miR-375 is activated by ASH1 and inhibits YAP1 in a lineage dependent manner in lung cancer.

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Running title: Lineage-dependent ASH1-miR-375-YAP1 regulation

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Abstract

Lung cancers with neuroendocrine (NE) features are often very aggressive but the underlying molecular mechanisms remain elusive. The transcription factor ASH1/ASCL1 is a master regulator of pulmonary NE cell development that is involved in the pathogenesis of lung cancers with NE features (NE-lung cancers). Here we report the definition of the microRNA miR-375 as a key downstream effector of ASH1 function in NE-lung cancer cells. miR-375 was markedly induced by ASH1 in lung cancer cells where it was sufficient to induce NE differentiation. miR-375 upregulation was a prerequisite for ASH1-mediated induction of NE features. The transcriptional co-activator YAP1 was determined to be a direct target of miR-375. YAP1 showed a negative correlation with miR-375 in a panel of lung cancer cell lines and growth inhibitory activities in NE-lung cancer cells. Our results elucidate an ASH1 effector axis in NE-lung cancers that is functionally pivotal in controlling NE features and the alleviation from YAP1-mediated growth inhibition.
Introduction

Lung cancer has long been the leading cause of cancer-related death in economically developed countries, and a better understanding of the molecular pathogenesis of this fatal disease is greatly anticipated for preventive and/or therapeutic breakthroughs (1). Accumulated evidence strongly suggests that alterations of miRNA expressions are involved in the development of human cancers (2-5). Our previous studies identified let-7 as a miRNA family with growth inhibitory activities, which were also found to be frequently down-regulated in lung cancers in association with poor prognosis (6). In marked contrast to the tumor suppressor-like let-7 miRNA family, the miR-17-92 miRNA cluster plays roles as oncogene-type miRNAs in the development of lung cancers (7, 8).

Lung cancer is classified into 2 major classes, small cell lung cancer (SCLC) and non-SCLC (NSCLC), of which SCLC characteristically exhibits neuroendocrine (NE) features and an aggressive clinical course. In addition, a small proportion of NSCLCs such as large cell NE carcinoma (LCNEC) also share such characteristics. Therefore, it is conceivable that elucidation of the underlying mechanisms involved in the acquisition of those characteristics in lung cancers with NE features may provide important clues for a better understanding of carcinogenic processes. Along this line, we previously reported that A549 lung adenocarcinoma cells exhibited NE properties when introduced with achaete-scute homologue 1 (ASH1/ASCL1), a proneural basic helix-loop-helix (bHLH) transcription factor (9), while ASH1 knockdown elicited prominent apoptosis in SCLC lung cancer cell lines (10). We also found that ASH1 mediates lineage-survival signal in SCLC at least in part through its transcriptional repressor activity toward putative tumor suppressor genes including DKK1 and E-cadherin (9). However, to date, virtually nothing is known about the possible involvement of miRNAs downstream of this dual function transcription factor, which is crucially involved in the biology of SCLC.

In this study, we investigated whether miRNAs are also governed by ASH1 and have roles downstream of ASH1 downstream in the development of lung cancers with NE features.
Consequently, miR-375 was identified as a miRNA directly and highly transactivated by ASH1. The involvement of miR-375 in acquisition of NE phenotypes and growth regulation in lung cancers with NE features is also discussed.

Materials and Methods

Cells and expression constructs. An A549 lung adenocarcinoma cell line without NE differentiation and a typical SCLC cell line, ACC-LC-172, as well as A549 cells stably transduced with ASH1-expressing (A549-ASH1) or empty (A549-VC) lentiviruses (9) were maintained in RPMI-1640 with 5% fetal bovine serum. ASH1-expressing lentiviral and plasmid vectors were constructed with CSII-CMV-MCS-IRE2-Blasticidin and pcDNA3 (Invitrogen), respectively, as previously described (9). YAP1 cDNA was purchased from OriGene (Rockville, MD) and inserted into CSII-EF-MCS-IRE2-Venus. The lentivirus vectors were kindly provided by Dr. H. Miyoshi (RIKEN BioResource Center). Venus (improved YFP) was provided by Dr. A. Miyawaki (RIKEN Brain Science Institute).

