Small, noncoding RNAs are short untranslated RNA molecules, some of which have been associated with cancer development. Recently, we showed that a class of small RNAs generated during the maturation process of tRNAs (tRNA-derived small RNAs, hereafter “tsRNAs”) is dysregulated in cancer. Specifically, we uncovered tsRNA signatures in chronic lymphocytic leukemia and lung cancer and demonstrated that the ts-4521/3676 cluster (now called “ts-101” and “ts-53,” respectively), ts-46, and ts-47 are down-regulated in these malignancies. Furthermore, we showed that tsRNAs are similar to Piwi-interacting RNAs (piRNAs) and demonstrated that ts-101 and ts-53 can associate with PiwiL2, a protein involved in the silencing of transposons. In this study, we extended our investigation on tsRNA signatures to samples collected from patients with colon, breast, or ovarian cancer and cell lines harboring specific oncogenic mutations and representing different stages of cancer progression. We detected tsRNA signatures in all patient samples and determined that tsRNA expression is altered upon oncogene activation and during cancer staging. In addition, we generated a knockdown cell model for ts-101 and ts-46 in HEK-293 cells and found significant differences in gene-expression patterns, with activation of genes involved in cell survival and down-regulation of genes involved in apoptosis and chromatin structure. Finally, we overexpressed ts-46 and ts-47 in two lung cancer cell lines and performed a clonogenic assay to examine their role in cell proliferation. We observed a strong inhibition of colony formation in cells overexpressing these tsRNAs compared with untreated cells, confirming that tsRNAs affect cell growth and survival.

Significance

We found that tRNA-derived small RNAs (tsRNAs) are dysregulated in many cancers and that their expression is modulated during cancer development and staging. Indeed, activation of oncogenes and inactivation of tumor suppressors lead to a dysregulation of specific tsRNAs, and tsRNA-KO cells display a specific change in gene-expression profile. Thus tsRNAs could be key effectors in cancer-related pathways. These results indicate active crosstalk between tsRNAs and oncogenes and suggest that tsRNAs could be useful markers for diagnosis or targets for therapy. Additionally, ts-46 and ts-47 affect cell growth in lung cancer cell lines, further confirming the involvement of tsRNAs in cancer pathogenesis.

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**tsRNA signatures in cancer**

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mutations located mainly in the genomic region of ts-101 in lung cancer samples (4). By using a custom tsRNA microarray chip, we determined the presence of tsRNA signatures in CLL and lung cancer. Ts-46 and ts-47 were the most down-regulated tsRNAs in these malignancies (4, 16). Here we describe the results of our recent experiments aimed at clarifying the role of tsRNAs in cancer and finding tsRNA signatures in different malignancies.

Results

tsRNA Signatures in Cancers. We studied tsRNA regulation/signatures in cancer by hybridizing total RNA samples from patients to our custom tsRNA microarray chip (4). Previously, we found a signature of 17 tsRNAs differentially expressed in CLL and a signature of six tsRNAs in lung cancer. Specifically, we identified ts-46 and ts-47 as tsRNAs that were strongly down-regulated in both malignancies (4). Thus, we examined the expression profile of tsRNAs in other cancer samples. We profiled 14 paired samples from seven patients with colon adenoma and 16 paired samples from eight patients with colon adenocarcinoma cancer. We found a signature of eight tsRNAs characterizing adenomas and a signature of seven tsRNAs for adenocarcinomas (Fig. 1 A and B and Fig. S1 A and B). Ts-53 and ts-101 were down-regulated in adenomas but not in adenocarcinomas, suggesting that they may have a role in the initial phases of transformation. Ts-40 was up-regulated in both comparisons; thus this tsRNA could be an oncogenic tsRNA in colon cancer development. Ts-36, which was previously correlated with cell proliferation (9), shows a twofold increase in its expression level in carcinomas but not in adenomas, indicating that this tsRNA could have a role in the final stages of the malignant transformation of colon cells. Then we studied samples from breast cancer and ovarian cancer patients. In breast cancer, we found only two tsRNAs significantly dysregulated: ts-66 and ts-86. Ts-66 was up-regulated in cancer, whereas ts-86 was down-regulated (Fig. 1C and Fig. S1C). In ovarian cancer we found a signature of 10 tsRNAs; among these, ts-29 was overexpressed more than twofold in cancer samples as compared with normal controls (Fig. 1D and Fig. S1D). To identify common sets of tsRNAs potentially playing an oncogenic or tumor-suppressor role in multiple malignancies, we examined the signatures from all the profiled types of cancer, including our previous results from CLL and lung cancer (4, 16). Our analysis revealed that five tsRNAs are down-regulated in both CLL and lung cancer (ts-46, ts-47, ts-49, ts-53, and ts-101), whereas ts-4 is up-regulated in these two malignancies. Ts-53 and ts-101 are also down-regulated in ovarian cancer. Thus, these two tsRNAs are nonresponsive to androgen and ovarian cancer. Importantly, a signature of 31 tsRNAs can discriminate among different cancers and tissue types (Fig. 2).

