MicroRNA-33a Mediates the Regulation of High Mobility Group AT-Hook 2 Gene (HMGA2) by Thyroid Transcription Factor 1 (TTF-1/NKX2–1)§##

Received for publication, April 3, 2013, and in revised form, April 24, 2013 Published, JBC Papers in Press, April 26, 2013, DOI 10.1074/jbc.M113.474643

Shawn J. Rice†, Shao-Chiang Lai§¶1, Lauren W. Wood§¶2, Kaitlin R. Helsley§¶3, E. Aaron Runkle†, Monte M. Winslow**, and David Mu**†

From the †Penn State Cancer Institute, Penn State University, Hershey, Pennsylvania 17033, the §Leroy T. Canoles Jr. Cancer Research Center, Eastern Virginia Medical School, Norfolk, Virginia 23501, the ¶Department of Microbiology and Molecular Cell Biology, Eastern Virginia Medical School, Norfolk, Virginia 23501, the **Department of Pathology, University of Pennsylvania, Philadelphia, Pennsylvania 19104, and the ††Department of Genetics, Stanford University School of Medicine, Stanford, California 94305

Background: TTF-1 inhibits lung cancer progression via HMGA2 down-regulation.

Results: TTF-1 up-regulates miR-33a, which in turn directly represses HMGA2.

Conclusion: The signaling axis of TTF-1 to HMGA2, important in controlling lung cancer metastasis, is mediated by miR-33a.

Significance: This study explains the mechanism of HMGA2 suppression by TTF-1.

In lung cancers, TTF-1 displays seemingly paradoxical activities. Although TTF-1 is amplified in primary human lung cancers, it inhibits primary lung tumors from metastasizing in a mouse model system. It was reported that the oncogenic proepithelial mesenchymal transition (EMT) high mobility group AT-hook 2 gene (HMGA2) mediates the antimetastatic function of TTF-1. To gain mechanistic insight into the metastasis-critical signaling axis of TTF-1 to HMGA2, we used both reverse and forward strategies and discovered that microRNA-33a (miR-33a) is under direct positive regulation of TTF-1. By chromatin immunoprecipitation, we determined that TTF-1 binds to the promoter of SREBF2, the host gene of miR-33a. The 3′-untranslated region (UTR) of HMGA2 contains three predicted binding sites of miR-33a. We showed that the first two highly conserved sites are conducive to HMGA2 repression by miR-33a, establishing HMGA2 as a genuine target of miR-33a. Functional studies revealed that enforced expression of miR-33a inhibits the mobility of lung cancer cells, and this inhibition can be rescued by overexpression of the form of HMGA2 without the 3′-UTR, suggesting that TTF-1 keeps the prometastasis gene HMGA2 in check via up-regulating miR-33a. This study reports the first miRNAs directly regulated by TTF-1 and clarifies how TTF-1 controls HMGA2 expression. Moreover, the documented importance of SREBF2 and miR-33a in regulating cholesterol metabolism suggests that TTF-1 may be a modulator of cholesterol homeostasis in the lung. Future studies will be dedicated to understanding how miRNAs influence the oncogenic activity of TTF-1 and the role of TTF-1 in cholesterol metabolism.

MicroRNAs (miRNAs)2 are small RNAs that do not encode proteins (1–4). The biological consequences of microRNA expression perturbation are manifested by a wide range of cellular or organismal phenotypes, including cancers (2). In the lung, miRNAs play critical roles in both development and tumorigenesis (5–8). Over 100 miRNAs are dynamically regulated during organogenesis of a normal murine lung (9). Selected examples of miRNAs influencing lung development include miR-17–92, as evidenced by the lung developmental defect in the knock-out mice (10), and miR-302/367, which regulates lung endoderm (11). The better characterized let-7 miRNA family also shows decreased expression in advanced lung cancers (12). Let-7s target a number of oncogenes, including HMGA2 (13–15). The let-7-directed repression of HMGA2 is robust, probably due to the fact that the 3-kb HMGA2 3′-UTR contains seven let-7 binding sites. Human tumor-associated gene translocation of HMGA2 eliminates its 3′-UTR, thus liberating HMGA2 from the let-7-directed repression (13, 14).

Despite advancements in our understanding of the miRNA biology in lung cancer (16), the extent of the interconnection between miRNA-based networks and critical lung cancer genes remains poorly characterized. In this regard, we focus on a master regulator of the lung developmental transcription program termed thyroid transcription factor 1 (TTF-1 or NKX2–1). In addition to being indispensable to fetal lung organogenesis and morphogenesis (17), TTF-1 also contributes to adult lung tumorigenesis based on the genetic evidence that TTF-1 is part of a recurrent multigenic ampiclon in lung cancers (18–21). Subsequent studies have identified RORI and LMO3 as indispensable downstream mediators of TTF-1 in lung adenocarcinomas (22, 23). Seemingly at odds with the observation that TTF-1 is a lung oncogene, Tff-1 was also found to prevent primary lung adenocarcinomas from metastasizing in a mouse model system (24). Moreover, a loss of the Tff-1 allele cooper-

---

† To whom correspondence should be addressed: Leroy T. Canoles Jr. Cancer Research Center, Department of Microbiology and Molecular Cell Biology, Eastern Virginia Medical School, Norfolk, VA 23501. E-mail: mud@evms.edu.

‡ This article contains supplemental Table 1.

§ This work was supported, in whole or in part, by National Institutes of Health, NCI, Grant CA127547 (to D. M.).

The abbreviations used are: miRNA, microRNA; QPCR, quantitative real-time PCR; LNA, locked nucleic acid; dox and Dox, doxycycline.
ates with oncogenic Kras<sup>G12D</sup>, causing pulmonary tumors in transgenic mice that were phenotypically similar to human mucinous adenocarcinomas (25, 26). In view of the multifaceted activities of TTF-1 in lung biology, we believe that mapping the connections between the miRNA network and the TTF-1-directed transcriptional program would provide novel entry points to investigate the lung biology orchestrated by TTF-1. To this end, we have recently reported the discovery of the first miRNA (i.e. miR-365) that directly regulates TTF-1 expression via binding to the 3′-UTR (27). In this study, we concentrate on searching for the miRNAs acting downstream to TTF-1 and have uncovered multiple microRNAs that are directly regulated by TTF-1. One such miRNA, miR-33a, was chosen for a comprehensive characterization in view of the fact that it scored in both reverse and forward screens. The results unambiguously place miR-33a under the positive transcriptional control of TTF-1. Moreover, we discovered that the HMGA2 oncogene, known to be repressed by TTF-1 (24), is a direct target repressed by miR-33a. Loss- and gain-of-function analyses validate miR-33a as a mediator of the repression by TTF-1 (TTF-1 → miR-33a → HMGA2). In light of our observations, we believe that TTF-1 utilizes miR-33a as a means to abate HMGA2 expression. Considering the known activities of miR-33a outside of cancer biology (e.g. cholesterol metabolism (28)), the results of this study are expected to carve out novel directions for future research on TTF-1-orchestrated lung biology.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Expression Vectors**—The human lung cancer cell lines NCI-H358, NCI-H441, A549, NCI-H1299, and BEAS-2B were acquired from the American Type Culture Collection (ATCC), and maintained as described previously (29). Mouse 394T4-bc37 (shLuc) and 394T4-E1 (shTtf-1) cells were provided by Dr. Monte Winslow (24) and maintained in DMEM supplemented with 10% fetal bovine serum, penicillin, and streptomycin. The pGL4.10 SPB promoter reporter construct and pcDNA3.1 TTF-1 and TTF-1 homeodomain deletion mutant expression vectors were constructed previously (29). The SREBF2 (−998 to −3, relative to the transcription start site) and C9ORF5 (−1000 to −5) promoters were PCR-amplified from human genomic DNA using primers listed in Table 1 and cloned into the promoterless luciferase vector pGL4.10 Basic (Promega). Deletion mutants of miR-33a binding sites were derived from a psiCHECK2 vector containing the 3′-UTR of HMGA2 fused to the 3′-end of a Renilla luciferase gene, kindly provided by Dr. Marcus Peter (30). Mutation constructs were created using the QuickChange site-directed mutagenesis kit (Stratagene) according to the manufacturer’s protocol; the primers are listed in Table 1. The human HMGA2 cDNA expression plasmid was obtained from Dr. Jian-Jun Wei (31), and the murine HmgA2 cDNA was from Addgene (Cambridge, MA).

