Global microRNA depletion suppresses tumor angiogenesis

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Global microRNA depletion suppresses tumor angiogenesis

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MicroRNAs delicately regulate the balance of angiogenesis. Here we show that depletion of all microRNAs suppresses tumor angiogenesis. We generated microRNA-deficient tumors by knocking out Dicer1. These tumors are highly hypoxic but poorly vascularized, suggestive of deficient angiogenesis signaling. Expression profiling revealed that angiogenesis genes were significantly down-regulated as a result of the microRNA deficiency. Factor inhibiting hypoxia-inducible factor 1 (HIF-1), FIH1, is derepressed under these conditions and suppresses HIF transcription. Knocking out FIH1 using CRISPR/Cas9-mediated genome engineering reversed the phenotypes of microRNA-deficient cells in HIF transcriptional activity, VEGF production, tumor hypoxia, and tumor angiogenesis. Using multiplexed CRISPR/Cas9, we deleted regions in FIH1 untranslated regions (UTRs) that contain microRNA-binding sites, which derepresses FIH1 protein and represses hypoxia response. These data suggest that microRNAs promote tumor responses to hypoxia and angiogenesis by repressing FIH1.

Keywords: microRNA; Dicer; angiogenesis; hypoxia; CRISPR/Cas9; gene regulation

Supplemental material is available for this article.

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MicroRNAs are a class of 20- to 22-nucleotide (nt) small RNAs that regulate diverse biological processes (Bartel 2009). The majority of genes in the mammalian genome are regulated by one or more microRNAs [Friedman et al. 2009; Ebert and Sharp 2012]. Individual microRNAs and microRNA families have been reported to regulate multiple hallmarks of cancer, such as cell proliferation, apoptosis, metastasis, and angiogenesis, serving as oncogenes or tumor suppressor genes [He et al. 2005; Calin and Croce 2006].

MicroRNAs can serve as both positive and negative regulators of angiogenesis. For example, miR-15/16 and miR-221/222 suppress tumor-induced vasculature formation by targeting VEGF, c-kit, and eNOS mRNAs [Cimmino et al. 2005; Hua et al. 2006]. On the other hand, miR-17-92, let-7, and miR-210 positively regulate tumor angiogenesis by inhibiting genes encoding endogenous angiogenesis inhibitors [Kuehbacher et al. 2007; Fasanaro et al. 2008; Suarez et al. 2008; Suarez and Sessa 2009], suggesting that the angiogenic switch is delicately balanced by multiple families of microRNAs.

Here we report that global microRNA depletion breaks the balance of the angiogenic switch. MicroRNA-deficient tumors are highly hypoxic but poorly vascularized. The reduced angiogenic capacity in microRNA-deficient cancer cells is primarily mediated by derepression of FIH1 [factor inhibiting HIF-1 [hypoxia-inducible factor 1]], which inhibits HIF transcriptional activity.

Results

We set out to study tumor angiogenesis in a non-small-cell lung cancer [NSCLC] model driven by the KrasG12D oncogene accompanied by p53 loss [Kumar et al. 2009]. Because the maturation of the vast majority of microRNAs requires Dicer1 [Bernstein et al. 2003; Gurtan et al. 2012], we generated Dicer1 knockout cancer cells to deplete the global microRNA population (Fig. 1A). Northern blot showed that mature microRNAs, such as miR-21a, let-7g, and miR-125b, are abundant in Dicer1+/− cells but undetectable in Dicer1−/− cells [Fig. 1B]. We performed small
RNA sequencing (small RNA-seq) to capture microRNAs (thus also considered as microRNA-seq) and detected >100 mature microRNAs in Dicer1+/− cells, with miR-21a-5p as the most abundant, followed by miR-182-5p and let-7 family members (Fig. 1C,D; Supplemental Table S2A). Dicer1+/−/− cells have a >100-fold decrease in mature microRNA levels compared with Dicer1+/−/− cells, whereas the hairpin precursors (pre-microRNAs) are expressed at similar levels in both genotypes (Fig. 1C,D; Supplemental Table S2B). We therefore considered Dicer1+/−/− and Dicer1+/−/− NSCLC cells induced tumors when injected into immunocompromised mice. Immunohistochemistry (IHC) showed that cancer cells stained positive for Dicer protein in tumors induced by Dicer1+/−/− cells but not in those induced by Dicer1+/−/− cells, whereas tumor-associated host tissue stained positive (Fig. 1E). We then performed analyses of tumor hypoxia and angiogenesis in Dicer1+/−/− and Dicer1+/−/− tumors. Hypoxyscale [Raleigh et al. 1996; Varghese et al. 1976] staining showed that Dicer1+/−/− tumors have higher levels of hypoxia than Dicer1+/−/− (Fig. 2A). Because hypoxic regions represent tissues with low oxygen levels, where normal cells activate a hypoxia-inducible response to provoke tumor angiogenesis (Carmeliet and Jain 2000; Weinberg 2007; Konisti et al. 2012), we expected to see more active angiogenesis with higher hypoxia. Surprisingly, immunofluorescence (IF) staining of an endothelial cell-specific marker, Isolectin B4, showed that Dicer1+/−/− cells have significantly reduced tumor-associated vasculature (Fig. 2B). Quantitation of Isolectin B4 and Ki67 staining demonstrated that the proliferation of tumor-associated ECs was significantly decreased in Dicer1 knockouts (Fig. 2B). These data suggested that microRNA-deficient tumors have reduced angiogenesis despite being highly hypoxic.
To understand the underlying gene regulation of tumor angiogenesis in microRNA-deficient cells, we profiled the transcriptomes of microRNA-competent and microRNA-deficient cells using messenger RNA sequencing (mRNA-seq) and identified populations of differentially expressed genes (Fig. 3A; Supplemental Table S1). The activity of microRNA families was analyzed from the degrees of derepression of their predicted target genes upon Dicer1 loss (Fig. 3B; Supplemental Fig. S1b,c). Many microRNA families exhibited strong silencing activity, as their target genes showed significant up-regulation upon microRNA loss (Fig. 3B; Supplemental Fig. S1b,c). Globally, of the 153 conserved microRNA families, 43 showed significant activity in these cells (Fig. 3B; Supplemental Table S3). The most active in Dicer1+/− cells are miR-29, miR-202-3p, let-7/miR-98, miR-17, miR93/295, and miR-125 families [Fig. 3B]. The relative activities of microRNAs on the predicted target mRNA transcriptome significantly correlate with their abundance in microRNA-seq data (Supplemental Fig. S1a), suggesting that depletion of the highly abundant microRNAs led to significant derepression of their targets.