tsRNAs Expression Is Modulated by Oncogenes. By using our custom tsRNA microarray chip, we investigated tsRNA expression patterns in human lymphocytes with and without activation of the MYC oncogene and found a signature of 15 tsRNAs; among these ts-47 was the most down-regulated by MYC activation (Table S1). Given these results, we examined the effect of other oncogenes on tsRNA expression. Because the tsRNA signature in breast cancer showed only two dysregulated tsRNAs, we hypothesized that cell lines derived from breast tissue would provide a good model to study the effect of specific oncogenes on tsRNA expression. Thus we used cell lines derived from MCF10A (normal breast epithelial cells) carrying activating mutations of the HRAS, KRAS, and PIK3CA genes and two cell lines from different stages of breast cancer: MCF7 cells, representing an early-stage, noninvasive, luminal type of breast cancer and displaying hormone sensitivity through the expression of estrogen receptor (ER), and MDA-MB-231 cells, representing a later stage of breast cancer from a metastatic, invasive, triple-negative basal breast cancer not responsive to hormonal therapy and poorly responsive to chemotherapy (Table S2). When performing an unsupervised analysis to compare all these cell lines together, we detected a signature of 50 tsRNAs able to cluster into eight groups following a hierarchical order: PIK3CA-mutated cells and HRAS-transformed cells clustered together in the first subtree; normal cells, early-stage breast cancer, and late-stage breast cancer cells clustered in a second subtree; and KRAS-mutated cells and cell lines harboring both KRAS and PIK3CA mutations clustered in a third subtree (Fig. 3D). This comparison shows that the activation of KRAS significantly affects tsRNA expression profiles in breast cells. In particular, nine tsRNAs (ts-2, ts-14, ts-38, ts-90, ts-32, ts-8, ts-21, ts-66, and ts-62) show a remarkable down-regulation in all cell lines with KRAS mutations (KRAS-KI, DKI2, and DKI5), whereas ts-55, ts-42, ts-29, and ts-30 are down-regulated in cell lines with mutations on both KRAS and PIK3CA (DKI2 and DKI5). Surprisingly, ts-46 is up-regulated in all cell lines with a PIK3CA mutation (MCF10A-H1047R, DKI2, and DKI5), and ts-47 is up-regulated in all cell lines with a KRAS mutation (KRAS-KI, DKI2, and DKI5), HRAS and PIK3CA mutated cells (MCF10A-H1047R and MCF10-AT1) are clustered together and show a profile very different from that of normal, KRAS, and KRAS’ PIK3CA cells. For example ts-24, ts-11, and ts-10 are strongly down-regulated only in HRAS and in PIK3CA mutated cells. We then performed two additional unsupervised analyses by separating the cell lines according to their origin (Fig. S2): one comparison among all MCF10A-derived cell lines and another between the two breast cancer cell lines and normal breast cells. We confirmed that the signatures and the clusters obtained in the first comparison are also observed when we grouped all the cell lines together and the two additional unsupervised analyses by separating the cell lines from two HEK293_KO-ts46 clones, two HEK293_KO-ts101 clones, and two HEK293_KO-ts46+47 clones we found a signature of 17 tsRNAs differentially expressed in breast cancer not responsive to hormonal therapy and poorly responsive to chemotherapy (Table S3).