**RNA Oligonucleotide Reagents and Transfection**—All siRNAs, miRNA mimics, and inhibitors were purchased from Dharmacon. Cells were transiently transfected with plasmid DNA, siRNA (non-targeting negative control, D-001210-01; TTF-1 A/B/C, D019105–03/04/17), antisense oligonucleotide miRNA inhibitors (non-targeting negative control, IN-001001–01; hsa-miR-33a inhibitor, IH-300509–08), or miRNA mimics (non-targeting control, CN-001005–01; hsa-miR-33a (C-300509–07) using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. Transfection efficiency was monitored with pMAXGFP plasmid or BLOCK-iT fluorescently labeled negative control oligonucleotides (Invitrogen) and judged to be >90% for each cell line.

**RNA Isolation and Reverse Transcription (RT)-Quantitative Real-time PCR (QPCR) Analysis**—Total RNA (miRNA and mRNA) was isolated from cells using TRIzol (Invitrogen). For mRNA quantification, RNA was reverse transcribed using the High Capacity cDNA synthesis kit (Applied Biosystems). The resultant cDNA was evaluated by real-time PCR using the primers listed in Table 1 and Brilliant II SYBR Green master mix (Stratagene) with a StepOne Plus real-time PCR system (Applied Biosystems). MicroRNAs were quantified using miRCURY<sup>TM</sup> (Exiqon) or qScript (Quanta Biosciences) miRNA cDNA synthesis kits followed by real-time PCR analysis using locked nucleic acid (LNA) miRNA-specific PCR primers and miRCURY<sup>TM</sup> SYBR Green master mix (Exiqon) or PerfeCta miRNA assays and PerfeCta SYBR Green master mix (Quanta Biosciences), respectively.

**MicroRNA Array Profiling**—Total RNA was harvested for profiling using the Cell and Plant miRCURY<sup>TM</sup> RNA isolation kit (Exiqon). The quality of the total RNA was verified by an Agilent 2100 bioanalyzer profile. Total RNA (700 ng) from sample and reference was labeled with Hy<sub>3</sub><sup>TM</sup> and Hy<sub>5</sub><sup>TM</sup> fluorescent label, respectively, using the miRCURY<sup>TM</sup> LNA Array power labeling kit (Exiqon) following the procedure described by the manufacturer. The Hy<sub>3</sub><sup>TM</sup>-labeled samples and a Hy<sub>5</sub><sup>TM</sup>-labeled reference RNA sample were mixed pairwise and hybridized to the miRCURY<sup>TM</sup> LNA Array version 5th Generation (Exiqon), which contains capture probes targeting all miRNAs for humans, mice, or rats registered in miRBASE version 16.0 at the Sanger Institute. The hybridization was performed according to the miRCURY<sup>TM</sup> LNA array manual using a Tecan HS4800 hybridization station. After hybridization, the microarray slides were scanned and stored in an ozone-free environment (ozone level below 2.0 ppb) in order to prevent potential bleaching of the fluorescent dyes. The miRCURY<sup>TM</sup> LNA array microarray slides were scanned using the Agilent G2565BA microarray scanner system (Agilent Technologies), and the image analysis was carried out using ImaGene version 9.0 software (BioDiscovery). The quantified signals were background-corrected (Normexp with offset value 10 (32)) and normalized using quantile normalization.

**Western Blotting**—Proteins were harvested from cells as described previously (29). Cell lysates (10–20 μg) were fractionated by SDS-PAGE and electrophoretically transferred to nitrocellulose membranes. Membranes were blocked in nonfat dry milk (5%) and incubated overnight with primary antibody against TTF-1 (clone 8G7G3/1, Santa Cruz Biotechnology, Inc. (Santa Cruz, CA)), HMGA2 (Biocheck), GAPDH (Cell Signaling), or HSP90 (BD Transduction Laboratories). Proteins were detected with appropriate HRP-conjugated secondary antibody (Thermo Scientific) and chemiluminescent substrates.
MicroRNAs Regulated by TTF-1

TABLE 1
List of PCR primers used in this study

| Name                      | Direction | Sequence                                  |
|---------------------------|-----------|-------------------------------------------|
| Mouse RT-QPCR primers     |           |                                           |
| Gapdh                     | Forward   | 5'-GCTTTGATACATATTACCCATAGTTAC-3'         |
|                           | Reverse   | 5'-TTTGAGTTATGATTTTTATAGTTAC-3'          |
| Ttf-1                     | Forward   | 5'-AAACCTGCGGCGAGACT-3'                  |
|                           | Reverse   | 5'-TGCTTGGAGCAGCATCGACAT-3'              |
| Srebf1                    | Forward   | 5'-CCACAGGTTGAGCACCTGCA-3'               |
|                           | Reverse   | 5'-ACCTGTCGAGATGATCCA-3'                 |
| Srebf2                    | Forward   | 5'-AACCTGCGGCGAGACT-3'                   |
|                           | Reverse   | 5'-ACTGTCGAGATGATCCA-3'                  |
| Hmga2                     | Forward   | 5'-GGGGGACACAGCTTAAATTACTG-3'            |
|                           | Reverse   | 5'-GCTTTGATACATATTACCCATAGTTAC-3'       |
| Human RT-QPCR primers     |           |                                           |
| G6PDH                     | Forward   | 5'-GGTGACGTAAGAGGATGAGTTA-3'             |
|                           | Reverse   | 5'-GCTTTCGAGATGATCCA-3'                  |
| TTF1                      | Forward   | 5'-CCCTCATGGCCACCTTCTG-3'                |
|                           | Reverse   | 5'-GCCACATTTGACCAAAATC-3'                |
| SREBF1                    | Forward   | 5'-GCTGCCCTTCACTAGAAGCTATCA-3'           |
|                           | Reverse   | 5'-GCCACATTTGACCAAAATC-3'                |
| SREBF2                    | Forward   | 5'-GCTTGACGTAAGAGGATGAGTTA-3'            |
|                           | Reverse   | 5'-GCTTGACGTAAGAGGATGAGTTA-3'            |
| HMGA2                     | Forward   | 5'-GCCACATTTGACCAAAATC-3'                |
|                           | Reverse   | 5'-GCCACATTTGACCAAAATC-3'                |
| ChIP QPCR primers         |           |                                           |
| C9orf5 (distal)           | Forward   | 5'-GGACAAGGAAAGCTTAC-3'                  |
|                           | Reverse   | 5'-GGACAAGGAAAGCTTAC-3'                  |
| C9orf5 (proximal)         | Forward   | 5'-GGACAAGGAAAGCTTAC-3'                  |
|                           | Reverse   | 5'-GGACAAGGAAAGCTTAC-3'                  |
| SREBF2 (distal)           | Forward   | 5'-GGACAAGGAAAGCTTAC-3'                  |
|                           | Reverse   | 5'-GGACAAGGAAAGCTTAC-3'                  |
| SREBF2 (proximal)         | Forward   | 5'-GGACAAGGAAAGCTTAC-3'                  |
|                           | Reverse   | 5'-GGACAAGGAAAGCTTAC-3'                  |
| Sp8 (distal)              | Forward   | 5'-GGACAAGGAAAGCTTAC-3'                  |
|                           | Reverse   | 5'-GGACAAGGAAAGCTTAC-3'                  |
| Sp8 (proximal)            | Forward   | 5'-GGACAAGGAAAGCTTAC-3'                  |
|                           | Reverse   | 5'-GGACAAGGAAAGCTTAC-3'                  |
| HMGA2 3'UTR psiCHECK2 site-directed mutagenesis primers* | Forward | 5'-GGACAAGGAAAGCTTAC-3' |
| Δ1                        | Forward   | 5'-GGACAAGGAAAGCTTAC-3'                  |
|                           | Reverse   | 5'-GGACAAGGAAAGCTTAC-3'                  |
| Δ2                        | Forward   | 5'-GGACAAGGAAAGCTTAC-3'                  |
|                           | Reverse   | 5'-GGACAAGGAAAGCTTAC-3'                  |
| Δ3                        | Forward   | 5'-GGACAAGGAAAGCTTAC-3'                  |
|                           | Reverse   | 5'-GGACAAGGAAAGCTTAC-3'                  |