Reporter assay. A 1028bp-long putative promoter fragment spanning from 992bp upstream of the pre-miR-375 sequence to 3bp upstream of the mature miR-375 sequence was amplified from human genomic DNA and cloned into a pGL4.10 basic reporter (pGL4-375P in Fig. 1B). pGL4-Δ1, Δ2, and Δ3 truncated reporter plasmids were constructed by re-amplification of the pGL4-375P plasmid, with each containing 103bp, 324bp and 681bp regions 5’ to the pre-miR-375 sequence (Fig. 1B and Supplementary Fig. S1B). E-box-deletion mutant reporters were also constructed by PCR-mediated in vitro mutagenesis of pGL4-Δ3. Each of these miR-375 promoter reporters was transfected into A549 cells using Lipofectamine 2000 (Invitrogen) together with an ASH1-expression vector, pcDNA3-ASH1, or control empty vector, pcDNA3, with the renilla luciferase reporter pRL-TK used as an internal control.

microRNA microarray and gene expression microarray analysis. Microarray analysis was
conducted to examine miRNA expression profiles using a Human miRNA Microarray, pre-commercial version 6.0 (Agilent) with 470 miRNA probes, according to the manufacturer’s instructions. A549 cells were infected with an ASH1-expressing or empty lentivirus and harvested 4 days later. miRNA microarray data were log2-transformed and normalized to the 75th percentile. Microarray analysis using a Whole Human Genome 4 x 44K Microarray G4112F (Agilent) was also conducted to examine changes in expression of potential target genes of miR-375 by transfection of Pre-miR-375 or Pre-miR-NC#2 (Ambion) in A549 cells, which were then harvested at 12, 24, 48, and 96 hours after transfection. RNA samples were prepared using an RNeasy kit (Qiagen) as previously described.

**Quantitative RT-PCR.** Quantitative RT-PCR analysis was performed using primers for chromogranin A (CHGA), chromogranin B (CHGB), secretogranin II (SCG2), secretogranin III (SCG3), ASH1, YAP1, and β-actin (Supplementary Table S1), along with Power SYBR Green PCR Master Mix (Applied Biosystems), and an ABI Prism7500 (Applied Biosystems), as previously described. Expression levels were calculated using the standard curve method and normalized with the expression of β-actin. The expression of miR-375 was determined by quantitative RT-PCR analysis using a TaqMan MicroRNA Assay and TaqMan MicroRNA RT Kit (Applied Biosystems). The expression level of miR-375 was normalized with that of the non-coding RNA RNU48.

**Quantitative ChIP assay.** ChIP assays were performed as described previously, using ChIP E1/2 and ChIP E4/5 primers, which were designed for amplification of genomic fragments containing E1 and E2 E-boxes and E4 and E5 E-boxes, respectively (Fig. 1B). ChIP analysis using “distant” primers for amplification of a genomic region about 5.3kb downstream of pre-miR-375 (Supplementary Fig. S1) as well as CHGB primers were used as negative and positive controls, respectively. The primer sequences are shown in Supplementary Table S1. The amounts of chromatin immunoprecipitated genomic DNA were measured using the ΔΔCt method to compare various ChIP primers and β-actin primers and the results of quantification were obtained as fold changes of A549-ASH1 against
A549-VC. Antibodies against acetylated histone H3 (H3Ac), tri-methylated H3 lysine 4 (H3K4me3), and tri-methylated H3 lysine 27 (H3K27me3) were purchased from Upstate.