Pathway analysis showed that mRNA genes down-regulated upon microRNA depletion are highly enriched in angiogenesis functions, including gene sets with clustered functions such as angiogenesis, vasculature development, blood vessel morphogenesis, cardiovascular system development and function, cell migration, and migration of ECs [Fig. 3C; Supplemental Tables S4, S5]. These genes significantly overlap with genes regulated by the α subunit of HIF-1α (also known as HIF1A) [Fig. 3D]. MicroRNAs generally act as repressors of their target genes [Bartel 2009]; thus, genes down-regulated upon microRNA loss are likely due to indirect effects.

To investigate this indirect regulation of angiogenesis by microRNAs, we examined the expression of genes known to negatively regulate HIF. Among all known HIF antagonists, FIH1 (also known as Hif1an) is the most highly up-regulated in microRNA-deficient cells [Fig. 3E]. Consistently, FIH1 protein level is also highly up-regulated.
upon microRNA depletion [Figs. 4B, 5A,C]. FIH1 hydroxylates an asparagine residue of HIF-1α in its C-terminal transactivation domain (McNeill et al. 2002; Koivunen et al. 2004). This hydroxylation blocks the association of HIF with the transcriptional coactivators CBP/p300 and thus inhibits transcriptional activation (Mahon et al. 2001; Lando et al. 2002).

FIH1 is a microRNA target with an annotated 5116-base-pair (bp) 3' untranslated region (UTR) predicted to harbor 473 microRNA-binding sites, including five let-7 sites and four miR-125 sites (Supplemental Fig. S1d). These results led us to hypothesize that FIH1 derepression might be responsible for the lower level of HIF transcriptional activity upon loss of microRNAs.

To test the role of FIH1 derepression, we generated FIH1 knockout and FIH1;Dicer1 double-knockout NSCLC cells. We adopted a recently developed genome-editing technology based on the RNA-guided nuclease Cas9 from the microbial CRISPR adaptive immune system (Fig. 4A; Cong et al. 2013; Mali et al. 2013). Using an algorithm that minimizes predicted genomic off-target sites [Hsu et al. 2013], we designed a single-guide RNA (sgRNA) targeting a genomic region almost immediately after the start codon of FIH1 on chromosome 19 (Fig. 4A). We transfected microRNA-deficient cells with a construct coexpressing the human codon-optimized Streptococcus pyogenes Cas9 and the FIH1 targeting sgRNA. Following incubation to allow genome editing, single cells were isolated by fluorescence-activated cell sorting (FACS) using flow cytometry into 96-well plates. Individual colonies were isolated and expanded to establish clonal cell lines. FIH1 protein level was determined by Western blot, and seven out of 12 (58%) of the clones completely lost FIH1 protein (Fig. 4B; Supplemental Fig. S8), suggesting that these clones are FIH1-null mutants.

We genotyped the clonal FIH1-null mutants by PCR-amplifying the targeted FIH1 locus followed by Illumina sequencing (Miseq). This revealed multiple FIH1-disrupting mutations, most of which are small deletions causing frameshifts (Fig. 4C; Supplemental Fig. S2). Clonal cell lines showed either one or two isoforms of the FIH1 alleles,
suggesting that these are either homozygous or trans-
heterozygous FIH1-null cells, respectively (Fig. 4C; Supple-
mental Figs. S2, S3). We also amplified and sequenced
the top two predicted off-target loci,
Evc2 and Fam126, in these cell lines. Neither locus showed any insertion or
deletion, suggesting minimal off-target activity (Supple-
mental Fig. S4). The
FIH1 knockout cell lines in the
Dicer1<sup>-/-</sup> and Dicer1<sup>-/-</sup> background are termed FIH1
knockout [Dicer1<sup>-/-</sup>;FIH1<sup>-/-</sup>] and Dicer1;FIH1 double
knockout [Dicer1<sup>-/-</sup>;FIH1<sup>-/-</sup>].

