marked cellular pleomorphism (20 $\times$). (E) Orthotopic ATC invading neck muscles (20 $\times$). (F) Orthotopic ATC with perineural invasion (20 $\times$). (G) Orthotopic ATC invading adjacent tracheal wall (60 $\times$). (H) Cervical lymph node metastasis (arrow) in subcapsular sinus and lymphovascular invasion (arrowhead) (20 $\times$). (I) and (J) Multifocal lung micrometastases characterized from 8505c cells with high-grade pleomorphism (20 $\times$ and 60 $\times$). Histological sections of mouse thyroid tissue stained by immunohistochemistry. (K) Orthotopic human ATC shows an average of 90% MIB-1 (Ki-67) proliferative index in tumor cells. (L) Positivity for nuclear p53 in almost all tumor cells (endothelial and normal thyroid follicular cells are negative). (M) Weak and focal positivity for nuclear thyroid transcription factor-1 (TTF-1) (normal thyroid follicular cells are strongly positive). (N) Loss of nuclear staining for PAX8 (normal thyroid follicular cells are strongly positive) detected weakly in the cytoplasm. (O) Focal and weak staining for pancytokeratin by the tumor cells (all magnifications, 40 $\times$). T, tumor cells; N, normal thyroid follicular cells. Color images available online at www.liebertonline.com/thy.

and lymphovascular invasion, similar to the behavior of human ATC. Metastases to cervical lymph nodes were seen in 50% (5/10) of the mice (Fig. 3H), and multiple micrometastases to lungs were observed in six lung samples that underwent detailed histological analysis (Fig. 3I and J). All subcutaneous thyroid tumors showed the same pathological features (high-grade nuclear atypia, marked cellular pleomorphism, necrosis, and high mitotic index) seen in the 8505c orthotopic thyroid tumors.

Immunohistochemically, orthotopic tumors showed a very high (3+) proliferative index MIB-1 (Ki-67) (90% proliferative index in the tumor cells) (Fig. 3K). In addition, all of the tumor nuclei were positive for mutated p53 protein (Fig. 3L), which is known to be a marker of aggressiveness in human undifferentiated tumors. Further, immunophenotyping of the orthotopic tumor revealed a thyroid-specific phenotype, with a weak nuclear expression of TTF-1 (Fig. 3M), absent nuclear staining for PAX8, and a weak PAX8 staining in the cytoplasm (Fig. 3N). We also found a weak and focal pancytokeratin (Fig. 3O) expression with absence of melanoma marker HMB-45 (data not shown), thus confirming the epithelial nature of this orthotopic tumor. Galectin-3 and HBME-1 markers generally expressed in human PTC were negative in this orthotopic ATC (data not shown).

Discussion

An orthotopic model of thyroid cancer is an important tool in developing novel therapeutics for combating aggressive thyroid cancers. While PTC has an excellent overall survival with standard therapy, there remains considerable work to be done on ATC, which remains among the most lethal of all types of malignancies, not just thyroid malignancies (2). ATC rapidly invades the neck and often spreads to other organs, primarily the lungs. ATC originates from a well-differentiated thyroid carcinoma. In fact, when PTC and ATC occur together, they generally share B-Raf^{V600E} mutation, supporting the notion that many ATCs actually represent progressive malignant degeneration of a preexisting well-differentiated PTC (34). We chose to establish our orthotopic model of ATC with the 8505c human cancer cell line originating from a patient with such a transformation from PTC to ATC.

A xenograft model of 8505c cells has been previously reported by Kotchetkov *et al.* (35). In the present study, 5 $\times$ 10⁶ cells were subcutaneously injected into the right flank of 7-week-old female CD-1 nude mice, and therapeutic intervention was initiated when the tumors reached 100 mm³ in size. Tumor growth rate and variability in the tumor volume among different animals prior to the point of intervention are

not reported; it is also not clear how long it took for the implanted tumors to grow to 100 mm³. Any comparison in terms of tumor growth between our subcutaneous model and that of Kotchetkov *et al.* is difficult because they reported time points of tumor growth only after the subcutaneous tumor reached 100 mm³ and the model was in nude mice not SCIDs. However, subsequent growth of these flank tumors, from 100 mm³ to 200 mm³ in approximately 30 days, was certainly slower than in our orthotopic model.

In our study the orthotopic tumors grew to greater than 240 ± 68.8 mm³ 30 days following the injection of only 0.5 × 10⁶ cells, 10-fold less than the number of tumor cells used in the study by Kotchetkov *et al.* The data from that study, therefore, underscore the importance of the tumor microenvironment in the promotion of tumor growth and progression. In both studies, the subcutaneous 8505c tumors showed features of ATC, such as high-grade cell atypia and pleomorphism. Although Kotchetkov *et al.* make no mention of lung metastases, in our study only orthotopic placement of the tumors led to lung metastases. In our study subcutaneous tumor size variability was generally very low (18.4 ± 3.5 mm³), and so we used a small number of mice in this group. Subcutaneous implantation resulted in small tumors, 228.5% less compared to orthotopic placement at 35 days posttumor implantation. This difference in overall growth confirms the crucial role of an appropriate microenvironment (i.e., endothelial and lymphatic vessels and stromal cells) and organ-specific angiogenesis in facilitating initial tumor growth and perhaps later aggressiveness. On the contrary, subcutaneous tumor models lack specific interactions between tumor cells and their native environment that influences the molecular, pathologic, and clinical features of the tumor.