* The Kpn1 cut site is underlined.

Luciferase Reporter Assays—Promoter reporter assays were carried out in 96-well plates as described previously (29). Briefly, cells were co-transfected with a firefly luciferase reporter construct and a Renilla luciferase control vector pGL4.73 (Promega). Twenty-four hours after transfection, firefly and Renilla luciferase values were quantified using Dual-Glo luciferase assay (Promega) on a GloMax-96 plate reader (Promega). Firefly luciferase values were normalized to Renilla luciferase values and expressed as relative values. For 3'-UTR-based reporter studies, cells were seeded onto 24-well plates and co-transfected with psiCHECK2 reporter constructs (300 ng) and RNA oligonucleotides on the following day. Firefly and Renilla luciferase values were measured 48 h after transfection, and Renilla luciferase signals were normalized to firefly luciferase signals.

Chromatin Immunoprecipitation (ChIP)—ChIP was performed on NCI-H441 cells as described previously (29), using either a TTF-1 antibody (H190) or a normal rabbit IgG (Santa Cruz Biotechnology, Inc.). Target sequences were detected by QPCR using Brilliant II SYBR Green master mix (Stratagene). QPCR signals of the immunoprecipitated chromatin were normalized to the signal from the total lysate (input) for each primer pair. Primers used for ChIP analyses are listed in Table 1.

Transwell Migration and Invasion Assays—For Boyden chamber migration assays, 5 × 10^4 transfected cells were seeded in triplicate onto upper chamber of a migration insert with 8-μm pore size (catalog no. 354578, BD Biosciences) in serum-free media; media supplemented with 5% fetal bovine serum, as a chemoattractant, was added to the lower well. After 24 h, cells from the top of the chamber membrane were removed, and the remaining cells on the bottom of the membrane were fixed with methanol and stained with hematoxylin. Average nuclei were determined in five ×100 fields using a Nikon Eclipse microscope and NIS Elements D software (Nikon), with nuclei counted blind and manually. Invasion assays were performed in the same manner as the migration assay but utilized Matrigel-coated inserts with 8-μm pore size (catalog no. 354480, BD Biosciences).

Statistical Analysis—GraphPad Prism version 5 software was used to perform statistical analyses, including Student’s t test, when comparing two groups (control and experimental). One-way analysis of variance with Tukey’s post-test was used to compare more than two groups, and a two-way analysis of vari-

16350 JOURNAL OF BIOLOGICAL CHEMISTRY
Suppresses metastasis formation of primary lung adenocarcinoma activity of Ttf-1 in human lung cancer cells as well. Activation of oncogenic Kras and p53 deletion (24), holds true in NCI-H358 Ttf-1 protein expression (Fig. 1). Small interfering RNA (siRNA) lead to a greater than 2-fold derepression in HMGA2 protein expression (NCI-H358). Interestingly, both human lung adenocarcinoma cell line with endogenous two independent small interfering RNAs (siRNAs) against HMGA2 gene, in a mouse model system. By gene expression profiling and quantitative test was used to analyze time course experiments. Representative experiments repeated at least twice are shown as mean ± S.D. Data were considered statistically significant when p was <0.05 (*, p < 0.05; **, p < 0.01; ***, p < 0.001).

RESULTS

TTF-1 Represses HMGA2 in Human Lung Cancer Cells—Winslow et al. (24) reported a surprising finding, that Ttf-1 suppresses metastasis formation of primary lung adenocarcinomas driven by mutant Kras and p53 loss (Kras<sup>G12D/; p53<sup>−/−</sup></sup>) in a mouse model system. By gene expression profiling and functional analyses, it was determined that a pro-EMT oncogene, Hmga2, is a downstream target of Ttf-1 and that repression of Hmga2 expression by Ttf-1 is a basis to the antimetastatic activity of Ttf-1 (24). To test if the Ttf-1/Hmga2 relationship is conserved in human lung cancer cells, we used two independent small interfering RNAs (siRNAs) against TTF-1 to knock down the endogenous expression of TTF-1 in a human lung adenocarcinoma cell line with endogenous HMGA2 protein expression (NCI-H358). Interestingly, both siTTF-1s lead to a greater than 2-fold derepression in HMGA2 protein expression (Fig. 1A). This observation suggests that the Ttf-1/Hmga2 axis, originally discovered in the murine lung adenocarcinoma cells derived from tumors initiated by somatic activation of oncogenic Kras and p53 deletion (24), holds true in human lung cancer cells as well.

Although it is known that HMGA2 is subject to regulation by the let-7 family of miRNAs (33), let-7s do not appear to play a role in mediating the repression of Hmga2 by Ttf-1 in murine lung cancer cells (24). Thus, we hypothesized that there are other miRNAs mediating the Ttf-1/Hmga2 regulation. To this end, we utilized a reporter plasmid where the 3′-UTR of human HMGA2 was fused to the 3′-end of the Renilla luciferase gene in the pscCHECK2 vector backbone (30). This reporter plasmid was transfected into the NCI-H358 human lung cancer cell line in which the endogenous TTF-1 expression was knocked down using two independent siTTF-1s. The results demonstrate that a decrease of TTF-1 expression leads to a concomitant increase in the luciferase activity of Luc-HMGA2/3′-UTR (Fig. 1B). This derepression of HMGA2 3′-UTR upon TTF-1 knockdown implicates the involvement of HMGA2 3′-UTR in the TTF-1-induced silencing of HMGA2.