We then investigated the roles of microRNA loss
and FIH1 in HIF transcriptional activity. Using a HIF-
responsive element [HRE] reporter assay, we found that
microRNA-deficient cells have significantly lower levels

**Figure 4.** (A) Schematic representation of FIH1 targeting using the CRISPR/Cas9 system. [Top panel] Gene structure model of FIH1 in mouse chromosome 19 [not drawn to scale]. [Bottom panel] sgRNA sequence and targeted region sequence. The start codon [ATG] and the PAM [CGG] are indicated. (B) Western blot of FIH protein from clonal cell lines after sg-FIH1 and Cas9 transfection showing loss of FIH1 protein expression in seven out of 12 cell lines. (C) Miseq of the FIH1 exon1 genomic PCRs of clonal cell lines in a Dicer1<sup>-/-</sup> background.
of HIF transcriptional activity as compared with heterozygotes [Fig. 5B]. Knocking down FIH1 using siRNAs reduced FIH1 protein by ~80% [Fig. 5A], leading to increased HIF transcription activity in microRNA-deficient cells but with little effect in microRNA-competent cells [Fig. 5B]. Complete FIH1 knockout led to loss of FIH1
protein, which was rescued by transfection of a plasmid carrying a human FIH1 coding region transgene [Fig. 5C]. Knocking out FIH1 in the microRNA-competent background did not alter HIF transcriptional activity, whereas knocking out FIH1 in the microRNA-deficient background increased HIF transcriptional activity back to a level comparable with that of the parental microRNA-competent, FIH1 wild-type cells [Dicer1+/+;FIH1+/+] [Fig. 5D]. Re-expression of human FIH1 protein in Dicer1;FIH1 double-mutant cells suppressed HIF transcriptional activity [Fig. 5D].

As direct evidence that the up-regulation of FIH upon Dicer loss was due to loss of microRNAs, reintroducing the FIH1 targeting microRNAs let-7 and miR-125 into Dicer1−/−;FIH1+/+ cells decreased FIH1 protein level [Fig. 5E] and augmented HIF transcriptional activity [Fig. 5E], whereas transfection of miR-16, which does not target FIH1, did not change HIF transcriptional activity [Fig. 5E]. In Dicer1;FIH1 double-knockout cells, reintroduction of let-7 or miR-125 had an insignificant effect compared with control siRNA [Fig. 5F]. These data suggest that FIH1 is epistatic to Dicer1, i.e., it acts downstream from Dicer-dependent microRNAs in HIF transcriptional activity.

VEGF is a major direct target of HIF and is a key regulator of angiogenesis (Leung et al. 1989; Carmeliet and Jain 2000). We used ELISA to quantitatively measure the level of VEGF. MicroRNA-deficient cells produced less VEGF as compared with their parental microRNA-competent cells [Fig. 5G]. Dicer1;FIH1 double-knockout cells produced more VEGF than their FIH1 wild-type counterparts, at a level close to Dicer1+/− [Fig. 5G]. These data suggest that FIH1 is also downstream from Dicer1 in VEGF production.

Dicer1;FIH1 double-knockout cells were then assayed for tumor hypoxia and angiogenesis in vivo. We subcutaneously injected Dicer1+/+, Dicer1−/−, and Dicer1−/−;FIH1−/− NSCLC cells into the flanks of nude mice and then harvested tumor samples. The Dicer1;FIH1 double-knockout tumors were significantly less hypoxic as compared with the parental Dicer1 knockout, similar to that of Dicer1+/− [Fig. 6A,B]. This result was confirmed with HypoxyProbe IHC [Supplemental Fig. S5a,c]. We then analyzed tumor vasculature using Isolectin B4 IF. Dicer1;FIH1 double-knockout tumors had significantly increased vasculature density compared with the Dicer1 single knockout, at a level similar to Dicer1+/− [Fig. 6A,C]. This result was confirmed with IHC using an independent EC marker, CD31 [Supplemental Fig. S5b,d]. These data revealed that FIH1 knockout reversed the defects of microRNA loss in tumor hypoxia and angiogenesis, suggesting that FIH1 is the major target downstream from microRNAs for these phenotypes in vivo.

Because the primary effect of microRNA repression is mediated by the 3′ UTR (Bartel 2009), we set out to test direct repression by microRNAs specifically on the FIH1 locus. We mutagenized the FIH1 3′ UTR by CRISPR/Cas9 using a combination of multiple sgRNAs targeting the FIH1 3′ UTR [Supplemental Fig. S6a]. We FACS-sorted single cells after mutagenesis and isolated multiple clonal cell lines. We PCR-amplified the genomic region of the

