Delineation of a cellular hierarchy in lung cancer reveals an oncofetal antigen expressed on tumor-initiating cells

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Abstract

Poorly differentiated tumors in non-small cell lung cancer (NSCLC) have been associated with shorter patient survival and shorter time to recurrence following treatment. Here we integrate multiple experimental models with clinicopathological analysis of patient tumors to delineate a cellular hierarchy in NSCLC. We demonstrate that the oncofetal protein 5T4 is expressed on tumor-initiating cells and associated with worse clinical outcome in NSCLC. Coexpression of 5T4 and factors involved in the epithelial-to-mesenchymal transition was observed in undifferentiated but not in differentiated tumor cells. Despite heterogeneous expression of 5T4 in NSCLC patient-derived xenografts, treatment with an anti-5T4 antibody-drug conjugate resulted in complete and sustained tumor regression. Thus, the aggressive growth of heterogeneous solid tumors can be blocked by therapeutic agents that target a subpopulation of cells near the top of the cellular hierarchy.
Introduction

Non-small cell lung cancer (NSCLC) is the leading cause of cancer-related deaths, due to high incidence and lack of effective therapies (1). NSCLC has heterogeneous pathologies and molecular profiles, which presents challenges to molecular targeting and treatment (2-4). The degree of tumor cell differentiation is an independent prognostic factor for clinical outcome: less differentiated tumors are associated with increased risk of death in both squamous cell carcinoma and adenocarcinoma (5). Strikingly, less differentiated tumors are also associated with increased risk of recurrence after resection (5). The molecular and cellular bases for these associations are not understood.

Investigation of the cellular heterogeneity in tumors has encouraged new frameworks, such as the cancer stem cell hypothesis, for understanding the cellular hierarchy as it relates to disease progression and for designing therapeutic strategies (6-8). These experiments have demonstrated that capacities for self-renewal and differentiation vary among cells in a given tumor. The types and relative proportions of various cell subpopulations in solid tumors are likely major determinants of disease progression and therapeutic responsiveness in cancer patients. In many cases, only certain subpopulations of cancer cells from a tumor can initiate tumor growth in immunodeficient mice. Herein these cells are referred to as tumor-initiating cells (TICs); they are also known in the literature as cancer stem cells and cancer-initiating cells (6-8). TICs have been isolated, enriched or identified in tumors of brain, breast, colon, head and neck, lung, melanoma, pancreas, prostate, and the hematopoietic system, typically with antibodies against cell surface markers (9). In general the tumors grown from TICs
recapitulate the cellular heterogeneity of the original tumor, which underscores the
developmental plasticity of many TICs and the differentiation hierarchy in many tumor
tissues.

Tumor cell subpopulations and their distinct roles in disease progression can be studied using a variety of complementary approaches (8). The isolation of defined cell subpopulations from tumors, xenografts and established cell lines based on cell surface markers allows for an in depth molecular analysis of rather homogenous subpopulations. The culturing of cancer cells in defined serum-free media and/or three-dimensional matrices has been shown to maintain karyotype, transcriptome and signaling that are reflective of the tissue of origin (10, 11). Patient-derived xenografts (PDX) are established directly from freshly resected tumors and exhibit rich architectures that reflects the original patient tumors. PDXs are complex yet tractable preclinical models that can be used to study cellular heterogeneity and evaluate the efficacy of therapeutic agents.

Here we apply these emerging frameworks and technologies to delineate a cellular hierarchy in NSCLC and apply the new knowledge to drug discovery. By integrating novel experimental models of NSCLC with the clinicopathological analysis of patient tumors, we identify the 5T4 oncofetal antigen as a marker of undifferentiated, highly tumorigenic cells and of worse clinical outcome. We demonstrate preclinical efficacy of an anti-5T4 antibody-drug conjugate (ADC) and suggest that this therapeutic strategy could be applied to other surface markers expressed on tumor-initiating cells.
Materials and Methods

Isolation and serum-free culture of human lung tumor cells. Human lung tumor tissue (sample 87426A1) was obtained from Asterand in accordance with appropriate consent procedures, washed in PBS and minced. Fragments were disaggregated with Collagenase III (250 U/ml) at 37°C for 2-3 hrs. Partially digested tissue fragments were passed through steel mesh screens. Cells were sieved through a 40 μm cell strainer (BD Falcon) and treated with red blood cell lysis buffer (Roche). Dead cells were excluded by magnetic separation (Miltenyi Biotec). The culture was established and maintained in serum-free BEBM complete medium (Lonza), which was changed 50% every other day.