**In situ hybridization.** We employed an FITC-labeled LNA probe for mmu-miR-375 and a scrambled sequence (Exiqon). Probes were diluted to 40 nM in hybridization buffer (Ambion). In situ hybridization was performed according to the manufacturer’s protocol, as previously described (11). In brief, after deparaffinization, neutral formalin-fixed specimens on slides were incubated in proteinase K solution (20 μg/ml) at 37°C. After fixing the specimens with 4% paraformaldehyde, endogenous peroxidase activities were quenched in methanol containing H2O2 [0.3 % (v/v)], then the probes were hybridized overnight at 37°C. After washing with SSC with 50% formamide, a CSA II biotin-free catalyzed signal amplification system (DAKO) was used to visualize miRNA expression as brown precipitates. Nuclear staining was done with hematoxylin.

**Immunohistochemical analysis.** Slides were subjected to an antigen retrieval procedure using Immunosaver (Nisshin EM) and then endogenous peroxidase activities were quenched. Next, the slides were incubated with rabbit polyclonal anti-synaptophysin antibody (Dako) followed by goat anti-rabbit immunoglobulins/HRP (Dako), and then visualized with liquid DAB (Dako). Nuclear counterstaining was done with hematoxylin.

**Transfection of Pre-miR-375 and LNA.** Both Pre-miR-375 and Pre-miR-NC#2 were purchased from Ambion. Antisense and scramble oligonucleotides against mature miR-375 were synthesized using locked nucleic acids (LNAs)(Greiner). Each of oligonucleotides was introduced into A549 cells at 10~15 nM using 2.5 μl/ml of Lipofectamine RNAiMax (Invitrogen) according to the instructions of supplier.

**Results**

This study was initiated to investigate the potential involvement of miRNAs downstream of ASH1 in acquisition of characteristics of lung cancers with NE features. To this end, we first performed
genome-wide expression profiling of miRNAs in order to search for those significantly affected by ASH1 transduction in a lung cancer cell line without NE features. As a result, we identified twelve up-regulated (>5-fold) and eight down-regulated (>5-fold) miRNAs in ASH1-transduced A549 cells (Table 1), of which miR-375 was found to be the most highly up-regulated, which was also verified by quantitative RT-PCR using an miR-375-specific TaqMan probe (Fig. 1A). An ASH1-lentivirus was also transduced into four other NSCLC cell lines, three of which showed marked miR-375 induction (Supplementary Fig. S2A). A survey of the genomic region harboring miR-375 indicated that this miRNA resides in an intergenic region between the CCDC108 and CRVBA2 genes at chromosome 2q35 (Supplementary Fig. S1A), while a region about 1kb in length was found to be evolutionally highly conserved (shown in red). Four of the 5 conserved E-boxes were CACCTG, while the other (E2) was CATCTG. To verify the promoter activity and responsiveness to ASH1, luciferase reporter constructs of the putative miR-375 promoter and its various mutants were co-transfected with an ASH1-expression vector, pcDNA3-ASH1, or an empty vector into A549 cells. The pGL4-375P showed marked transactivation by ASH1 (Fig. 1B). pGL4-Δ3 containing 3 E-boxes (E1 to E3) showed robust responsiveness to ASH1, whereas pGL4-Δ2 with a further deletion failed to respond. Reporters, each of which contained a single E-box deletion mutation (pGL4-ΔE1, ΔE 2, and ΔE 3), demonstrated moderate reductions in ASH1 responsiveness, while pGL4-ΔE123, carrying a deletion of all 3 E-boxes (E1 to E3), lost responsiveness to a level similar to that of pGL4-Δ2, indicating their crucial involvement in ASH1 responsiveness. To further confirm the promoter activity of this region, ChIP assays with antibodies against various histone H3 modifications were performed using A549 cells infected with either ASH1-carrying or empty viruses (Fig. 1C). Consequently, specific induction of activating histone modifications (H3Ac and H3K4me3) in the genomic regions encompassing these 3 E-boxes were clearly shown in ASH1-expressing A549 cells. In addition, a ChIP assay with an anti-myc-tag antibody against the myc-tagged ASH1 protein indicated a direct interaction of ASH1 with the E1/2 region (Supplementary Fig. S3).
The association between ASH1 and miR-375 expression was then analyzed in fetal mouse lung (Fig. 2A). While neuroepithelial bodies (NEBs), known to consist of ASH1-expressing pulmonary NE cells and epithelial progenitor cells(12), showed positive immunohistochemical staining for the NE marker synaptophysin, in situ hybridization demonstrated co-expression of miR-375, showing that ASH1-miR-375 signal is associated with NE differentiation. We also noted that miR-375 was also detectable in pancreatic islet cells despite a lack of ASH1 expression, suggesting other mechanisms for its induction in the pancreas (Supplementary Fig. S4A). We observed histological type-dependent expression of miR-375 in the present lung cancer cell lines (Fig. 2B). A high level of expression was specifically detected in SCLC cell lines, which generally express ASH1 and have NE features (Fig. 2B). A moderate expression of miR-375 was observed in large cell carcinoma cell lines, while miR-375 was detected in a few exceptional adenocarcinoma cell lines, but in none of the squamous carcinoma cell lines. In addition, a positive correlation was seen between miR-375 and ASH1 expression in the lung cancer cell lines (data not shown). Our in situ hybridization analysis revealed a positive signal for miR-375 in the ACC-LC-172 SCLC cell line, while A549 did not show any signals (Supplementary Fig. S4B & S4C).