**Figure 6.** [A] IF of sections of tumors induced by Dicer1 heterozygous, Dicer1 knockout, and Dicer1;FIH1 double-knockout cells. Channels are Hoechst (blue), Hypoxyprobe (green), and Isolectin B4 [Iso.B4, red]. Images were captured by a confocal microscope under 20× magnification with 0.5× digital zoom. Bar, 50 μm. (B) Quantification of the levels of hypoxia intensity indicated by relative HypoxyProbe staining intensity. Quantification was performed blind on three randomly chosen fields using CellProfiler. (C) Quantification of vasculature density indicated by relative Isolectin B4 intensity (percentage). Quantification was performed blind on three randomly chosen fields using CellProfiler. Data represent mean ± SEM. The asterisk denotes statistical significance (t-test, P < 0.01).
FIH1 3' UTR of these clonal cell lines, which revealed various monoallelic or biallelic deletions in the FIH1 3' UTR (Supplemental Fig. S6b). These FIH1-3' UTR mutants showed elevated levels of FIH1 protein as a result of partial loss of microRNA repression (Supplemental Fig. S6c). Among these mutants, we focused on the cell line that has the largest deletion in the FIH1 3' UTR. We performed Illumina sequencing and Sanger sequencing for this clone. Sequencing revealed that it harbors a homozygous 3770-bp deletion, which deletes the majority of the microRNA-binding sites in the 3' UTR (Fig. 7A,B). This cell line showed a high level of protein up-regulation compared with FIH1 3' UTR wild type (Supplemental Fig. S6c, mutant 4). We performed the HRE assay and found that this mutant showed down-regulation of HIF transcriptional activity to a level comparable with microRNA-deficient cells (Fig. 7C). These data strongly indicate that microRNAs directly repress FIH1 to regulate HIF transcriptional activity.

**Figure 7.** [A] Sanger sequencing of genomic PCR of a clonal FIH1 3' UTR mutant cell line harboring a homozygous 3.7-kb deletion generated using CRISPR/Cas9. [B] Captured Illumina sequencing of the FIH1 3' UTR of the mutant cell line harboring a homozygous 3.7-kb deletion. [C] HRE assay of representative FIH1 3' UTR mutant cell lines showing repressed HIF transcription activity. Data represent mean ± SEM. The asterisk denotes statistical significance (t-test, \( P < 0.01 \)). [n.s.] Not significant.
We tested the tumor growth rate of Dicer1⁰⁻, Dicer1⁻⁻, Dicer1⁻⁻;FIH1⁻⁻, and Dicer1⁻⁻;FIH1⁻⁻UTR-mutant cell lines. The FIH1 3' UTR mutant, which derepresses FIH1, grows more slowly than its parental cell line, Dicer1⁻⁻; at a rate similar to Dicer1⁻⁻ [Supplemental Fig. S7]. These data suggest that derepression of FIH1 by loss of either microRNAs or microRNA-binding sites suppresses tumor growth. The Dicer1⁻⁻;FIH1⁻⁻ double mutant grows more slowly than Dicer1⁻⁻; at a rate similar to Dicer1⁻⁻ [Supplemental Fig. S7], implying that loss of FIH1 in this context may have effects other than angiogenesis that affect tumor growth. In general, tumor hypoxia, angiogenesis, and HIF signaling are delicately controlled by multiple components and may not linearly translate into tumor growth (Semenza 2003; Schofield and Ratcliffe 2004; Kaelin 2008; Kaelin and Ratcliffe 2008).

We went on to test the dependence of regulation by FIH1 on the asparagine residue at the C-terminal domain of HIF1A. We generated a HIF1A-N803A mutant construct and reintroduced it into the Dicer1⁻⁻ cells by transfection. HIF1A-N803A transfection leads to a significantly higher increase of HRE activity as compared with HIF1A wild type in the presence of high levels of FIH1 [Supplemental Fig. S9]. These data and the previous results suggest that FIH1 is actively repressing the wild-type HIF1A through hydroxylation of N803 in these NSCLC cells.

FIH1 has been recently shown to have multiple targets (Zheng et al. 2008; Janke et al. 2013), in particular, FIH1 has been shown to target NOTCH for transactivation in other cellular systems (Zheng et al. 2008; Wilkins et al. 2009). In RNA-seq data from the NSCLC cell lines, the vast majority (36 out of 38) of expressed NOTCH target genes are not significantly altered upon FIH1 derepression following loss of microRNA regulation [Supplemental Fig. S10; Supplemental Table S6]. These data suggest that NOTCH is not a major target of FIH1 in these cell lines.

A previous study (Dayan et al. 2006) documented that CA9 [Car9 in mice] and PHD3 [Egln3 in mice] are sensitive to FIH1. We analyzed the expression of these genes in our RNA-seq data set and found that Car9 and Egln3 are down-regulated as FIH1 is derepressed upon microRNA loss in Dicer1⁻⁻ cells. In the list of FIH1-sensitive and FIH1-insensitive genes in the study by Dayan et al. (2006), we found that the FIH1-sensitive genes are mostly (12 out of 14, 86%) down-regulated in Dicer1⁻⁻ cells [compared with random, χ² test, P = 0.02], and FIH1-insensitive genes behave randomly [χ² test, P = 0.61] [Supplemental Table S7]. These data suggest that FIH1-sensitive genes are modulated by FIH1 and microRNAs.

Discussion

Mutations and misregulation of Dicer1 have been implicated in tumorigenesis in mouse models of NSCLC (Kumar et al. 2007, 2009), sarcoma (Ravi et al. 2012), and retinoblastoma (Nittner et al. 2012) as well as various human cancers (Kumar et al. 2009; Heravi-Moussavi et al. 2012). One of the first indications that microRNAs control malignant properties of human cancers was the strong correlation between low levels of let-7 microRNAs and progression of NSCLCs (Takamizawa et al. 2004; Lu et al. 2005). Mouse models of this disease recapitulated this correlation (Kumar et al. 2007, 2008). Thus, it was unexpected to find that Dicer-null NSCLC cells are defective for angiogenesis due to an inability to respond to hypoxia. This highlights the complexity of regulation of systems of genes by microRNAs in relationship to cancer. For example, let-7 microRNA can act as a tumor suppressor by suppressing growth rates through repression of the RAS pathway and sets of oncofetal genes (Gurtan et al. 2013), while at the same time, let-7 has oncogenic activity by promoting angiogenesis by repressing the activity of FIH1. Let-7 and miR-125 suppress FIH1, which suppresses the transcriptional activity of HIF and thus angiogenesis in response to hypoxia. In fact, FIH1 is highly responsive to changes in microRNA levels, with an extensive 3' UTR with hundreds of target sites for multiple microRNA families. Furthermore, we show that deletions in the 3' UTR of FIH1 increase its expression and accordingly decrease the activity of HIF.