Air-liquid interface cultures. Millicell 1 μM PET hanging cell culture inserts (Millipore) were seeded with 2.5x10^5 cells from 87426A1 culture. After 1-2 days, the upper and lower chambers were rinsed with PBS, and CnT-23 medium (Millipore) containing 50 nM retinoic acid and 1 mM CaCl_2 was added only to the lower chamber, leaving cells exposed to the air. Lifted cultures were fed every other day with fresh medium or harvested in Buffer RLT (Qiagen) for RNA isolation or TBS/0.5% NP40 for immunoblot analysis. For gene expression profiling, “undifferentiated” values are averages of triplicate samples, and “differentiated” values, due to limited sample, were averaged from differentiation timepoints of 8, 16 and 24 days.

Animal studies. All animal studies were approved by the Pfizer Pearl River Institutional Animal Care and Use Committee according to established guidelines. To assess
tumorigenic potential of sorted cells, the cells were implanted in 50% Matrigel or where noted, 50% growth factor-reduced Matrigel (BD Biosciences) subcutaneously between the shoulder blades. 60257A1 cells were implanted in NOD-SCID mice and tumor incidence was recorded after nine weeks. For H460T and HCC2429, 100 sorted cells were implanted per nu/nu mouse. Tumors were measured at least once a week with tumor volume = 0.5 x width^2 x length. The p-values are based on two-sided t-test. Efficacy studies are described in Supplemental Information.

Clinical samples

This study was approved by the MD Anderson institutional review board. Tissue specimens were collected between 1997 and 2002 from 320 lung cancer tumors (204 adenocarcinomas and 116 squamous cell carcinomas), and selected specimens were used for construction of tissue microarrays. The primary antibodies used for immunohistochemistry were mouse monoclonal anti-CD24 (Clone SN3b, LabVision), rabbit polyclonal anti-CD44 (Sigma), and mouse monoclonal anti-5T4 (MAB4975, R&D). The statistical analysis was based on membrane expression levels as described in Supplementary Data.

Additional Materials and Methods can be found in the Supplementary Data.
Results

A cell line model reveals 5T4 expression in tumor-initiating cells

To identify a model of tumor heterogeneity in NSCLC, we screened several cell lines for heterogeneous expression of cell surface markers that had previously been used to enrich or isolate TICs (6, 9). Staining of the H460T cell line with anti-CD24 and anti-CD44 antibodies revealed distinct subpopulations of cells with a stable distribution over many passages in culture (Figure 1A and data not shown). The delineation between CD24<sup>-/lo</sup> and CD24<sup>hi</sup> was readily apparent; the threshold for CD44 was based on the isotype control. To determine whether these subpopulations had different tumorigenic capacities, we isolated cells of each subpopulation by fluorescence-activated cell sorting (FACS) and implanted them subcutaneously into immunocompromised mice. CD24<sup>-/lo</sup>CD44<sup>hi</sup> cells formed large tumors rapidly, whereas CD24<sup>hi</sup>CD44<sup>hi</sup> cells exhibited slower tumor growth (p < 0.001; Figure 1B). Tumors from CD24<sup>-/lo</sup>CD44<sup>hi</sup> cells were also larger than tumors from CD24<sup>-/lo</sup>CD44<sup>lo</sup> cells (p < 0.02; Figure S1A). In vitro, CD24<sup>-/lo</sup>CD44<sup>hi</sup> cells migrated more efficiently than CD24<sup>hi</sup>CD44<sup>hi</sup> cells and exhibited multipotency in that they gave rise to less tumorigenic CD24<sup>hi</sup>CD44<sup>hi</sup> cells (Figure S1 and data not shown). Together these results indicated that the CD24<sup>-/lo</sup>CD44<sup>hi</sup> combination of surface markers enriched for TICs in H460T. Similar results were observed for another NSCLC cell line, HCC2429 (data not shown).