We next investigated the functional significance of ASH1-inducible miR-375 in terms of biologic phenotypes of ASH1-positive lung cancer cells. To this end, we conducted genome-wide expression profiling analysis of miR-375-transfected A549 cells (Fig. 3A). Two hundred fifty-three genes exhibited greater than 2-fold changes in their expression levels between 12 and 96 hours after transfection. Interestingly, we noted that multiple NE-related genes were gradually induced at 48 and 96 hours after miR-375 introduction, which was also confirmed by quantitative RT-PCR analysis results (Fig. 3B). Since this finding strongly suggests that miR-375 alone is capable of inducing NE markers in the absence of ASH1, we then investigated whether miR-375 is required for NE-marker induction by ASH1. A549 cells were first infected with an ASH1-expressing lentivirus and subsequently transfected with miR-375 antisense or scramble LNAs, which resulted in marked
inhibition of ASH1-mediated induction of various NE markers in the presence of miR-375 antisense, but not negative control, miR-375 scramble (Fig. 3C). These findings clearly demonstrated that ASH1-inducible miR-375 is required for NE-marker induction by ASH1 in lung cancer cells. To verify the specificity of NE-marker induction by miR-375, we also transfected unrelated miRNAs into A549 cells. As shown in Supplementary Fig. S2B, various unrelated miRNAs scarcely induced CHGB expression, suggesting the specificity of miR-375-mediated NE-marker induction. In addition, miR-375 was transfected into two other NSCLC cell lines and two immortalized normal lung epithelial cell lines, HPL1D and BEAS2B, which confirmed CHGB induction at varying degrees in all four cell lines (Supplementary Fig. S2C).