Gene and mirRNA Expression Are Dysregulated in tsRNA-KO Cell Models. To understand better whether tsRNAs have a role in regulating gene expression, we generated ts-101 and ts-46 KO stable cell lines from HEK293 cells by using CRISPR technology. Affymetrix gene-expression profiling was performed using RNA from two HEK293 KO-ts46 clones, two HEK293 KO-ts101 clones, and two WT controls transfected with the empty vector. We found 270 genes differentially expressed in the ts-46 clones (211 coding genes plus 59 noncoding genes) and 216 genes differentially expressed in the ts-101 clones (170 coding genes plus 46 noncoding genes) compared with the WT controls (Fig. S3 and Table S3). Among up-regulated genes in the ts-46 and ts-101 KO clones we found miR-222, IRS4, MAP3K9, PLK4,
Among down-regulated genes we found TAF9B, METTL23, FILIP1, OCLN, BMP2, TAX1BP1, RASGRF2, MIR613, OGN, SMC1A, and all the components of the H3A1 histone subfamily (HIST1H3A, HIST1H3B, HIST1H3C, HIST1H3D, HIST1H3E, HIST1H3F, HIST1H3G, HIST1H3H, HIST1H3I, and HIST1H3J). As expected mir-4521 (corresponding to ts-101) is strongly down-regulated in ts-101 clones. The down-regulation of all members of the H3A1 histone subfamily encoding for one of the components of the nucleosome core suggests that tsRNAs can interfere with chromatin configuration and thus with the epigenetic control of gene expression. Additionally, we performed a functional enrichment study by using the Ingenuity Pathway Analysis (IPA) software to evaluate whether cancer-related pathways are altered in ts-46 and ts-101 KO cells. Ts-46 and ts-101 KO cells showed a significant activation of networks associated with cell proliferation and inhibition of networks associated with apoptosis. Indeed, in ts-46 KO cells several pathways related to cell transformation and cancer development are over-activated, including ILK signaling (18), integrin signaling.
Evidence of the Tumor Suppression Function of ts-46 and ts-47: An Inhibiting Effect on Colony Formation in Lung Cancer Cell Lines. We previously carried out a clonogenic formation experiment to determine if ts-53 can function as tumor suppressor in lung cancer cell lines A549 and H1299. We indeed observed that exogenous expression of ts-53 resulted in a decrease in colony formation, indicating that ts-53 can function as a tumor suppressor. Considering that ts-46 and ts-47 are also down-regulated in both CLL and lung cancer, we studied their effect on the colony-formation ability of lung cancer cell lines. We overexpressed ts-46 and ts-47 in the lung cancer cell lines H1299 and A549 and performed a colony assay by seeding the same number of transfected cells for each condition and observed a significant decrease in the colony-formation ability of the cells overexpressing ts-46 or ts-47 (Fig. 4).