Discovery of miRNAs That Mediate the Ttf-1−Hmga2 Relationship in Murine Lung Cancer Cells—Our working hypothesis was that TTF-1 may directly up-regulate certain miRNAs, which in turn repress HMGA2 expression via direct binding to the 3′-UTR of HMGA2. To identify these putative miRNA regulators, we utilized the non-metastatic (TnonMet) murine primary lung tumor cells (394T4) generated and modified by Winslow et al. (24) to stably express a small hairpin RNA (shRNA) against Ttf-1 (394T4-shTtf-1). The Ttf-1 knockdown and the corresponding Hmga2 up-regulation in 394T4-shTtf-1 cells elicited a higher metastatic phenotype, whereas the control cells, 394T4-shLuc carrying an shRNA against luciferase (shLuc), were non-metastatic and thus similar to the parental 394T4 cells (24). We reasoned that the putative miRNA(s) responsible for suppressing Hmga2 in a Ttf-1-dependent manner in the 394T4-shLuc (Ttf-1<sup>high</sup>) cells would be down-regulated in 394T4-shTtf-1 (Ttf-1<sup>low</sup>) cells due to Ttf-1 knockdown. To identify these miRNA regulators that are present in both mice and humans, we created a custom QPCR array that contains 44 LNA-based QPCR probes in duplicates (Exiqon). Each of the LNA probes detects an miRNA predicted by TargetScan version 5.2 (34) to bind to human HMGA2 3′-UTR (Table 2). Approximately 57% (i.e. 25) of the 44 human miRNA probes are able to detect the mouse counterparts based on sequence conservation. This QPCR array was employed to quantify the murine miRNA expression differences between 394T4-shLuc (Ttf-1<sup>high</sup>) and 394T4-shTtf-1 (Ttf-1<sup>low</sup>) cells. Twelve murine miRNAs were scored in the QPCR array assay, and the results are presented as ratios of miRNA expression levels (shLuc/shTtf-1; Fig. 2A). Interestingly, two human miRNA probes (hsa-miR-33a and hsa-miR-495) detected high expression ratios, suggesting that the two corresponding murine miRNAs may be positively regulated by Ttf-1. Indeed, secondary confirmational studies utilizing an independent miRNA detection system (Quanta Biosciences) validated the QPCR array data for mmu-miR-33 (i.e. Ttf-1 knockdown repressing mmu-miR-33 expression) (Fig. 2B). (Note that mmu-miR-33 is the mouse homolog of human hsa-miR-33a. The two miRNAs are 100% identical in sequence. Hereafter, hsa-miR-33a and mmu-miR-33 are referred to as miR-33a and miR-33, respectively.) We did not pursue the second positive hit of the QPCR array screen (mmu-miR-495) because this miRNA, unlike miR-33, was not predicted to target the mouse Hmga2 3′-UTR by TargetScan; therefore, mmu-miR-495 is not likely to be involved in the Ttf-1-mediated repression of Hmga2 in the murine lung cancer cells.

Searching for TTF-1-regulated miRNAs in a Human Lung Epithelial System with Inducible TTF-1 Expression—To substantiate the candidacy of miR-33a as a TTF-1-regulated miRNA and to explore TTF-1-regulated miRNAs globally in human cells, we took on a gain-of-function strategy using a human lung epithelial cell system in which the expression of a
MicroRNAs Regulated by TTF-1

TABLE 2
Probe list of the custom HMGA2-3’-UTR-targeting QPCR array

| miRNA    | Comment                      | miRNA    | Comment                      |
|----------|------------------------------|----------|------------------------------|
| 1 hsa-let-7b | Included in analysis          | 22 hsa-miR-26a | Included in analysis          |
| 2 hsa-let-7c | Included in analysis          | 23 hsa-miR-760 | Included in analysis          |
| 3 hsa-let-7d | Included in analysis          | 24 hsa-miR-98  | Included in analysis          |
| 4 hsa-let-7e | Included in analysis          | 25 hsa-miR-98  | Included in analysis          |
| 5 hsa-let-7f | Included in analysis          | 26 hsa-miR-202 | Excluded, human-specific      |
| 6 hsa-let-7i | Included in analysis          | 27 hsa-miR-211 | Excluded, human-specific      |
| 7 hsa-miR-186 | Included in analysis          | 28 hsa-miR-337-3p | Excluded, human-specific      |
| 8 hsa-miR-26b | Included in analysis          | 29 hsa-miR-33b | Excluded, human-specific      |
| 9 hsa-miR-33a | Included in analysis          | 30 hsa-miR-376c | Excluded, human-specific      |
| 10 hsa-miR-495 | Included in analysis        | 31 hsa-miR-450b-5p | Excluded, human-specific      |
| 11 hsa-miR-532-3p | Included in analysis      | 32 hsa-miR-509-3-5p | Excluded, human-specific      |
| 12 hsa-miR-9 | Included in analysis          | 33 hsa-miR-520d-5p | Excluded, human-specific      |
| 13 hsa-miR-137 | Not detected                | 34 hsa-miR-522  | Excluded, human-specific      |
| 14 hsa-miR-196a | Not detected               | 35 hsa-miR-524-5p | Excluded, human-specific      |
| 15 hsa-miR-204 | Not detected                | 36 hsa-miR-548c-3p | Excluded, human-specific      |
| 16 hsa-miR-361-5p | Not detected             | 37 hsa-miR-554  | Excluded, human-specific      |
| 17 hsa-miR-370 | Not detected                | 38 hsa-miR-556-5p | Excluded, human-specific      |
| 18 hsa-miR-539 | Not detected                | 39 hsa-miR-570  | Excluded, human-specific      |
| 19 hsa-let-7a  | Excluded, replicate Ct>1.5 cycles | 40 hsa-miR-573  | Excluded, human-specific      |
| 20 hsa-let-7g  | Excluded, replicate Ct>1.5 cycles | 41 hsa-miR-578  | Excluded, human-specific      |
| 21 hsa-miR-129-5p | Excluded, replicate Ct>1.5 cycles | 42 hsa-miR-579  | Excluded, human-specific      |
| 22 hsa-miR-196b | Excluded, replicate Ct>1.5 cycles | 43 hsa-miR-588  | Excluded, human-specific      |
| 23 hsa-miR-26a  | Excluded, replicate Ct>1.5 cycles | 44 hsa-miR-608  | Excluded, human-specific      |
| 24 hsa-miR-760  | Excluded, replicate Ct>1.5 cycles | 28 hsa-miR-337-3p | Excluded, human-specific      |
| 25 hsa-miR-98  | Excluded, replicate Ct>1.5 cycles | 29 hsa-miR-33b | Excluded, human-specific      |
| 26 hsa-miR-202  | Excluded, human-specific miRNA | 30 hsa-miR-376c | Excluded, human-specific miRNA|
| 27 hsa-miR-211  | Excluded, human-specific miRNA | 31 hsa-miR-450b-5p | Excluded, human-specific miRNA|
| 28 hsa-miR-337-3p | Excluded, human-specific miRNA | 32 hsa-miR-509-3-5p | Excluded, human-specific miRNA|
| 29 hsa-miR-33b | Excluded, human-specific miRNA | 33 hsa-miR-520d-5p | Excluded, human-specific miRNA|
| 30 hsa-miR-376c | Excluded, human-specific miRNA | 34 hsa-miR-522 | Excluded, human-specific miRNA|
| 31 hsa-miR-450b-5p | Excluded, human-specific miRNA | 35 hsa-miR-524-5p | Excluded, human-specific miRNA|
| 32 hsa-miR-509-3-5p | Excluded, human-specific miRNA | 36 hsa-miR-548c-3p | Excluded, human-specific miRNA|
| 33 hsa-miR-520d-5p | Excluded, human-specific miRNA | 37 hsa-miR-554 | Excluded, human-specific miRNA|
| 34 hsa-miR-522 | Excluded, human-specific miRNA | 38 hsa-miR-556-5p | Excluded, human-specific miRNA|
| 35 hsa-miR-524-5p | Excluded, human-specific miRNA | 39 hsa-miR-570 | Excluded, human-specific miRNA|
| 36 hsa-miR-548c-3p | Excluded, human-specific miRNA | 40 hsa-miR-573 | Excluded, human-specific miRNA|
| 37 hsa-miR-554 | Excluded, human-specific miRNA | 41 hsa-miR-578 | Excluded, human-specific miRNA|
| 38 hsa-miR-556-5p | Excluded, human-specific miRNA | 42 hsa-miR-579 | Excluded, human-specific miRNA|
| 39 hsa-miR-570 | Excluded, human-specific miRNA | 43 hsa-miR-588 | Excluded, human-specific miRNA|
| 40 hsa-miR-573 | Excluded, human-specific miRNA | 44 hsa-miR-608 | Excluded, human-specific miRNA|