To study the direct effects of miR-375, we analyzed changes in the expression profiles of target genes for miR-375 predicted with TargetScan4.1 (http://www.targetscan.org/) and observed leftward shifts of the expression profile histograms, especially at 24 and 48 hours after miR-375 transfection (Fig. 4A), which indicated moderate but significant down-regulation of the predicted target genes of miR-375. In contrast, histograms of genes residing in chromosome 1 did not show any shifts, confirming specificity. Among the potential target genes affected by miR-375, transcriptional co-activator YAP1 was the most significantly repressed after miR-375 transfection (Fig. 4B), which was also confirmed by western blotting analysis (Fig. 4C). Two potential miR-375-binding sites were also noted within the 3'-UTR of YAP1 mRNA, thus we performed a luciferase assay using YAP1 3'-UTR reporters (miR-375x1 and miR-375x2) containing either 1 or 2 potential miR-375 binding sites (Fig. 4D). A549 cells transfected with these reporter constructs along with either Pre-miR-375 or negative control Pre-miR-NC#2 showed significant suppression of luciferase activity in a target site-dependent manner. The YAP1 3’-UTR reporter with deletion of the potential miR-375 binding site abrogated miR-375-mediated suppression of luciferase activity (Supplementary Fig. S5A). The specificity of the miR-375 target sites was also supported by our findings of lack of suppression of the wild-type YAP1 3’-UTR reporter activity by various unrelated miRNAs (Supplementary Fig.
S5B). The relationship of miR-375 with YAP1 was also substantiated by the significant negative correlation (R = 0.793, p<0.0001) between miR-375 and YAP1 in a panel of 33 lung cancer cell lines and 3 immortalized normal airway epithelial cell lines (Fig. 4E, left). In addition, we observed a histologic type-dependent expression pattern with low YAP1 expression in SCLC and abundant expression in NSCLC cell lines, indicating an expression pattern opposite to that of ASH1, which has abundant expression in SCLC (Fig. 4E, right). These relationships among ASH1, miR-375, and YAP1 were also observed in primary lung cancer specimens (Fig. 4F), suggesting the existence of robust regulatory relationships in the ASH1-miR-375-YAP1 pathway in lung cancers with NE features.

The negative correlation between miR-375 and YAP1 found in a histologic type-related manner prompted us to investigate YAP1 functions in lung cancer cells of both histologic types. A549 adenocarcinoma and ACC-LC-172 SCLC cell lines were infected with YAP1-expressing or an empty lentivirus expressing the fluorescent protein Venus from an internal ribosomal entry site. Fluorescent microscopic examination revealed marked reduction of the fluorescence-positive population, which was indicative of successful infection by the YAP1-expressing virus, in contrast to robust growth in fluorescence-negative uninfected ACC-LC-172 cells (Fig. 5A), which we also confirmed by FACS analysis (Fig. 5B). In contrast to ACC-LC-172, A549 cells appeared to be tolerant to the introduction of YAP1, since the fluorescence-positive YAP1-infected population gradually increased (Fig. 5B). Time-courses of fluorescent signals in lentivirus-infected A549 and ACC-LC172 cells are shown in Supplementary Fig. S6A. YAP1-virus infection significantly inhibited the increase of fluorescent signals in ACC-LC-172 cells, but not in A549, suggesting a lineage-dependent growth suppressive effect of YAP1. These findings were also confirmed by western blotting analysis using lysates harvested on day 9, which showed scarcely detectable HA-tagged exogenous YAP1 protein expression in ACC-LC-172 as opposed to the abundant exogenous YAP1 expression in A549 cells (Fig. 5C). Cleavage of caspase-7 and caspase-3 was detected in YAP1-introduced ACC-LC-172 cells (Fig. 5D), while propidium iodide-stained dead
cells were frequently observed in YAP1-introduced fluorescence-positive cells in ACC-LC-172 (Supplementary Fig. S6B), but not in A549 (data not shown). YAP1 introduction also resulted in growth suppression in two other SCLC cell lines, NCI-H69 and ACC-LC-48 (Supplementary Fig. S7), which confirmed the results observed in ACC-LC-172.

**Discussion**

The present results clearly show that ASH1 directly transactivates *miR-375*, resulting in an NE lineage-specific up-regulation of *miR-375* in lung cancers. Although NeuroD1 and Pdx1 potentially bind to the E-box in the *miR-375* promoter (13), their expression was rarely detected and showed no correlations with *miR-375* expression (data not shown). Therefore, we believe that ASH1 plays a major role as a transcriptional activator of *miR-375*. Our finding of NEB-specific expression of *miR-375* also supports the existence of an ASH1-*miR-375* signaling axis in the lung. It is important to note that *miR-375* was recently reported to be detectable in pancreatic islet cells under the regulation of NeuroD1 and Pdx1, as well as in pituitary and adrenal glands (13-16). Herein, we clearly demonstrated the functional importance of three E-boxes (E1, E2, and E3) in ASH1-mediated induction of the promoter activity of *miR-375*. Furthermore, a recent report described the promoter activity of a similar genomic region 5’ to murine *miR-375* in a β-cell line, though its responsiveness to potential activators such as NeuroD1 and ASH1 was not examined (17).