To establish the clinical relevance of these results, we examined CD24 and CD44 expression in patient samples. By immunohistochemistry, CD24<sup>-/lo</sup>CD44<sup>hi</sup> membrane
expression was associated with less differentiated tumor histology (p = 0.0456). CD24−/loCD44hi membrane expression was also associated with worse overall survival in male squamous cell carcinoma (p = 0.0310; Figure S2A). In addition, analysis of published mRNA data (12) revealed that CD24−/loCD44hi was associated with worse overall survival in adenocarcinomas (p = 0.0501; Figure S2B). Taken together, these results demonstrated that the TIC markers identified in H460T and HCC2429 were also poor prognosis markers in NSCLC, which indicates the clinical relevance of the cell line models.

From a therapeutic perspective, it is not feasible to target the CD24−/loCD44hi combination of markers. Therefore we sought to identify novel TIC markers in NSCLC that could be potential therapeutic targets. We generated gene expression profiles from triplicate samples of sorted CD24−/loCD44hi and CD24hiCD44hi cells from H460T (Figure S1E). The 5T4 (TPBG) gene had significantly higher expression in the more tumorigenic CD24−/loCD44hi cells compared to the CD24hiCD44hi cells (Figure 1C and Table S1). Consistent with this result, in NSCLC clinical samples, 5T4 mRNA expression was significantly higher in CD24−/loCD44hi versus “Not CD24−/loCD44hi” tumors (12) (p = 0.0293; Figure S2C).

5T4 is a single-pass transmembrane protein that is preferentially expressed in many tumor types compared to normal tissues (13) and has been explored as a cancer target. During embryonic development, 5T4 functions in EMT and cell migration, at least in part by interacting with CXCR4 and/or the actin cytoskeleton (14-17). Given the oncofetal characteristics of 5T4 and its increased expression in the tumorigenic CD24−/loCD44hi subpopulation of H460T, we focused on 5T4 as a potential TIC marker in NSCLC.
Immunofluorescence studies in H460T revealed that staining for 5T4 and CD24 were mutually exclusive, consistent with the gene expression data, and moreover that most if not all CD24^lo cells expressed 5T4 (Figure 1D). This result was extended by flow cytometry (Figure S3) and immunoblot analysis (Figure 1E).

Based on the expression of 5T4 in normal progenitor cells during embryonic development (18), we hypothesized that 5T4 expression is associated with a less differentiated state and that inducing differentiation of tumor cells would reduce 5T4 expression. Sorted cells were treated with retinoic acid and subjected to immunoblot analysis. Indeed, 5T4 expression was dramatically reduced in treated CD24^loCD44^hi cells (Figure 1E).

To test tumorigenicity based on 5T4 expression, we sorted cells from a clonal line of H460T and implanted them into mice. As predicted, 5T4^hi cells were more tumorigenic than 5T4^lo cells (p < 0.03; Figure 1F), which indicated that 5T4 can independently enrich for TICs in this system. Based on all of these results, we sought to further establish 5T4 as a novel TIC antigen by using PDXs.

**5T4 enriches for tumorigenicity in a patient-derived xenograft**

We established NSCLC PDX lines by implanting fragments of freshly resected tumors (squamous cell carcinoma) subcutaneously in immunocompromised mice. Each line was propagated by explanting xenograft fragments into naive animals and was maintained exclusively in vivo. In each PDX line, the histology of the xenograft resembled that of the original tumor (data not shown).
To determine whether 5T4 expression enriched for tumorigenic capacity in PDXs, 5T4\textsuperscript{hi} and 5T4\textsuperscript{-lo} cells were sorted from dissociated 60257A1 PDXs and implanted into animals. Cells from this PDX line exhibited a wide continuous range of 5T4 expression levels, and the highest and lowest 15\% were assigned 5T4\textsuperscript{hi} and 5T4\textsuperscript{-lo} (Figure 2A). 5T4\textsuperscript{hi} cells yielded substantially higher tumor incidence than 5T4\textsuperscript{-lo} cells in three independent experiments (Table 1). Based on limiting dilution analysis, 5T4\textsuperscript{hi} cells were \textasciitilde 30-fold more tumorigenic than 5T4\textsuperscript{-lo} cells when implanted in 50\% growth factor-reduced Matrigel (Table 1). To determine if tumorigenicity was dependent on growth factors, as reported previously (19), the experiment was repeated two additional times in regular Matrigel. The tumorigenicity of 5T4\textsuperscript{-lo} cells was slightly enhanced in these conditions but was still significantly lower than that of 5T4\textsuperscript{hi} cells (Table 1). Tumors that grew from sorted 5T4\textsuperscript{hi} cells showed heterogeneous 5T4 expression (Figure 2B). Thus the results in the PDX are consistent with those from H460T and demonstrate that 5T4\textsuperscript{hi} expression can enrich for TICs in NSCLC.