human TTF-1 transgene could be turned on by doxycycline (i.e. a doxycycline (dox)-on system) (29). The host cells of this inducible system are the premalignant, viral oncogene-immortalized human lung epithelial cells, BEAS-2B (35). We chose this cell strain due to the fact that it lacks endogenous TTF-1 expression (36), thus maximizing the miRNA expression per-
changes were dependent on TTF-1 expression but independent of dox treatment. For validation, LNA-based probes to miR-33a and miR-32 were used in RT-QPCR to confirm the array profiling data. The results, corroborating the array profiling observations, indicate a 1.7–2-fold increase of miR-33a and miR-32 upon TTF-1 induction (Fig. 3).

Identification of miR-33a and miR-32 as Direct Transcriptional Targets of TTF-1—In view of the biochemical property of TTF-1 as a transcription factor (17, 37, 38), we surmised that TTF-1 would activate the promoter of the respective genes hosting miR-33a and miR-32. To approach this issue, we cloned the promoter region (<1 kb) of SREBF2 (NM_004599, the host gene of hsa-miR-33a) and C9ORF5 (NM_032012, the host gene of hsa-miR-32) into the 5′-end of a promoterless luciferase reporter vector. Luciferase assays were performed 24 h after transfection (n = 4). y axis, fold change in relative luminescence units (RLU).

Figure 4. TTF-1 binds to and activates transcription from the promoters of the miR-32 and miR-33a host genes. A, diagram of the promoter regions of the miR-32 and miR-33a host genes, C9ORF5 and SREBF2, respectively. Locations for negative control (NC) and promoter PCR primer pairs used in the ChIP analyses are shown relative to the respective gene’s predicted transcriptional start site (TSS). B, A549 cells were transfected with the indicated expression vector (empty vector (EV), TTF-1, or a TTF-1 homeodomain deletion mutant, HDD) and a luciferase promoter reporter construct for C9ORF5 (miR-32) or SREBF2 (miR-33a). Luciferase assays were performed 24 h after transfection (n = 4). y axis, fold change in relative luminescence units (RLU).
cell line commonly used to study transcriptional activity of exogenous TTF-1 (39). The data in Fig. 4B show that both promoters respond to TTF-1 transactivation, whereas the homeodomain deletion mutant of TTF-1 failed to activate the promoters, consistent with the notion that the homeodomain-dependent DNA binding activity of TTF-1 is essential for this function (40). The positive luciferase data are in line with the thesis that SREBF2 is under TTF-1 transcriptional control. To further test this relationship, we used two siTTF-1s to knock down endogenous TTF-1 in the NCI-H441 human lung adenocarcinoma cell line, which has high endogenous TTF-1 expression (29). RT-QPCR analyses show that TTF-1 knockdown induced a concomitant 40–50% decrease in SREBF2 RNA (Fig. 4C).

To determine whether TTF-1 directly bound to the promoters of the respective host genes of miR-33a and miR-32, we conducted ChIP using an anti-TTF-1 antibody. The immunoprecipitated chromatin from NCI-H441 cells were analyzed using two genomic location probes: a proximal probe at 1 kb and a distal probe at 5 kb upstream to the transcription start site (Fig. 4A, TSS). In both cases, the proximal QPCR probes detected quantitative recovery of promoter DNA sequences for both host genes with TTF-1. In view of the positive results for miR-33a in both loss- and gain-of-function analyses using mouse and human cells, miR-33a stood out as a strong candidate miRNA under direct TTF-1 control.

TTF-1-induced Down-regulation of HMG2 Is Mediated by miR-33a—Both human HMG2 and mouse Hmga2 were ranked by TargetScan (34) as potential target genes of hsa-miR-33a and mmu-miR-33, respectively (Table 3). In addition, there are three putative binding sites for miR-33a and one binding site for miR-32 in the HMG2 3'UTR (Fig. 5A). In view of these data, we initiated studies to validate HMG2 as an authentic target gene of miR-33a and miR-32. An miR-33a mimic oligonucleotide or an miRNA known to target HMG2 (let-7d) was cotransfected with a luciferase reporter of HMG2 3'UTR into a human lung cancer cell line, NCI-H1299. We chose NCI-H1299 because the endogenous HMG2 expression in NCI-H1299 is responsive to let-7 regulation (13), implicating the existence of a functional miRNA-dependent surveillance of HMG2 in NCI-H1299 cells. Chemiluminescence measurement revealed a 50% reduction of reporter activity in the miR-33a transfectants (Fig. 5B); the positive control let-7d induced a stronger inhibition of HMG2 3'UTR reporter (≈80%), which is probably due to the fact that there are seven let-7 binding sites in the 3'UTR of HMG2 (Fig. 5A). Interestingly, although the miR-32 mimic elicited a slight response in the reporter assay, this response appeared to be independent of the predicted miR-32 site (Fig. 5C). Thus, we conclude that the predicted binding site of miR-33 in the 3'UTR of HMG2 may be nonfunctional. We then proceeded to measure the endogenous RNA and protein expression of HMG2 following miR-33a or let-7d transfection in NCI-H1299 cells (Fig. 5, D and E). In addition, we also measured the HMG2 protein level following miR-33a transfection in the murine lung cancer cells (394T4-shTtf-1; Fig. 5F). Overall the results corroborate the notion that HMG2 is a target gene of miR-33a.

To substantiate that the Ttf-1-induced up-regulation of mmu-miR-33 in the 394T4 murine cells was responsible for repressing Hmga2, we first treated 394T4-shLuc (Ttf-1high) cells with an miR-33a miRNA inhibitor (anti-miR-33a, which targets both hsa-miR-33a and mmu-miR-33) and found that the Hmga2 protein level increased by ≈2-fold (Fig. 6A). To extend this observation to human lung cancer cells, we next treated NCI-H358 cells with anti-miR-33a. Inhibition of miR-
33a in these cells again resulted in a ~3-fold increase in HMGA2 protein (Fig. 6B), corroborating the results seen with the murine 394T4-shLuc cells. Finally, we analyzed the impact of anti-miR-33a on the Hmga2 repression imposed by exogenous TTF-1. To this end, we transfected human TTF-1 cDNA into a murine Ttf-1 knockdown background (394T4-shTtf-1 cells). The stably expressed shRNA against the mouse Ttf-1 gene in the 394T4-shTtf-1 cells does not down-regulate the human TTF-1 gene due to the fact the particular shRNA targeting sequence (CGCCATGTCTTGTTCTACCTT) is unique to mouse Ttf-1. The expression of human TTF-1 clearly conferred repression of Hmga2 (Fig. 6C). Importantly, anti-miR-33a abolished the Hmg2 protein repression imposed by the exogenous human TTF-1 (Fig. 6C), proving that miR-33a is a critical mediator of the TTF-1-induced Hmga2 repression.