Down-regulation of *miR-375* has been reported in a few other types of cancer (18-20). Interestingly, *miR-375* was suggested to play tumor suppressor roles in those cancer types, while target genes for *miR-375* thus far reported include PDK1, 14-3-3ζ (19), HuD (21), and JAK2 (22). Also, hepatocellular carcinoma was recently added to the list of cancers with *miR-375* down-regulation and YAP1 has been suggested to be a target gene relieved by that down-regulation. Consistent with those findings, we observed moderate down-regulation of PDK1, 14-3-3ζ, HuD and JAK2 when *miR-375* was introduced to A549 cells, though YAP1 showed the most significant
down-regulation in our experimental settings (Supplementary Fig. S8B). In addition, we did not observe clear growth inhibition of A549 cells stably introduced with a lentivirus expressing \textit{miR-375} (Supplementary Fig. S8A), even though YAP1 was effectively down-regulated by \textit{miR-375} (Fig. 4B \textit{~} 4D). Along this line, it is notable that \textit{miR-375} knockout mice were shown to be hyperglycemic in association with decreased β-cell mass as a result of impaired proliferation of β-cells (15) and that \textit{miR-375} was demonstrated to regulate a number of genes other than YAP1, which potentially control cellular growth and proliferation of pancreatic islets (15). It is also interesting that estrogen receptor α (ERα)-expressing breast cancers showed ERα-signal dependency and a high expression of \textit{miR-375} (23). ERα binds the \textit{miR-375} promoter and induces its expression. \textit{miR-375} in turn represses the dexamethasone-induced RAS1 (RASD1) gene, which negatively regulates ERα expression, suggesting the existence of a positive feedback loop between ERα and \textit{miR-375}, as well as a growth-promoting role of \textit{miR-375} in ERα-positive breast cancers (23). Taken together, it is conceivable that \textit{miR-375} plays distinct roles in various cancers, depending on the cellular context and transcriptomes including its potential target genes, and that down-regulation of YAP1 by ASH-1-transactivated \textit{miR-375} promotes rather than inhibits growth of SCLC cells.

YAP1 has several domains including a TEAD-binding region and two WW domains, while it lacks a DNA-binding domain and functions as a transcriptional co-activator through interactions with DNA-binding transcription factors (24-26). YAP1 interacts with the TEAD family through a TEAD-binding domain, and transactivates growth-promoting genes, while it also binds to PPXY motif-containing molecules including p73 through WW-domains, thus enhancing p73-dependent apoptosis in response to DNA damage (27-29). It has been reported that phosphorylation by AKT or repression by ΔNp63 down-regulates the proapoptotic activity of YAP1 (30), and that PML is also involved in regulation of p73-YAP apoptotic signaling through sumoylation and stabilization of YAP (31). Therefore, accumulating evidence enhances the notion that YAP1 exerts both oncogenic and tumor-suppressive activities in a context-dependent manner (32, 33). The present findings show that
YAP1 moderately promotes NSCLC proliferation when overexpressed, whereas it significantly suppresses SCLC growth, suggesting its lineage-dependent dual roles in lung cancers.

In conclusion, we identified miR-375 as a direct transcriptional target for ASH1 and showed that it has a crucial role for mediating signals required for ASH1-mediated induction of NE features in lung cancers. In addition, the present findings indicate that miR-375 directly down-regulates YAP1, while we also found that it demonstrates NE lineage-specific growth inhibitory activities in lung cancers. A future study of the downstream genes of the ASH1-miR-375 axis will be of great interest in order to fully elucidate the underlying signaling networks involved in NE differentiation and highly malignant behaviors of NE lung cancers including SCLC.