To uncover molecular differences between 5T4\textsuperscript{hi} and 5T4\textsuperscript{-lo} cells, we generated gene expression profiles from cells sorted on three independent occasions from 60257A1. Strikingly, the Clara cell secretory protein (CCSP, also known as uteroglobin, SCGB1A) was expressed 20\–fold higher in 5T4\textsuperscript{hi} cells compared to 5T4\textsuperscript{-lo} cells (Figure 2C). CCSP has been characterized as a marker of lung stem cells in normal tissue and adenocarcinoma (20). In addition, several genes associated with tumorigenesis, invasion and stem cells were expressed at higher levels in 5T4\textsuperscript{hi} cells, including carcinoembryonal antigens CEACAM6 and CEACAM1, cathepsin S, gelsolin, and IL8 (Figure 2C). In contrast, low levels of CD133 were comparable in both populations (Figure S4A). In
summary, the gene expression profiles distinguished the 5T4<sup>hi</sup> and 5T4<sup>-</sup><sub>lo</sub> populations and offered potential explanations for their different tumor-initiating capacities.

**5T4 and markers of EMT are expressed in cells near the top of the tumor hierarchy**

Immunohistochemical analysis of our PDXs revealed a range of 5T4 expression levels and patterns. Three of six PDX lines exhibited heterogeneous expression of 5T4, and in two of the three, 5T4 expression was coupled to tumor architecture in that expression was higher at the tumor-stroma interface (Figure 3A, 3B, and Figure S4B). Notably, in 37622A1 a pattern similar to 5T4 was observed for vimentin, a marker of EMT, and Ki67, a proliferation marker (Figure 3A). Coimmunostaining confirmed that 5T4<sup>hi</sup> cells were typically positive for Ki67 in this PDX line (Figure 3B).

When a serum-free culture was established from the 37622A1 PDX (Figure 3C), the cells exhibited uniformly high 5T4 expression, whereas the PDX exhibited a broader expression range (Figure 3D). Since the culture media only supports growth of undifferentiated cells, this result was consistent with the hypothesis that 5T4 is highly expressed on TICs. Strikingly, when the cultured cells were implanted into animals, the resulting tumors exhibited heterogeneous 5T4 expression (Figures 3E and S4C). This result suggested that 5T4<sup>hi</sup> cells gave rise to 5T4<sup>-</sup><sub>lo</sub> cells during tumor growth by a mechanism that potentially requires tumor-stroma interaction or other microenvironmental cues.

To further investigate the cellular hierarchy in NSCLC, we developed a novel three-dimensional (3D) model of lung cancer differentiation. In a serum-free culture
established directly from freshly resected tumor (sample 87426A1), cells could either be maintained in the undifferentiated state submerged in stem cell media or be induced to differentiate at the air-liquid interface with retinoic acid, but notably without the use of serum (Figure 4A). The air-liquid interface is considered a physiological environment for lung cells and used to study differentiation of immortalized bronchial epithelial cells (21). The 87426A1 culture efficiently formed a 3D multi-layered epithelium upon exposure to the air-liquid interface for 18 days (Figure 4B).

Immunoblot analysis demonstrated that 5T4 levels were high in the undifferentiated state and decreased quickly and dramatically upon differentiation (Figure 4C). This result was analogous to retinoic acid treatment of sorted H460T cells (Figure 1E).