Overall, orthotopic tumor placement appears to better mimic the microenvironment, morphology, growth, and metastatic patterns of human cancer. Although the subcutaneous model is technically easier to generate, it is not always the most relevant to clinical cancer (8). There is widespread use of subcutaneous tumor models, especially in therapeutic screening studies. This suggests that correlations between subcutaneous tumor model data and clinical activity are good, but some studies provide the opposite view (8). One major challenge of these models is that they do not clearly reproduce the primary site of the common human cancers and nor do they represent the common sites of metastasis. A general view among researchers involved in the drug discovery process is that models that more closely reflect the biological and morphological features of cancer growth and metastasis in humans will better predict potential clinical activity (8).

Our data show that the 8505c orthotopic thyroid cancer model might mimic the biological behavior of human ATC better than subcutaneous tumors models. While orthotopic models of thyroid cancer have been developed and published previously, many of the cell lines used are found to be not only cross-contaminated and redundant but also derived from melanoma or colon cancer cell primaries (27). For example, DRO and ARO cells were developed into an orthotopic thyroid cancer model; however, these cell lines are respectively melanoma or identical to different KAT cell lines (i.e., KAT-5, KAT-4) and genetically match the colon cancer cell line HT-29 (19,20,27). Many cell lines thought to be distinct are likely derivatives of the cell lines (e.g., BHP5-16, BHP14-9, and NPA-87) used to generate orthotopic models of human PTC, which

are likely of melanoma origin rather than thyroid (1,27). Given these findings, we felt the importance of establishing an orthotopic model of HTC with a cell line that is confirmed to be of thyroid origin (27). The 8505c cell line is unique, not cross-contaminated, and originated from a patient with an advanced thyroid carcinoma (27). These cells harbor two common point mutations known to be important in progression and aggressiveness of HTC, p53 (codon 248) and B-Raf^{V600E}. Importantly, our orthotopic tumor demonstrated an immunoprofile specific to thyroid follicular cell origin, with TTF-1 expressed in tumor nuclei. TTF-1 is one of the most commonly used markers in diagnostic pathology, with a very high sensitivity and specificity in the diagnosis of tumors of pulmonary and thyroid lineage (36). TTF-1 mRNA levels are higher (5- to 25.2-fold) in the 8505c cell line compared to other human ATC cell lines but lower (1.7- to 7.4-fold) than in normal thyroid tissue (27). Our orthotopic tumors showed weak nuclear TTF-1 staining. The divergence among mRNA and protein levels may implicate posttranscriptional mechanisms of regulation. In our study we performed immunohistochemistry, which is a semiquantitative method that provides reliable information on the protein status.

Another thyroid-specific marker, PAX8, a tissue-specific transcription factor, is expressed in the thyroid follicular cells, contributing to the maintenance of the differentiated phenotype. Both PAX8 and TTF-1 typically localize to the nuclei in normal human thyroid cells, as well as in benign thyroid tumors and well-differentiated neoplasms of follicular cell origin like PTC and FTC, and both show a gradual decrease in their nuclear localization during thyroid tumor dedifferentiation (37). They are only weakly positive in anaplastic tumors. These two transcription factors are present together only in the thyroid follicular cell, suggesting that this unique combination could play a role in the expression of the thyroid-specific phenotype (38).

In our orthotopic tumor cells, PAX8 was detected weakly in the cytoplasm but not in the nucleus, similar to the findings in tissue from human ATCs and according to its low mRNA levels detected in the 8505c cells (27,37). Moreover, proliferative index and mitotic index in the orthotopic thyroid tumors were very high, clearly demonstrating their aggressive and metastatic behavior.

Our orthotopic model of aggressive thyroid cancer using 8505c cells engineered with GFP might provide a specific and sensitive molecular system for the detection also of small tumors as well as cervical lymph node and lung micrometastases. Manipulation of the 8505c cells with the introduction of common mutations may further increase the aggressiveness of the model, while knockdown of some of the known oncogenes in the primary cell line will allow further stratification of the important determinants of the invasive and metastatic properties of these cells. Knowledge of these genetic and molecular alterations as they relate to orthotopic thyroid cancer models will provide more information on the tumor biology of thyroid cancer aggressiveness and progression, and ultimately could lead to improved clinical treatments.

In conclusion, this novel orthotopic model of advanced HTC is technically feasible, easily reproducible, and might serve as an effective tool in preclinical studies of anti-oncogenic or antiangiogenic compounds. In addition, this orthotopic ATC model provides an important tool to test the

effects of therapeutic interventions on the primary tumor and pulmonary metastases.

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Disclosure Statement

All authors certify that they have no competing financial interests pertaining to any of the data or statements given in this article.

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Address correspondence to:
Sareh Parangi, M.D.

Assistant Professor of Surgery
Harvard Medical School
Massachusetts General Hospital
Wang ACC 460
15 Parkman Street
Boston, MA 02115

E-mail: sparangi@partners.org