Discussion
While studying the miR-4521/3676 cluster in CLL, we found that these two miRNAs are tsRNAs, which we now call ts-101 and ts-53, respectively (16). We showed that ts-53 and ts-101 are deleted in 17p- CLL and that ts-53 targets TCL1 (16) in an miRNA fashion. Later, we found that ts-46 and ts-47 are down-regulated in CLL and lung cancer and showed that tsRNAs can act like piRNAs by interacting with Piwi proteins. Thus, tsRNAs can interfere with the epigenetic regulation of genes (4). The data presented here combined with these previous reports indicate that tsRNAs play a key role in the onset and progression of several types of cancers. Indeed, by analyzing the data from CLL, lung, colon, breast, and ovarian cancer samples, we found a cancer-specific signature of 31 tsRNAs able to discriminate these cancers (Fig. 2). We previously described CLL and lung cancer signatures (4, 16). In this study we report that tsRNA signatures also can be identified in adenomas and carcinomas of the colon, breast invasive ductal carcinoma, and ovarian cancers (Fig. 1). Indeed, we found that tsRNA expression differs in normal and tumor tissues, although these differences could reflect discrepancies related to the transformed phenotypes or cell-type compositions among the samples. To minimize this possibility, we used paired samples from colon and breast cancer patients, with the samples of normal tissue harvested from the normal tissue surrounding the cancer samples. However, for the comparisons of ovary samples, it was not possible to obtain a specimen of
normal tissue from the same patient. Thus, ovary normal tissue representing the different origins of all cancer sample histotypes were collected from cervical cancer patients undergoing radical hysterectomy. The analysis of colon samples shows eight tsRNAs dysregulated in adenomas and seven in carcinomas. Only two tsRNAs, ts-66 and ts-86, were found to be dysregulated in breast cancer. Interestingly, when the tsRNA expression profile of breast cell lines was compared with that of prostate cell lines, ts-66 was always dysregulated: It was down-regulated in breast cancer cell lines but was up-regulated in the prostate AR− late-stage cell line compared with AR+ early-stage and normal cells. Because tRNA halves and piRNAs were recently shown to be involved in hormone response in breast and prostate cancer (32, 33), it is possible that tsRNAs also may be implicated in the response to sex hormones in hormone-related cancers. Last, we found a signature of 10 tsRNAs dysregulated in ovarian cancers.

To verify that tsRNA expression can be dysregulated by activation of specific oncopgenes, we studied the effect of MYC activation in lymphocytes and found that ts-47, which is lost in CLL and lung cancer, is strongly down-regulated by MYC activation, indicating that MYC may be involved in cancer by turning off an epigenetic silencer belonging to the tsRNA gene family (Table S1). We then studied the effects of other oncopgenes on tsRNAs by using well-established breast-derived cell lines carrying specific mutations that activate key oncopgenes such as HRAS, KRAS, and PIK3CA and two cancer cell lines. The results of these experiments suggest that tsRNAs could be key effectors in pathways regulated by these oncopgenes and that these molecules could have important roles in the cell transformation process and in cancer development/progression. Additionally, the oncopgene-driven dysregulation of tsRNA could be involved in feedback loops, as previously shown for miRNAs (34). Indeed, when profiling the gene-expression patterns of ts-46 and ts-101 KO cells, we found that pathways such as PTEN and ceramide signaling are inhibited. PTEN is a major negative regulator of the PI3-kinase pathway, and recent studies indicate that ceramide signaling induces apoptosis by reducing the activity of p42/44-MAPK and Akt (35, 36). Pathways involved in cell growth and cancer development are up-regulated in ts-101 and ts-46 KO cells (Fig. S4). In both KO cell types the S1P and glutamate receptor signaling pathways are significantly overactivated, and production of S1P promotes tumor growth, resistance to apoptosis, and metastasis. Interestingly, ceramide and S1P counter-regulate the phosphorylation of Bax and Bad to control apoptosis and cell survival (37); thus a simultaneous activation of S1P and inhibition of the ceramide signal can significantly damage the cell growth control system. Therefore, ts-46 could control cell growth by interfering with the regulation of the S1P/ceramide pathways (22). Additionally, ts-46 KOs show an increase of (i) ILK signaling, associated with tumor growth and metastasis (18), (ii) integrin signaling, associated with cell survival (19, 38), and (iii) PDGF and mTOR signaling, well-known cancer-related pathways. Furthermore, in ts-101 KO cells, genes related to the chromatin structure are down-regulated. We previously showed that ts-53 can interact with Piwil2, a protein involved in DNA methylation (4); thus these results support our hypothesis that tsRNAs could be involved in the epigenetic control of gene expression. In light of a very recent study reporting that tRF-5031c [named td-piR(glu) in this study] interacts with Piwi proteins and with DNA- and histone-methyltransferases affecting methylation of genes and chromatin condensation (39), it is possible that tsRNAs could also have a role in DNA and histone methylation, affecting chromatin functionality and structure.