To obtain a global view of the cellular hierarchy in NSCLC, gene expression profiles were generated from undifferentiated and differentiated cells. Consistent with the above results, 5T4 expression decreased and CD24 expression increased during differentiation (Figure 4D, 4E). These data were confirmed by flow cytometry (Figure S5). The profiles revealed striking patterns of genes involved in EMT and angiogenesis. The EMT markers vimentin, fibronectin, Slug, and Twist were expressed at significantly higher levels in undifferentiated cells (Figure 4D); in contrast, the epithelial differentiation markers mucin and involucrin as well as transglutaminase and cytokeratins were expressed at significantly higher levels in differentiated cells (Figure 4E). The coexpression of 5T4 and vimentin in undifferentiated cells was consistent with the similar expression patterns of 5T4 and vimentin in 37622A1 PDX (Figure 3A). Interestingly, angiogenesis factors including VEGF-A, VEGF-C, PDGF-A, and PIGF and uPA, were
expressed at significantly higher levels in undifferentiated cells (Figure 4F). These results are noteworthy given the higher 5T4 expression at the tumor-stroma interface in two of our PDX lines.

An unbiased meta-analysis revealed several gene signatures with significant expression changes during differentiation (Supplemental Table S2). Gene sets with higher expression in undifferentiated cells included signatures of poor clinical prognosis, stem cells, oncogenic signaling, and developmental signaling. Gene sets with higher expression in differentiated cells included signatures of better clinical prognosis, differentiated tumors, and differentiated cells. Taken together, these results demonstrate a cellular hierarchy in a primary NSCLC culture and support the concept that less differentiated tumor cells can have a more malignant phenotype.

**Correlation between 5T4 expression and clinicopathologic features and disease outcomes**

We sought to determine whether 5T4 expression in NSCLC tumors correlated with clinicopathologic features and disease outcomes. In a panel of surgically resected tumors, 5T4 expression levels and patterns varied, and each sample was assigned an overall score. The distribution of 5T4 expression according to clinicopathological characteristics is shown in Table S3. Notably, membranous 5T4 expression was higher in poorly and moderately versus well differentiated tumors (p = 0.0005 and p = 0.0197 respectively, with overall ANOVA yielding p = 0.0022; see Table S5). In addition, high membranous 5T4 expression was associated with shorter survival time (p = 0.0283, Figure 5A; and p =
0.078 in multivariate analysis, Table S5) and shorter time to recurrence (p = 0.0495, Figure 5B; and p = 0.034 in multivariate analysis, Table S4). When the cohort was stratified by 5T4hi and 5T4-lo, there was no difference in the impact of patient treatment on clinical outcome (not shown). These observations are consistent with our experimental results that indicate that 5T4 is associated with less differentiated, more tumorigenic cells in NSCLC.

**An antibody-drug conjugate is specifically cytotoxic to 5T4-expressing cells**

Taken together, these results provided the rationale for targeting 5T4-expressing cells with an antibody-drug conjugate (ADC) even in tumors with heterogeneous 5T4 expression. Unconjugated anti-5T4 monoclonal antibody has not exhibited any in vitro activity or in vivo efficacy (22). To generate the ADC, anti-5T4 antibody (13), previously shown to internalize into cells (22), was conjugated to the enediyne natural product calicheamicin, a DNA damaging agent (23) (Figure 6A). To demonstrate specificity for 5T4-expressing cells, in vitro experiments were performed on sorted H460T cells. In a proliferation assay, the 5T4-enriched population was >10-fold more sensitive to the ADC than the 5T4-low population (Figure 6B). In a colony formation assay in which cells were treated with drug and then seeded at low density, the 5T4-enriched population was similarly >10-fold more sensitive to the ADC than the 5T4-low population (Figure 6C). In contrast, no difference in sensitivities was observed when the two populations were treated with calicheamicin alone, anti-CD22 calicheamicin ADC, camptothecin, 5-
fluoruracil, or ionizing radiation (data not shown). These results demonstrated the specificity of the anti-5T4 ADC.