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References


Genome Biol. 2007;8:R173.


Table 1. Up- and down-regulated miRNAs in ASH1-transduced A549 cells

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<th>A549-VC#</th>
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# normalized signal intensity.
Figure legends

Figure 1. Characterization of the putative miR-375 promoter.

(A) Induction of miR-375 by ASH1. The expression of miRNAs in ASH1-infected A549 were determined using microRNA quantitative RT-PCR analyses.

(B) Reporter assay. Putative miR-375 promoter reporters were transfected into A549 cells together with an ASH1-expression vector, pcDNA3-ASH1, or a control empty vector, pcDNA3. The reporter plasmids pGL4-Δ1, Δ2, and Δ3 contain promoter fragments that are -103bp, -324bp, and -681bp upstream from the start site of pre-miR-375, as indicated in Supplementary Fig. S1B. Deletions of conserved E-boxes (E1 ~E5) are indicated by "x" marks.

(C) Quantitative ChIP assay. ChIP assays with antibodies against various histone H3 modifications were performed using A549-ASH1 and ASH1-VC cells. PCR products of the ChIP E1/E2 and ChIP E4/E5 regions, indicated by arrows in (B), were measured. The ratios of A549-ASH1 cells against A549-VC cells are demonstrated as fold changes. PCR findings for both ChIP E1/E2 and ChIP E4/E5-demonstrated increases in activation of histone modifications (H3Ac and H3K4me3) in the miR-375 promoter, while distant primers did not show any activating histone modifications. CHGB, chromogranin B.

Figure 2. miR-375 expression in normal lung and lung cancers. (A) In vivo expression of miR-375 in neuroepithelial bodies (NEBs) from fetal mouse lung tissue. (a) Hematoxylin - Eosin (HE) staining
of normal fetal lung tissue containing 2 NEBs. The area with 2 NEBs enclosed by a red line is shown magnified in (a-d). [Inset of (a): 4x magnification. Black bar= 50 μm. (a)–(d): 40x magnification. Black bar= 10 μm]. (b) IHC staining with antibody against the neuroendocrine marker, synaptophysin. (c,d) In situ hybridization with a miR-375 antisense oligo (c) and scramble control oligo (d). Combinational analyses with IHC and in situ hybridization results demonstrated restricted expressions of synaptophysin, and miR-375 in NEBs.

(B) Expression levels of miR-375 in lung cancer cell lines. NL; normal lung, AD; adenocarcinoma, SQ; squamous cell carcinoma, LA; large cell carcinoma, SCLC; small cell lung cancer. The SCLC cell lines showed significantly elevated expression of miR-375, whereas only moderate expression was observed in most of the LA cells. Only a few AD cell lines showed the expression of miR-375. Over-expression of miR-375 in SCLC was statistically significant as compared with that expression in lung cancers of other histological types.

Figure 3. Gene expression profiling and NE induction after Pre-miR-375 transfection.

(A) The expression ratio of each gene of Pre-miR-375 transfected cells to those of Pre-miR-NC#2 transfected cells was analyzed using clustering. Time-course (12 ~ 96 hours) of gene expression profiles after Pre-miR-375 transfection. Two hundred fifty-three genes showed greater than 2-fold changes in expression level. Genes related to NE features were indicated. SYT1, synaptotagmin I;
STMN3, stathmin-like 3; HEY1, hairy/enhancer-of-split related with YRPW motif 1; SCG3, secretogranin III.

(B) Induction of NE markers by miR-375. The expressions of NE markers were measured using qRT-PCR. A549 cells transfected with Pre-miR-375 showed strong induction of NE markers as well as A549-ASH1 cells.