Analysis of miR-33a Binding Sites in the 3′-UTR of HMGA2—To analyze the relative contribution of the three miR-33a binding sites to HMGA2 repression by miR-33a, we mutated the three TargetScan-predicted miR-33a binding sites individually and in all possible pairwise combinations, affording a total of seven mutant reporter plasmids of HMGA2 3′-UTR for analysis (Fig. 7A). The luciferase reporter activities of individual mutant plasmids were evaluated following cotransfection into NCI-H1299 cells along with either an exogenous miR-33a mimic.

FIGURE 5. HMGA2 is a novel target of miR-33a in mouse and human lung cancer cells. A, diagram depicting the 3′-UTR of the human HMGA2 gene. Locations of predicted miRNA binding sites for let-7 (black), miR-33a (gray), and miR-32 (white) are marked with arrows, and seed site locations are listed below the arrows. B, NCI-H1299 cells were transfected with the HMGA2 3′-UTR reporter and either a scrambled control oligonucleotide (Scr) or an miR-33a mimic (miR-33a). Luciferase activity was read 48 h post-transfection (n = 3). C, HMGA2 3′-UTR is not a target for miR-32. NCI-H1299 cells were cotransfected with an miR-32 mimic or a negative control oligonucleotide (NC) and either a wild-type HMGA2 3′-UTR reporter construct (Wt) or an HMGA2 3′-UTR reporter with a deleted miR-32 binding site (ΔmiR-32). After 48 h, Renilla and firefly luciferase activities were assayed. Although miR-32 resulted in a slight inhibition of the HMGA2 reporter, deletion of the sole predicted miR-32 binding site did not cause a derepression of the HMGA2 reporter. RLU, relative luminescence units. D, quantification of endogenous HMGA2 mRNA after transfection of NCI-H1299 cells with a scrambled control oligonucleotide or an miR-33a mimic. *, p < 0.05; **, p < 0.01; ***, p < 0.001.

FIGURE 6. TTF-1 up-regulation of miR-33a suppresses HMGA2 expression in human and mouse lung epithelial cells. A, Western blot analysis of Hmga2, Ttf-1, and Hsp90 expression in murine 394T4-shLuc cells transfected with either a negative control (anti-miR-NC) or an miR-33a inhibitor (anti-miR-33a). B, Western blot analysis of HMGA2, TFF-1, and Hsp90 expression in the human NCI-H358 cells transfected with either a negative control or an miR-33a inhibitor. C, 394T4-shTff-1 cells were transfected as indicated, and the corresponding cell lysates were analyzed by immunoblotting for the expression of Tff-1, Hmg2, and Hsp90.
MicroRNAs Regulated by TTF-1

A

| Name | HMGA2 3'UTR |
|------|-------------|
| Wt   | CAAUGCA→CAAUGCA→AAUGCA | (262-268) (1318-1324)(1912-1918) |
| Δ1   | CAAUGCA→GGTACC→AAUGCA |
| Δ2   | CAAUGCA→GGTACC→AAUGCA |
| Δ3   | CAAUGCA→GGTACC→AAUGCA |
| Δ1/2 | GGTACC→GGTACC→AAUGCA |
| Δ1/3 | GGTACC→CAUGCA→GGTACC |
| Δ2/3 | GGTACC→CAUGCA→GGTACC |
| Δ1/2/3 | GGTACC→GGTACC→GGTACC |

B

![Graph showing RLU vs Reporter Construct](image)

oligonucleotide or a scrambled control oligonucleotide. Of the three single-site mutants (Δ1, Δ2, and Δ3), Δ1 showed the strongest derepression (Fig. 7B), whereas Δ3 was essentially identical to the wild-type reporter plasmid. This suggests that the relative contribution of the three miR-33a binding sites to repression of HMGA2 3’-UTR is in the following order: site 1 > site 2 > site 3. Data obtained with the three double mutants (Δ1/2, Δ1/3, and Δ2/3) generally agreed with the observation gathered from single mutants (Fig. 7B). It is intriguing to note that the derepression of losing both sites 1 and 2 (Δ1/2) appeared to be larger than the sum of the two single mutants (Δ1 + Δ2), indicating potential synergy between sites 1 and 2. Although miRNA binding sites are known to work synergistically in repressing target genes, the two miRNA binding sites would generally have to be close in distance for optimal cooperativity (34, 41). Therefore, in view of the long distance (>1 kb) between miR-33a sites 1 and 2 in the 3’-UTR of HMGA2, it is surprising that these two sites might work cooperatively.

miR-33a Impedes Motility of Human and Murine Lung Cancer Cells—Because Ttf-1 was shown to regulate metastatic dissemination (24), we chose to examine the migratory and invasive properties of human and murine lung cancer cells following modulation of TTF-1/miR-33a/HMGA2 levels. Initially, we compared the migration and invasiveness of the two murine lung cancer cell lines, 394T4-shLuc (Ttf-1high) and 394T4-shTtf-1 (Ttf-1low), using transwell migration (uncoated control inserts) and invasion (Matrigel-coated inserts) assays. Although the motility of the 394T4-shTtf-1 cells was greater than the shLuc cells, there was no difference in invasiveness between the treatment groups (Fig. 8, A and B). Transfection of the human NCI-H1299 cells with an exogenous miR-33a oligonucleotide impeded migration compared with a scrambled control oligonucleotide in the transwell migration assay. However, the invasiveness of cells as determined by the transwell assay was not altered by miR-33a transfection (Fig. 8, C and D). Therefore, we focused the subsequent studies on the relevance of the TTF-1 → miR-33a → HMGA2 axis to cellular motility. The assumption was that miR-33a could functionally replace TTF-1 in slowing migration due to repression of HMGA2, which promotes cell motility (31). The murine 394T4-shLuc cells (Ttf-1high), transfected with a mmu-miR-33 inhibitor (anti-miR-33a) or a negative control, were evaluated for migratory potential using the transwell assays. The anti-miR-33a conferred an increase in migration by 1.8-fold (Fig. 9A), indicating that derepression of Hmga2 by anti-miR-33a enhances cellular migration. Conversely, enforced overexpression of Hmga2 in the 394T4-shLuc cells increased its motility (Fig. 9B, lane 1 versus lane 2), phenocopying the cellular responses to anti-miR-33a. Transfection of the miR-33a mimic into 394T4-shLuc cells slowed down cellular motility, as shown in Fig. 9B (lane 1 versus lane 3). Importantly, stable overexpression of Hmga2 lacking 3’-UTR overcame the inhibitory effect of miR-33a on cellular migration (Fig. 9B, lane 3 versus lane 4).