Regression of tumors with heterogeneous 5T4 expression with an ADC

We hypothesized that a therapeutic agent that targets and eliminates 5T4
expression with an ADC

We hypothesized that a therapeutic agent that targets and eliminates 5T4hi-expressing TICs could ultimately cause regression of tumors despite heterogeneous expression of 5T4 within the tumor. The stable chemical linker between antibody and drug restricts the release of calicheamicin to cells that internalize the ADC (24). We assessed the efficacy of an anti-5T4 ADC on the growth of two PDX lines with heterogeneous 5T4 expression. In both lines 5T4 expression was predominant at the tumor-stroma interface, but the overall 5T4 expression level was higher in 37622A1 than 60274A1 (Figure S4B, S4D). 37622A1 tumors harbored a K-ras G13V mutation while 60274A1 tumors had wildtype K-ras (Figure 6D, 6E).

PDX tumors were treated with anti-5T4 ADC, anti-CD33 ADC or vehicle; the anti-CD33 ADC served as a negative control because these PDX lines do not express CD33 (data not shown). In 37622A1, treatment with anti-5T4 ADC regressed the tumors, and no regrowth was observed even three months after the last dose; in contrast, treatment with anti-CD33 ADC or vehicle did not inhibit tumor growth (Figure 6D). Similarly, in 60274A1 treatment with anti-5T4 ADC regressed the tumors despite the lower 5T4 expression in this line (Figure 6E). Treatment with calicheamicin (not conjugated to an antibody) has not shown any significant impact on tumor growth (22, 24, 25).
In contrast to the efficacy observed with anti-5T4 ADC, treatment of both PDXs with cisplatin at the maximum tolerable dose regressed tumors only transiently, and the tumors regrew after treatment was completed (Figures 6E and S6). These results highlight the superior long-term efficacy of an ADC that targets TICs as compared to a conventional chemotherapeutic.

Discussion

5T4 and the cellular hierarchy in NSCLC

Using an integrated strategy, we have delineated a cellular hierarchy in NSCLC and identified 5T4 as a clinically relevant marker that can enrich for TICs. We found that 5T4 expression was associated with higher tumorigenic capacity and the undifferentiated state; these results parallel the expression of 5T4 in cycling progenitor cells during embryonic development (18). The association of 5T4 expression with EMT in tumors, observed in the 87426A1 serum-free culture and the 37622A1 PDX, mirrors the association of 5T4 with EMT in normal development (15). Our results are consistent with a recent study that described the relationship between EMT and stem cell phenotypes in normal and cancerous tissues (26).

Expression of 5T4 has been associated with advanced disease and/or poor prognosis in colorectal, ovarian, and gastric cancers (27-29). We found that 5T4 is associated with less differentiated tumor histology, shorter time to recurrence, and worse overall survival in NSCLC. Interestingly, while CD133 was used to isolate NSCLC TICs
(30, 31) and was expressed in drug-tolerant PC9 cells (32), CD133 expression in NSCLC was not associated with patient survival (33). Based on the published observation that CD133⁺ cells are quiescent (30) and our observation in a different model that 5T4⁺ cells are proliferating, we speculate that CD133⁺ and 5T4⁺ might define different TIC populations even in the same NSCLC tumor -- analogous to the emerging evidence for distinct stem cell populations, e.g. quiescent vs. active/proliferating, in normal tissues (34).

The clinical relevance of the 5T4 marker suggests that 5T4⁺ TICs contribute directly to one or more aspects of disease progression in NSCLC. For instance, 5T4⁺ TICs may contribute to metastasis since that process might originate when cells in a primary epithelial tumor undergo EMT (35).

The morphological heterogeneity observed in NSCLC and other epithelial tumors is likely paralleled by a continuum of tumor-initiating capabilities. In other words, the term “TIC” refers to a quantitative trait that is influenced not only by cancer cell-autonomous signaling but also by cellular context, i.e. neighboring malignant cells and the tumor microenvironment. For instance, the tumorigenicity of defined cell populations can depend on parameters such as mouse strain and the use of Matrigel (19, 36). We found a similar effect in that the 5T4⁻/⁻ cells yielded higher tumor incidence when implanted with regular Matrigel as opposed to growth factor-reduced Matrigel. Furthermore we observed two examples of context-dependent TIC behavior: the uniformly 5T4⁺ cells in the PDX-derived culture gave rise to tumors with heterogenous 5T4 expression (Figure 3), and the uniformly 5T4⁺ cells in the tumor-derived culture gave rise to cells with dramatically reduced 5T4 expression under differentiation.
conditions in vitro (Figure 4). Xenografts and serum-free cultures can be complementary models to study tumor cell plasticity, hierarchy and stromal interaction.