Last, we show that ts-46 and ts-47 have an inhibiting effect on the ability of lung cancer cells to form colonies, as previously observed for ts-53. This effect suggests that the deficiency of expression of these tsRNAs can favor cell proliferation and cancer onset/progression (Fig. 4). We performed this experiment with two cell lines differing in p53 expression and KRAS mutation status, and obtained the same inhibitory effect in both cases. These tsRNAs therefore could counterbalance the oncogetic activity of KRAS mutation in lung cancer and positively affect the p53 pathway in cells in which this tumor suppressor is impeded.

All these findings indicate that tsRNAs have a key role in cancer onset and progression and suggest that tsRNAs can be studied as a class that may have oncogenic or tumor-suppressor functions in cancer.

Methods

Tissue Samples. This study was carried out in accordance with a protocol approved by the Institutional Review Board of The Ohio State University. Cancer samples and normal counterparts were obtained from patients enrolled in a clinical study of the role of ncRNAs in solid tumors at the Institute Regina Elena and University La Sapienza, Rome; patients’ written informed consent was obtained in accordance with the Declaration of Helsinki. Samples were grouped as follows: seven tumor samples and seven samples of normal surrounding tissue from colorectal adenomas patients; eight cancer samples and eight samples of normal surrounding tissue from colorectal

Fig. 4. Colony assay on lung cancer cell lines transfected with ts-46 or ts-47. Colony assay performed on H1299 (Left) and A549 (Right) cells. (Upper) The top wells were transfected with the empty vector, and the lower wells were transfected with the vector expressing ts-46. (Lower) The top wells were transfected with the empty vector, and the lower wells were transfected with the vector expressing ts-47. A graphic representation of the colony count is provided beside each plate.
adenocarcinoma patients; nine cancer samples and nine samples of normal surrounding tissue from breast cancer patients with invasive ductal carcinoma; nine cancer samples from nine ovarian cancer patients; and ten samples of normal ovary tissue from ten cervical cancer patients undergoing radical hysterectomy. Histology reports are shown in Table S4.

RNA was extracted using the standard TRIzol method (Invitrogen), and RNA quality was assayed using an Agilent 2100 Bioanalyzer.

Cell Cultures. Cell lines A549 (p53 WT, KRAS G12V), H1299 (p53 null and KRAS WT), and HKEC239 were cultured in RPMI (Sigma-Aldrich) supplemented with 10% FBS and 100 µg/mL gentamicin at 37 °C. P493-6 cells carrying a conditional, tetracycline-regulated MYC (40, 41) were grown in RPMI medium 1640 supplemented with 10% FCS, 100 U/mL penicillin, 100 µg/mL streptomycin, and 2 mM L-glutamine (Life Technologies). For repression of MYC, 0.1 µg/mL tetracycline was added to the culture medium.

MCF10A cells (nontransformed human breast epithelial cells) were grown in DMEM:F12 (HyClone SH30271), 5% (vol/vol) horse serum (Gibco no. 16050-060, lot no. 1075876), 10 µg/mL human insulin (Sigma I-1882), 20 ng/mL recombinant hEGF (PeproTech AF-100-150), 100 µg/mL bovine pituitary extract and 1.25 µg/mL EGF. LNCaP prostate cancer cells were grown in RPMI 1640 medium (Sigma) containing penicillin (100 units/mL), streptomycin (100 µg/mL), and 10% FBS. PC3 prostate cancer cells were grown in T-medium (80% DMEM (4.5 g/L glucose), 20% F12K (Gibco), 3 g/L NaHCO3 (Sigma), 13.6 g/L triiodothyronine (Sigma), 5 µg/mL transferrin (Sigma), 0.25 µg/mL biotin (Sigma), and 25 µg/mL adenine (Sigma)). At the time of use, 5 µg/mL insulin (Sigma), 5% FBS (Atlanta), 100 units/mL penicillin, and 100 µg/mL streptomycin (1%) (Gibco) were added (Table S2).

tRNA Nomenclature. All names and sequences of tRNAs in this study can be found in Table S5.

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