Several microRNA families generated by Dicer are known to be required for blood vessel formation during mouse embryonic development (Yang et al. 2005; Suarez et al. 2008). However, the delicate balance of the angiogenic switch is highly context-dependent (Carmeliet and Jain 2000), and the pleiotropic regulatory functions of microRNAs vary from cell type to cell type. Several microRNAs have been shown to regulate hypoxia response and angiogenesis in different cancer cell lines, such as miR-9 (Zhuang et al. 2012), miR-210 (Kelly et al. 2011), let-7 (Chen et al. 2013), and miR-29a (Wang et al. 2013). These microRNAs are thought to function in either a hypoxia-dependent manner via HIF (Kelly et al. 2011; Chen et al. 2013) or a hypoxia-independent manner via other pathways, such as TGF-β, JAK/STAT (Zhuang et al. 2012; Wang et al. 2013), or EGFR signaling (Shen et al. 2013).

Many microRNAs are predicted to actively repress FIH1, which hydroxylates the asparagine residue of HIF-1α at its transactivation domain, thus repressing HIF transcription activity (Mahon et al. 2001; McNeill et al. 2002). Tumor cells respond to hypoxia by activating the HIF pathway, which turns on the production of VEGF and other adaptive response genes (Carmeliet and Jain 2000). In a head and neck carcinoma model, it was shown that miR-31 suppresses FIH to activate the HIF pathway [Liu et al. 2010]. In the absence of Dicer1 and thus virtually all microRNAs, FIH1 is highly derepressed and inhibits the normal function of HIF-1α, thereby suppressing the response to hypoxia. This interference caused reduced tumor angiogenesis in Dicer1-null tumors even though the tumor hypoxia level is high. Using RNA-guided genome engineering technology based on CRISPR/Cas9, we showed that loss-of-function mutations in FIH1 reversed the defect of Dicer1-null cells in hypoxia response and tumor angiogenesis. Furthermore, deletion of several kilobases of the 3' UTR region in the FIH1 gene, which abolishes microRNA regulation and leads to derepressed FIH protein,
suppresses HIF signaling. FIH1 is repressed in the presence of normal microRNAs (Dicer1+/− cells) and thus is expressed at a low level. FIH1 knockout in Dicer1+/− cells has no effect in the HRE because the basal expression level is low, consistent with the fact that FIH1 knockout mice show no dysregulation of HIF1 [Zhang et al. 2010]. Only upon loss of microRNA repression by loss of either all microRNAs through Dicer1 deletion or microRNA-binding sites through 3′ UTR deletion is the effect of FIH1 on the HRE manifested.

We observed a global regulation of microRNAs on FIH1 in a panel of human cancer cell lines, with strong correlation in solid tumor types and no correlation with hematopoietic or lymphatic malignancies (data not shown). This may be explained by the fact that angiogenesis and hypoxia are more prominent in solid tumors compared with hematopoietic malignancies. Thus, microRNA regulation of FIH1 is general across many types of human cancers. The Dicer–microRNA–FIH pathway may have important roles in tumor hypoxia response and angiogenesis in human cancer.

Materials and methods

CRISPR-mediated genome editing of the FIH1 coding region

sgRNAs targeting FIH1 were designed using tools described at http://tools.genome-engineering.org (Cong et al. 2013; Hsu et al. 2013). The guided sequence with minimal off-target gene targeting and high on-target score was chosen [5′-TAGAGTAGAG ATGGCCGCCGA-3′]. The oligo pair [mFIH-ex1-g-F1 and mFIH-ex1-g-R1] was annealed and cloned into BbsI-digested vector px264Lon to generate a construct, sg-FIH-ex1-F1-pX264Lon. This construct was transfected to Dicer1−/− and Dicer1+/− NSCLC cells. Plasmid transfection was performed using Lipofectamine 2000 [Life Technologies]. After 48 or 72 h, cells were cloned using FACS, and colonies were expanded for genotyping, RNA analysis, and protein analysis. Mutations were genotyped by genomic PCR followed by sequencing.