**Therapeutic targeting of a tumor-initiating cell antigen**

5T4 is an oncofetal protein as defined by expression during embryonic development and in cancer, but little expression in normal adult tissues. Recent work demonstrated the importance of another oncofetal protein, L1CAM, in glioblastoma tumor-initiating cells (37). Unconjugated antibodies directed at TIC surface markers might affect TIC survival, differentiation, and/or interaction with stroma, but many of them are expected to have limited single agent activity in the clinic. Our data provides preclinical proof of concept for targeting TICs with ADCs. Strikingly, efficacy was observed in *KRAS* wildtype and *KRAS* mutant tumors, consistent with the ADC mechanism of action. Future studies could determine whether the anti-5T4 ADC strategy in NSCLC might transcend *KRAS* status, in contrast to anti-EGFR therapies (38).

The efficacy of a TIC-directed therapy will likely depend on the proportion and properties of the targeted TICs. Even if quiescent or slow-growing TICs are destroyed or differentiated, tumors with proliferating progenitor-like cells might not regress in the short term (8); on the other hand, immediate therapeutic impact might be achieved if we eliminate proliferating TICs that contribute to both pathological damage and disease progression. The dramatic, antigen-specific activity of the anti-5T4 ADC might be explained by the proliferative state of 5T4hi TICs, but further studies are needed to fully understand the mechanism, which might also involve a bystander effect (39) and/or drug-
induced 5T4 expression (e.g. as observed in 60274A1 PDX following cisplatin treatment; our unpublished data). Due to the continuous nature of 5T4 expression that we observed in some models, we cannot exclude the possibility that the anti-5T4 ADC might target both TICs and non-TICs in some tumors, which would be therapeutically advantageous.

In summary, our study delineates a cellular hierarchy in NSCLC, characterizes the undifferentiated highly tumorigenic cell subpopulation, and demonstrates the application of this knowledge to the development of anti-cancer therapeutics. Characterization of 5T4 as a TIC marker in NSCLC suggests an explanation for the long-term efficacy of the anti-5T4 ADC despite heterogeneous 5T4 expression in the tumors. Future studies could uncover additional molecules that, even if expressed in only a subpopulation of cells in the tumor, constitute appealing therapeutic targets against aggressive heterogeneous diseases.

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References


Table 1. Tumor incidence in animals implanted with 5T4\textsuperscript{hi} or 5T4\textsuperscript{lo} cells sorted from 60257A1 patient-derived xenografts in three independent experiments.

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\textsuperscript{1} n.d., not determined.
Figure Legends

Figure 1. Oncofetal antigen 5T4 is identified in a cell line model of tumor-initiating cells.
A, Distinct populations of H460T were revealed by flow cytometry with anti-CD24 and anti-CD44 antibodies.
B, CD24^{−/lo}CD44^{hi} and CD24^{hi}CD44^{hi} cells were sorted and implanted subcutaneously into mice. Values indicate mean ± SEM.
C, 5T4 mRNA signal intensities on gene microarrays probed with samples from sorted CD24^{−/lo}CD44^{hi} and CD24^{hi}CD44^{hi} cells. Values indicate mean ± standard deviation.
D, H460T cells were fixed, stained with anti-CD24-Qdot585 and anti-5T4-Qdot705, and imaged with a 40x objective.
E, Immunoblot analysis on sorted cells that were treated with retinoic acid or vehicle.
F, Tumor growth of 5T4^{hi} and 5T4^{−/lo} sorted cells. H460T clonal line CD24N-26 was used as described in the text. A representative experiment is shown. Values indicate mean ± SEM.