(C) Inhibition of NE markers by miR-375 anti-sense LN. First, infection was performed with an ASH1-expressing or empty lentivirus, then miR-375 antisense or scramble LNA was transfected into A549 cells. Strong inductions of NE markers by ASH1 were significantly inhibited by miR-375 antisense-LNA. AS, antisense; SC, scramble.

Figure 4. YAP1 inhibition by miR-375.

(A) Histogram of gene expressions at 4 time points after Pre-miR-375 transfection. Gene expressions at 12–96 hours after transfection are shown as log₂ ratio values between A549 cells transfected with Pre-miR-375 and Pre-miR-NC#2. Upper panel shows histogram of miR-375 predicted target genes. Lower panel shows a histogram of genes residing in chromosome 1, which was used as a control.

(B) Time-course of expression of each miR-375 predicted target gene after Pre-miR-375 transfection.

(C) Western blotting analysis of YAP1 protein. A549 cells transfected with Pre-miR-RNAs (15nM) were analyzed using Western blotting with the antibody against endogenous YAP1 protein. The intensity of YAP1 bands was determined with a densitometer and normalized with lamin B bands.
Pre-miR-375 transfection down-regulated the level of YAP1 protein by 58%. This down-regulation is demonstrated with a bar graph, in which the extent of down-regulation is also numerically indicated.

(D) YAP1 3’-UTR reporter assay. YAP1 mRNA contains 2 miR-375 target sites at the 3’-UTR. Two reporter constructs, miR-375x1 and miR-375x2, carry 1 and 2 miR-375 sites, respectively. A549 cells were transfected with reporter constructs and Pre-miR-RNAs (10nM). The ratio of luciferase activity of Pre-miR-375 transfectants to that of Pre-miR-NC#2 transfectants is shown. miR-375 significantly suppressed luciferase activity in a target site-dependent manner. The alignment of two miR-375 target sites with mature miR-375 is also shown. The nucleotide positions in 3’-UTR are indicated.

(E) Negative correlations of miR-375 and YAP1 expressions in lung cancer cell lines. Left panel, YAP1 and miR-375 expressions showed significant negative correlations among the tested lung cancer cell lines. Right panel, YAP1 was frequently over-expressed in AD and SQ cells, whereas its expression was strongly suppressed in SCLC. In contrast, most SCLC showed over-expression of miR-375, whereas miR-375 was scarcely expressed in the AD and SQ cell lines. (F) Correlations of ASH1, miR-375, and YAP1 expression levels in primary lung cancer specimens. ASH1 and miR-375 expressions were positively correlated, while inverse correlations were present between miR-375 and YAP1, as well as between ASH1 and YAP1.

**Figure 5.** Lineage-dependent growth-regulating effects of YAP1 in lung cancers.
(A) Fluorescent image of lentivirus-infected A549 and ACC-LC-172 obtained 9 days after infection. Cells were infected with a YAP1-expressing or empty lentivirus carrying the fluorescent protein Venus. YAP1- and empty-virus infection resulted in fluorescent signals in A549 cells. In contrast, fluorescence-positive cells were scarcely detected in ACC-LC-172 cells infected with the YAP1-virus, whereas non-infected cells grew well.

(B) Flowcytometry analysis of fluorescence-positive cells. Lentivirus-infected cells were analyzed at 3 and 9 days after infection. The fluorescence-positive population was moderately increased in the A549 cells infected with the YAP1-virus, whereas that population almost disappeared in ACC-LC-172 cells infected with the YAP1-virus.

(C) Western blotting analysis of YAP1. A549 abundantly expressed endogenous and exogenous (HA-tagged) YAP1. In contrast, ACC-LC-172 did not express endogenous YAP1. In addition, exogenous YAP1 was scarcely detected.

(D) Western blotting analysis of caspases. Five days after infection, activated cleavage of caspase-7 and caspase-3 was detected in YAP1-virus infected ACC-LC-172 cells. Normalized intensities of cleaved caspases are numerically indicated.
miR-375 is activated by ASH1 and inhibits YAP1 in a lineage dependent manner in lung cancer

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