The oligos to generate the guide sequence for FIH1 targeting were mFIH-ex1-g-F1 [5′-CACCGTGAGTAGAGATGGCCGCC GA-3′] and mFIH-ex1-g-R1 [5′-AAACTCGCCGCCATCTCTA CTCTAC-3′]. The primers used for FIH1 genotyping and sequencing were mFIH-F1 [GGGCCGTCCCTAGAGTAGAG], mFIH-R3 [GGTGAGAAACCTTTCCAAAT], mFIH_F7 [GCAGTACAGCGTGAACCCCAGATA], mFIH-R13 [TCCACCACCTCTCCTGC], mFIH_F6 [TGCTTCGTTGAT], mFIH_F5 [GCAATATTTCGTGGGCTTGT], mFIH_F4 [CTAGTATCTTGCAAG], mFIH_F2 [CTAAGCGAGTCGGCCTTATG], mFIH-F1 [GGGCCGTCCCTAGAGTAGAG], mFIH-R3 [GCGTACTGTTAA], mFIH-ex1-g-F1 (5′-GACAGAGGCTTGAGAGG), mFIH-ex1-g-R1 (5′-AAACTCGCCGCCATCTCTA CTCTAC-3′), and mFIH-ex1-g-R1 (5′-ATGGCGGCGA-3′). The oligo pair (mFIH-ex1-g-F1 and mFIH-ex1-g-R1) was annealed and cloned into BbsI-digested vector pX264Lon to generate a construct, sg-FIH-ex1-F1-pX264Lon. This construct was transfected to Dicer1−/− and Dicer1+/− NSCLC cells. Plasmid transfection was performed using Lipofectamine 2000 [Life Technologies]. After 48 or 72 h, cells were cloned using FACS, and colonies were expanded for genotyping, RNA analysis, and protein analysis. Mutations were genotyped by genomic PCR followed by sequencing.

Genomic PCR Miseq

Genomic PCR products were directly purified using DCC kit or gel-purified using a gel extraction kit [Zymo]. Barcoded DNA libraries were prepared using the Nextera XT kit [llumina]. Normalized libraries were subjected to Miseq following the manufacturer’s protocols.

Mouse and cell lines

NSCLC cell lines were derived using a previously described mouse model [Kumar et al. 2009]. Tumors were induced in the lung by intratracheal injection of Ad-Cre virus to activate KRas-G12D, p53−/−, Dicer1−/−. Forced Dicer1 homozygous deletion was induced in vitro by administration of adenovirus encoding Cre-GFP and FACS sorting of GFP− single cells or by infection with retrovirus encoding ER-Cre, treatment with 4-OH-tamoxifen, and FACS-sorting of single cells. Multiple paired single-cell clonal lines were derived. Clonal lines were genotyped for Kras and p53 as described previously [DuPage et al. 2009] and genotyped for Dicer1 using the following primers: MC113 [5′-AGCATGGGGGACCTCGTG CCTG-3′], MC93 [5′-CATGACTCTCTAATCAACT-3′], and MC94 [5′-GTCGAGATCTGGTTCAACAG-3′].

Murine sarcoma cell lines were derived by Ravi et al. (2012) and used as a genotyping control. Cells were cultured in standard DMEM + 10% FBS unless otherwise specified. Isogenic Dicer1 heterozygotes and Dicer1 knockout lines were infected with retrovirus encoding Luciferase-GFP, and stable clonal cell lines were established from FACS-sorting of GFP+ single cells.

Primers for off-target genotyping and sequencing were Fam126a_Fihex1f1OT 4 F [AGCAAATGGCAAATGTGGTC], Fam126a_Fihex1f1OT 4 R [GGAGAACCCCTCAGCAGA], Evc2_Fihex1f1OT 4 F [TGCTGAGATGGTATCTGCTG], and Evc2_Fihex1f1OT 4 R [CTTCCCTACAGCAGTCCAGT].
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In situ tumor induction was performed by subcutaneously injecting $10^5$ or $10^6$ NSCLC cells of specific genotypes into the flanks of nude mice [Mus musculus, nu/nu, 4- to 6-wk-old females, Charles River Laboratories]. Animals were monitored for tumor growth by physical examination or luciferase imaging with IVIS (Xenogen) after intraperitoneal injection of beetle luciferin [Promega, Caliper] at 165 mg/kg. At the endpoint of the experiments, tumors were harvested for molecular biology and/or histology.

All animal work were performed under the guidelines of the Division of Comparative Medicine (DCM) with protocols [0911-098-11 and 0911-098-14] approved by the Massachusetts Institute of Technology Committee for Animal Care (CAC) and were consistent with the Guide for Care and Use of Laboratory Animals, National Research Council, 1996 (institutional animal welfare assurance no. A-3125-01).

**Tumor collection**

Tumor samples were harvested at the experimental end points. Briefly, Hypoxyprobe [pimonidazole HCl] at a dose of 60 mg/kg body weight was intraperitoneally injected into mice. At 1 h after injection, the mice were sacrificed by lethal dose of CO$_2$. Tumor tissues were immediately dissected. A fraction of tumor tissues was frozen on dry ice and stored at −80°C for molecular analyses. Other fractions of the tissues were immediately fixed with 4% paraformaldehyde (PFA) overnight at 4°C, followed by 75% ethanol wash, paraffin-embedding, and sectioning. Slides were subjected to histology analysis.