Figure 2. 5T4 can enrich for tumor-initiating cells in NSCLC.
A, 5T4 expression in the 60257A1 PDX.
B, Immunohistochemistry of 5T4 in tumors that grew from 5T4^{hi} cells sorted from 60275A1.
C, mRNA signal intensities on gene microarrays probed with samples from 5T4^{hi} or 5T4^{−/lo} cells sorted from PDXs on three independent occasions. Values indicate the mean ±
Figure 3. Heterogeneous expression of 5T4 in NSCLC patient-derived xenografts.
A, Immunohistochemistry on serial sections of 37622A1 PDX, stained for 5T4, Ki67, and vimentin. Scale bar, 100 μM.
B, Confocal images of coimmunostaining of 5T4 and Ki67 in 37622A1 PDX. Scale bar, 100 μM.
C, Schematic of the establishment of an adherent serum-free culture from 37622A1 PDX.
D, Analysis of live Human Epithelial Antigen-positive cells in the PDX (red) and the PDX-derived culture (blue) compared to isotype control (gray).
E, Immunohistochemistry of 5T4 in a tumor xenograft that grew from the serum-free culture.

Figure 4. 5T4 and markers of EMT are associated with the undifferentiated state.
A, Schematic of serum-free culture under conditions that maintain the undifferentiated state or induce differentiation at the air-liquid interface.
B, Micrographs of the 87426A1 culture in the undifferentiated state (left) and after differentiation for 18 days (right). Scale bars, 100 μm.
C, 5T4 immunoblot analysis during differentiation.
D-F, mRNA signal intensities on gene microarrays probed with samples of undifferentiated and differentiated cells. Values represent mean ± standard deviation.
Figure 5. 5T4 expression in NSCLC is associated with worse clinical outcome.

A, 5T4 expression in NSCLC is associated with worse overall survival in patients.
B, 5T4 expression in NSCLC is associated with shorter time to recurrence in patients.

Figure 6. Long-term regression of patient-derived xenografts upon treatment with an anti-5T4 antibody-drug conjugate.

A, Schematic of an anti-5T4 calicheamicin conjugate.

B, The 5T4-enriched population from H460T is sensitive to anti-5T4-calicheamicin conjugate in a cell growth assay. Crosshairs indicate IC50 values of free calicheamicin.

C, The 5T4-enriched population from H460T is sensitive to anti-5T4-calicheamicin conjugate in a clonogenic assay. Crosshairs indicate IC50 values of free calicheamicin.

D-E, Long-term regression of PDXs by treatment with anti-5T4 ADC but not cisplatin. Animals were dosed on days 1, 5 and 9 after staging. Values indicate mean ± SEM. Asterisks indicate timepoints with statistically significant differences in tumor size. See also Figure S6.
Fig. 2

A

Control

5T4

5T4<sup>hi</sup> 5T4<sup>lo</sup> 15.1 14.7

B

C

CCSP/SCGB1A1  CEACAM6  CEACAM1

mRNA

5T4<sup>hi</sup> 5T4<sup>lo</sup>

5T4<sup>hi</sup> 5T4<sup>lo</sup>

5T4<sup>hi</sup> 5T4<sup>lo</sup>

CTSS  IL8  GSN

mRNA

5T4<sup>hi</sup> 5T4<sup>lo</sup>

5T4<sup>hi</sup> 5T4<sup>lo</sup>

5T4<sup>hi</sup> 5T4<sup>lo</sup>
Fig. 4

A

Undifferentiated

Differentiated

B


C

Days

0

1

2

4

10

21

5T4

Ponceau S

D

5T4 (TPBG)

SLUG

TWIST1

FIBRONECTIN

VIMENTIN

mRNA

Undiff

Diff

Undiff

Diff

Undiff

Diff

Undiff

Diff

Undiff

Diff

E

INVOLUCRIN

TGM1

MUCIN1

KERATIN4

CD24

mRNA

Undiff

Diff

Undiff

Diff

Undiff

Diff

Undiff

Diff

Undiff

Diff

F

VEGF-A

VEGF-C

PDGFA

PIGF

uPA

mRNA

Undiff

Diff

Undiff

Diff

Undiff

Diff

Undiff

Diff

Undiff

Diff
Fig. 5

A

Surviving Proportion

5T4<sup>lo</sup>

5T4<sup>hi</sup>

p = 0.0283

Years

B

Recurrence-free Proportion

5T4<sup>lo</sup>

5T4<sup>hi</sup>

p = 0.0495

Years
Delineation of a cellular hierarchy in lung cancer reveals an oncofetal antigen expressed on tumor-initiating cells

Marc Damelin, Kenneth G Geles, Maximillian T Follettie, et al.

Cancer Res  Published OnlineFirst May 3, 2011.

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