To complement these observations derived from the mouse lung cancer cells, we turned to the human NCI-H1299 cells, which are TTF-1low. A human HMGA2 cDNA lacking 3’-UTR and thus non-targetable by miR-33a was retrovirally transduced into NCI-H1299 cells for stable expression. Subsequently, either an miR-33a mimic oligonucleotide or a scrambled control RNA oligonucleotide was introduced via transfection. RT-QPCR analysis showed that miR-33a mimic reduced the endogenous HMGA2 RNA by 64% in NCI-H1299 cells (Fig. 9C). By the transwell assay, the miR-33a mimic transfection conferred a 40% reduction in motility of NCI-H1299 cells compared with a scrambled oligonucleotide (Fig. 9D, lane 1 versus lane 3). Importantly, this miR-33a-induced decrease in motility was rescued by the stable expression of the HMGA2 transgene (Fig. 9D, lane 3 versus lane 4). These data implicate HMGA2 as a major mediator of miR-33a-directed impediment of lung cancer cell migration.

DISCUSSION

The study of Winslow et al. reported that TTF-1-dependent suppression of HMGA2 is critical to the antimetastatic function...
MicroRNAs Regulated by TTF-1

The TTF-1 \(\rightarrow\) miR-33a \(\rightarrow\) HMGA2 signaling axis inhibits motility of lung cancer cells. A, the murine 394T4-shLuc (Ttf-1\(^{-\text{high}}\)) cells were transfected with a negative control oligonucleotide (NC) or an miR-33 inhibitor (anti-miR-33a) and allowed to migrate through uncoated transwell inserts for 22 h. Migrated cells were counted and normalized to negative control (\(n = 3\)). B, the murine 394T4-Ttf-1 cells stably expressing a transgene Hmga2 lacking 3' UTR were transfected with a scrambled control oligonucleotide (Scr Oligo) or an miR-33a mimic oligonucleotide (miR-33a mimic). A transwell migration assay was performed as in A. C, RT-QPCR analysis of HMGA2 mRNA expression of NCI-H1299 transfected cells. Human HMGA2 was stably expressed via retrovirus-mediated gene transfer. Subsequently, a scrambled control oligonucleotide or an miR-33a mimic oligonucleotide was transfected, and the RNA of total HMGA2 (endogenous plus exogenous) was quantified by RT-QPCR. D, the human NCI-H1299 cells stably expressing a transgene HMGA2 lacking 3' UTR were transfected with a scrambled control oligonucleotide or an miR-33a mimic oligonucleotide (miR-33a mimic). Transwell migration assay was performed as in A. Error bars, S.D. *p < 0.05; **p < 0.01; ***p < 0.001.

of TTF-1 (24). In that study, the potential involvement of miRNAs in Ttf-1-driven suppression of Hmga2 was focused on the let-7 family of miRNAs because of the known target/miRNA relationship between HMGA2 and let-7s (13–15). Using a reporter plasmid that reads out let-7 activity, the primary and metastatic Kras\(^{G12D}; p53^{KA}\) mouse lung cancer cells of different Ttf-1 expression status exhibited equivalent let-7 activity, suggesting that the let-7 miRNAs do not intervene in the Ttf-1-directed Hmga2 repression. However, considering the long 3' UTR (~3 kb) of HMGA2/Hmga2, we hypothesized that other miRNAs may be dispatched by Ttf-1 to repress Hmga2. Our earlier investigation identified the first miRNA (i.e., miR-365) that directly represses TTF-1 expression (27). This finding prompted us to initiate the present work to identify the miRNAs that are downstream to and directly regulated by TTF-1 using both loss- and gain-of-TTF-1-function strategies. A motivating factor was that the TTF-1-controlled miRNAs may target HMGA2, thus shedding mechanistic light on the TTF-1 \(\rightarrow\) HMGA2 signaling axis. Therefore, our initial reverse (loss-of-function) screen via a QPCR array was HMGA2 3' UTR-centric in that we compared the expression level of a series of miRNAs predicted to bind to HMGA2 3' UTR in the Ttf-1\(^{low}\) and Ttf-1\(^{high}\) mouse lung cancer cell lines created by Winslow et al. (24). For the forward (gain-of-function) miRNA screen, we employed a TTF-1-inducible human lung cell system in which a TTF-1-transgene is under dox control (29). In this system, we conducted an unbiased global screen for miRNAs whose expression was altered from the TTF-1\(^{off}\) to TTF-1\(^{on}\) state using a commercial microarray bearing probes to all known human, mouse, and rat miRNAs. Interestingly, hsa-miR-33a/mmu-miR-33 was scored in both types of screens as an miRNA positively regulated by TTF-1. Curiously, miR-32 was also identified in the global forward screen as an miRNA that is up-regulated by TTF-1. Indeed, by chromatin immunoprecipitation, TTF-1 was demonstrated to bind to the promoter region of the host genes of both miR-33a and miR-32. However, only miR-33a was validated as a genuine miRNA that targets Hmga2. Clearly, the functional consequences of the TTF-1 \(\rightarrow\) miR-32 regulation remain to be determined. Considering the documented expression alterations of miR-32 in lung cancer (downregulation (42)), multiple myeloma (up-regulation (43)), and prostate cancer (up-regulation (44)), it is likely that miR-32 may also relay functionally significant signaling from TTF-1 in lung cancer.