**IF and IHC**

Paraffin-embedded tumor tissues were sectioned at the thickness of 5 mm. For general histopathological evaluation, tissue sections were stained with the standard hematoxylin–eosin (H&E) method [Xue et al. 2010, 2012]. Tissue sections were blocked with 3% goat serum in PBS for 30 min and incubated with single or multiple primary antibodies overnight at 4°C. Primary antibodies included CD31 (BD), MAb1 (1:100; Hypoxyprobe, Inc.), and biotin-conjugated Isolentin B4 (1:400; Vector Laboratories). After washing with PBS, secondary antibodies were added to the sections and incubated for 1 h at room temperature. Secondary antibodies included an anti-rat Alexa 488 antibody [Invitrogen], an avidin-conjugated Alexa 555 antibody [Invitrogen], an anti-mouse Alexa 488, antibody [Invitrogen], and/or an avidin-conjugated horseradish peroxidase [HRP] antibody [Vector Laboratories]. Sections were washed with PBS, followed by counterstaining with DAPI [Vector Laboratories], and were mounted in Vectashield mounting medium [Vector Laboratories]. Some of the sections were counterstained with DAB chromogen [Invitrogen]. Stained tissue samples were analyzed using a Zeiss confocal LSM700 microscope, a conventional light microscope, or an EVOs microscope [Advanced Microscope Group].

Whole-mount staining was conducted according to published methods [Xue et al. 2010, 2012]. Briefly, tumor tissues were prepared into −2-mm slices and washed in PBS for 1 h, followed by incubation with 20 mg/mL proteinase K for 5 min. The tissues were further permeabilized with methanol for 30 min and blocked with 3% fat-free milk in PBS, respectively. Tumor tissues were stained with primary antibody against CD31 overnight at 4°C. After rigorous washing with PBS, the tissues were stained with anti-rat Alexa 488-conjugated secondary antibody for 2 h at room temperature. Stained slides were mounted in Vectashield mounting medium and imaged under a Zeiss confocal LSM700 microscope. Three-dimensional images of each sample were projected by acquiring seven layers of images at a 5-mm distance between layers. Quantitative analyses from nine different tissue sections were performed.

IF and IHC slides were quantified in a blind selection of images using CellProfiler [Lampropot et al. 2007], with pipeline implementation using custom python scripts. Images [Schneider et al. 2012], or the color range and histogram tools from Adobe Photoshop CS.

**Tumor grow rate analysis**

Tumor induction was performed by subcutaneous injection of $10^5$ NSCLC cells of the desired genotype to 6- to 8-wk-old female nude mice (n = 4 each group). Tumors were measured over time by calipers, and volumes were estimated as spheroids using the formula $V = \pi \times a \times b \times h/6$, where V, a, b, and h are volume, length, width, and depth of a tumor, respectively.

**Standard molecular biology**

All conventional molecular biology experiments, including DNA/RNA/protein extraction, PCR, RT–PCR, Western blot, and Northern blot were performed using standard molecular biology protocols with slight modifications or using commercially available kits [Promega, Qiagen, Zymo, Life Technologies, and TakaraBio]. Proteins were quantified with Bradford assay [Fisher] and/or BCA assay [Fisher], calibrated by BSA standard curves, and normalized before quantification by Western or ELISA. Antibodies were from various sources, including Dicer [Cell Signaling], Vinculin [Sharp Laboratory], CD31 [BD], and FIH1 [SIBT]. VEGF ELISA and angiogenesis protein array [R&D Systems] were performed according to the manufacturer’s protocols.

**MicroRNA Northern blot**

Total RNA was prepared from $1 \times 10^6$ to $5 \times 10^6$ cells or 50–200 mg of tumor tissues using Trizol reagent [Life Technologies] according to the manufacturer’s protocol. Five micrograms of RNA was mixed with an equal volume of formamide loading buffer, denatured for 5 min at 95°C, and run for 1 h at 35 W on 8% or 12% denaturing polyacrylamide gels [Sequagel, National Diagnostics] after 30 min of prerunning. A semidy transfer apparatus set to a 300-mA limit was used to transfer the RNA to a Hybond-N+ nylon membrane [GE Healthcare Life Sciences] for 1.5 h. Membrane containing RNA was then UV-cross-linked at 1.2 × 10$^9$ μJ in a Cross-linker 2400 [Stratagene] on top of Whatman paper. The membrane was prehybridized with UltraHyb buffer [Ambion] for 0.5 h and then probed overnight at 42°C with $[\gamma-32P]$-ATP 5’-end-labeled DNA probes [IDT] reverse complementary to mature microRNA sequences. The membrane was washed twice for 30 min in 2× SSC/0.1% SDS buffer and then exposed to films [GE] for 1, 24, or 48 h and imaged on a Typhoon PhosphorImager [Molecular Dynamics]. DNA oligo probes for U1 snRNA or U6 snRNA were used as loading controls.

**Oxygen conditioning and hypoxia setting**

Oxygen conditioning was performed similar to previously described methods [Metallo et al. 2012]. Briefly, a hypoxia chamber was setup with oxygen, carbon dioxide, and nitrogen to adjust oxygen levels. Hypoxia condition was set to 1% oxygen and 5% carbon dioxide unless otherwise noted.

**Luciferase assay**

HIF transcription activity reporter FHRE-luc vector was ordered from Addgene [Brunet et al. 1999]. Cells were cotransfected with FHRE-luc, pRL-CMV, and pCMV-GFP with/without additional constructs such as overexpression vectors or microRNAs.
MicroRNAs (mmu-let-7-g-5p, mmu-mir-125-b-5p, and mmu-mir-16-5p) for transfection were synthesized as customized siRNA (Dharmacon) based on the mature microRNA sequences from mirBase release 19, with a UU overhang at the 3′ end. Dharmacon nontargeting control siRNA was used as a negative control. siRNAs and microRNAs were transfected at 10−50 μM concentrations. Human FIH1 cDNA constructs that overexpress FIH1 or FIH1-GFP were ordered from ThermoScientific and Addgene [21399 and 21403] [Metzen et al. 2003] and transfected at 1 μg/mL. Human HIF1A cDNA construct was purchased from Thermo, and the N803A mutation was generated by oligo-based site-directed mutagenesis. Cells were lysed and assayed using a dual-luciferase kit (Promega). Data were first normalized to the constitutive Renilla luciferase and then to the control group under hypoxia.