The regulation of HMGA2 is complex. For example, loss of the gastrointestinal transcription factor Hnf4a causes derepression of Hmga2 in the Ttf-1-negative murine lung tumors (26). The data presented in this study unequivocally establish that
TTF-1 relies on miR-33a to hold HMGA2 in check. This mode of restraining the HMGA2 oncogene in lung cells appears conserved from mice to humans per our observations. In our experimental systems, it appears that miR-33a represents an important mediator of TTF-1-induced HMGA2 repression in view of our “add-back” experiment (Fig. 6C) in which the endogenous Hmga2 suppression imposed by the exogenous human TTF-1 in the background of Ttf-1¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬‐
genes. Cancer Res. 71, 349–359
32. Ritchie, M. E., Silver, J., Oshlack, A., Holmes, M., Miyagama, D., Holloway, A., and Smyth, G. K. (2007) A comparison of background correction methods for two-colour microarrays. Bioinformatics 23, 2700–2707
33. Fusco, A., and Fedele, M. (2007) Roles of HMGA proteins in cancer. Nat. Rev. Cancer 7, 899–910
34. Grimson, A., Farah, K. K., Johnston, W. K., Garrett-Engle, P., Lim, L. P., and Bartel, D. P. (2007) MicroRNA targeting specificity in mammals. Determinants beyond seed pairing. Mol. Cell 27, 91–105
35. Reddel, R. R., Ke, Y., Gerwin, B. I., McMenamin, M. G., Lechner, J. F., Su, R. T., Brash, D. E., Park, J. B., Rhim, J. S., and Harris, C. C. (1988) Transformation of human bronchial epithelial cells by infection with SV40 or adenovirus-12 SV40 hybrid virus, or transfection via strontium phosphate coprecipitation with a plasmid containing SV40 early region genes. Cancer Res. 48, 1904–1909
36. Hsi, D. S., Acharya, C. R., Balakumaran, B. S., Riedel, R. F., Kim, M. K., Stevenson, M., Tuchman, S., Mukherjee, S., Barry, W., Dressman, H. K., Nevins, J. R., Powers, S., Mu, D., and Potti, A. (2009) Characterizing the developmental pathways TTF-1, NKX2-8, and PAX9 in lung cancer. Proc. Natl. Acad. Sci. U.S.A. 106, 5312–5317
37. Boggaram, V. (2009) Thyroid transcription factor-1 (TTF-1/Nkx2.1/TTF1) gene regulation in the lung. Clin. Sci. 116, 27–35
38. Damante, G., Fabbro, D., Pellizzari, L., Civitareale, D., Guazzi, S., Polycarpou-Schwartz, M., Caucci, S., Quadriglioglio, F., Formisano, S., and Di Lauro, R. (1994) Sequence-specific DNA recognition by the thyroid transcription factor-1 homodomain. Nucleic Acids Res. 22, 3075–3083
39. Saito, R. A., Watabe, T., Horiguchi, K., Kohyama, T., Saitoh, M., Nagase, T., and Miyazono, K. (2009) Thyroid transcription factor-1 inhibits transforming growth factor-β-mediated epithelial-to-mesenchymal transition in lung adenocarcinoma cells. Cancer Res. 69, 2783–2791
40. Guazzi, S., Price, M., De Felice, M., Damante, G., Mattei, M. G., and Di Lauro, R. (1990) Thyroid nuclear factor 1 (TTF-1) contains a homeodomain and displays a novel DNA binding specificity. EMBO J. 9, 3631–3639
41. Saetrom, P., Heale, B. S., Snoe, O. J., Aagaard, L., Alluin, I., and Rossi, J. J. (2007) Distance constraints between microRNA target sites dictate efficacy and cooperativity. Nucleic Acids Res. 35, 2333–2342
42. Yanahara, N., Caplen, N., Bowman, E., Seike, M., Kumaizoto, K., Yi, M., Stephens, R. M., Okamoto, A., Yokota, J., Tanaka, T., Calin, G. A., Liu, C. G., Croce, C. M., and Harris, C. C. (2008) Unique microRNA molecular profiles in lung cancer diagnosis and prognosis. Cancer Cell 19, 189–198
43. Pichiorri, F., Suh, S. S., Ladetto, M., Kuehl, M., Palumbo, T., Drandi, D., Taccioli, C., Zanesi, N., Alder, H., Hagan, J. P., Munker, R., Volinia, S., Boccadoro, M., Garzon, R., Palumbo, A., Aqeilan, R. I., and Croce, C. M. (2008) MicroRNAs regulate critical genes associated with multiple myeloma pathogenesis. Proc. Natl. Acad. Sci. U.S.A. 105, 12885–12890
44. Ambis, S., Prueitt, R. L., Yi, M., Hudson, R. S., Howe, T. M., Petrocca, F., Wallace, T. A., Liu, C. G., Volinia, S., Calin, G. A., Yantis, H. G., Stephens, R. M., and Croce, C. M. (2008) Genomic profiling of microRNA and messenger RNA expression reveals deregulated microRNA expression in prostate cancer. Cancer Res. 68, 6162–6170
45. Antonica, F., Kasprzyk, D. F., Optiz, R., Iacovino, M., Liao, X. H., Dumitrescu, A. M., Refetoff, S., Peremans, K., Manto, M., Kyba, M., and Costa-glion, S. (2012) Generation of functional thyroid from embryonic stem cells. Nature 491, 66–71
46. Kimura, S., Hara, Y., Pineau, T., Fernandez-Salguero, P., Fox, C. H., Ward, M. A., and Peter, M. E. (2007) Let-7 expression defines two alternative genetic pathways in TGF-β signaling in mesenchymal progenitor cells. Cancer Res. 67, 3140–3147
47. Manfredi, J. J. (2010) The Mdm2-p53 relationship evolves. Mdm2 swings both ways as an oncogene and a tumor suppressor. Genes Dev. 24, 1580–1589
48. Radtke, F., and Raj, K. (2003) The role of Notch in tumorigenesis. Oncogene or tumour suppressor? Nat. Rev. Cancer 3, 756–767
49. Yang, L., Han, Y., Suarez Saiz, F., and Minden, M. D. (2007) A tumor suppressor and oncogene. The WTI story. Leukemia 21, 868–876
50. Liu, H., Radisky, D. C., Nelson, C. M., Zhang, H., Hata, J. E., Roth, A. A., and Bissell, M. J. (2006) Mechanism of Akt1 inhibition of breast cancer cell invasion reveals a protumorigenic role for TSC2. Proc. Natl. Acad. Sci.
51. Liu, H., Radisky, D. C., Yang, D., Xu, R., Radisky, E. S., Bissell, M. J., and Bishop, J. M. (2012) MYC suppresses cancer metastasis by direct transcriptional silencing of $\alpha_5$ and $\beta_1$ integrin subunits. *Nat. Cell Biol.* **14**, 567–574

52. Hosono, Y., Yamaguchi, T., Mizutani, E., Yanagisawa, K., Arima, C., Tomida, S., Shimada, Y., Hiraoka, M., Kato, S., Yokoi, K., Suzuki, M., and Takahashi, T. (2012) MYBP, a transcriptional target of TTF-1, inhibits ROCK1, and reduces cell motility and metastasis. *EMBO J.* **31**, 481–493

53. Moore, K. J., Rayner, K. J., Suárez, Y., and Fernández-Hernando, C. (2011) The role of microRNAs in cholesterol efflux and hepatic lipid metabolism. *Annu. Rev. Nutr.* **31**, 49–63

54. Rottiers, V., and Naár, A. M. (2012) MicroRNAs in metabolism and metabolic disorders. *Nat. Rev. Mol. Cell Biol.* **13**, 239–250

55. Brown, M. S., and Goldstein, J. L. (1997) The SREBP pathway. Regulation of cholesterol metabolism by proteolysis of a membrane-bound transcription factor. *Cell* **89**, 331–340

56. Horton, J. D., Goldstein, J. L., and Brown, M. S. (2002) SREBPs. Activators of the complete program of cholesterol and fatty acid synthesis in the liver.

**MicroRNAs Regulated by TTF-1**

57. Smith, B., and Land, H. (2012) Anticancer activity of the cholesterol exporter ABCA1 gene. *Cell Rep.* **2**, 580–590

58. Thomas, M., Lange-Grünweller, K., Weirauch, U., Gutsch, D., Aigner, A., Grünweller, A., and Hartmann, R. K. (2012) The proto-oncogene Pim-1 is a target of miR-33a. *Oncogene* **31**, 918–928

59. Ibrahim, A. F., Weirauch, U., Thomas, M., Grünweller, A., Hartmann, R. K., and Aigner, A. (2011) MicroRNA replacement therapy for miR-145 and miR-33a is efficacious in a model of colon carcinoma. *Cancer Res.* **71**, 5214–5224

60. Cirera-Salinas, D., Pauta, M., Allen, R. M., Salerno, A. G., Ramírez, C. M., Chamorro-Jorganes, A., Wanschel, A. C., Lasuncion, M. A., Morales-Ruiz, M., Suarez, Y., Baldan, A., Espugues, E., and Fernández-Hernando, C. (2012) Mir-33 regulates cell proliferation and cell cycle progression. *Cell Cycle* **11**, 922–933

61. Kuo, P. L., Liao, S. H., Hung, J. Y., Huang, M. S., and Hsu, Y. L. (2013) MicroRNA-33a functions as a bone metastasis suppressor in lung cancer by targeting parathyroid hormone related protein. *Biochim. Biophys. Acta* **1830**, 3756–3766