Expression profiling by mRNA-seq

Total RNAs were prepared from 1 × 10⁶ to ~5 × 10⁶ cells of specific genotypes [Dicer1 heterozygous or knockout] samples using Trizol reagent (Life Technologies). Samples were quality-controlled on BioAnalyzer to ensure RNA integrity. Before library prep, 1 μL of 1:50 diluted ERCC spike-in RNA (Life Technologies) was added to 1 μg of total RNA. Normalized spiked total RNA samples were then used to generate high-throughput sequencing libraries using the Tru-seq kit (Illumina) following the manufacturer’s instructions. Briefly, miRNAs were poly-A-purified from total RNAs, fragmented, and converted to cDNA using the dUTP second strand marking protocol outlined in Levin et al. (2010), with slight modifications [first strand synthesis incubation: 10 min at 25°C, 50 min at 42°C, 15 min at 70°C, and 4°C hold; second strand synthesis incubation: 1 h at 16°C]. Synthesized cDNA samples were cleaned up with a 1.5× SPRI reaction, eluted in 50 μL of EB, quality-controlled with the Bioanalyzer, and then adaptor-ligated and size-selected using automated SPRI works system [Beckman Coulter] to generate Illumina libraries with a size range of 200–400 bp. The libraries were amplified using the primers against the Illumina adapters PE1.0 and/or PE2.0 (PE1.0, 5′-AGATC CGGAAGACGCCTAGCAGAAATGCGCCAGACGG; and PE2.0, 5′-AGATC GGGAAGACGCCTAGCAGAAATGCGCCAGACGG).

The libraries were uniquely barcoded to each sample during the amplification step and then used to generate clustered flow cells and sequenced on a HiSeq-2000 machine.

Small RNA-seq (microRNA-seq)

MicroRNA-seq samples were prep from a subset of matched samples of the mRNA-seq RNA samples. Library preparation was performed using the small RNA library preparation kit (E7330, New England Biolabs) with size selection for 15–90 bp, a range including all potential mature microRNAs and precursors. Subsequent quality control (QC) sequencing steps were performed similarly following Illumina’s instructions.

mRNA and microRNA sequencing data processing

Raw fastq data of mRNA-seq were preprocessed using standard protocols.

Briefly, multiplexed barcoded reads from single-end or paired-end sequencing FASTQ files were bucketed by sample identity, and reads from both ends were processed for adapter removal using the fastx clipper utility from the Hannon laboratory FASTX-Toolkit suite [http://hannonlab.cshl.edu/fastx_toolkit]. The adapters used were PE1.0 and/or PE2.0 as above. For paired-end reads, in order to eliminate reads from the low end of the distribution of insert sizes and limit the number of short sequences contributing to mapping ambiguity, both ends of a read were dropped if one (or both) of the reads resulted in a sequence <15 bp after adapter stripping. The distribution of insert lengths was determined empirically by aligning 1.25 million reads from each sample against the mouse transcriptome (University of California at Santa Cruz [UCSC] build mm9) using the Bowtie short read alignment tool [Langmead et al. 2009]. All libraries passed all initial QCs and mapping QCs.

Processed reads of mRNA-seq were aligned to the UCSC transcriptome and then to the genome (UCSC build mm9 unless otherwise noted) using the TopHat spliced junction alignment tool [Trapnell et al. 2009]. Processed reads of microRNA-seq were first mapped to mouse mature microRNA sequences annotated in miRBase release 19, allowing for unique and repeat alignments with up to a single base pair mismatch per alignment. Reads that did not align to mature microRNA sequences were similarly mapped to microRNA hairpin sequences where possible, allowing for up to two mismatches per alignment. Mapping files [bam format] were used to quantify the relative gene expression level. In mRNA-seq, measurement was done in terms of reads per fragment per kilobase of transcript per million mapped reads [RPKM/FPKM] using Cufflinks [Trapnell et al. 2010]. In microRNA-seq, measurement was done in reads per million of mapped reads [RPM] using custom scripts. Gene differential expression analysis was performed using cuffdiff [Trapnell et al. 2010] as well as ANOVA using custom R scripts.

Gene set analyses

Gene and genomic annotation was based on mouse genome UCSC build mm9 unless otherwise specified. Expressed genes were classified using an FPKM cutoff based on the overall distribution. Genes associated with certain biological processes were retrieved from the Gene Ontology database [http://www.geneontology.org]. Gene ontology and gene set enrichment analyses were performed in DAVID (Huang et al. 2009), Ingenuity Pathway Analyses (IPA; Ingenuity Systems), and/or BioConductor (http://www.bioconductor.org). Transcription factor–target gene relationship data were retrieved from the literature knowledge base of the IPA and TRANSFAC databases [Matys et al. 2003]. The HIF antagonists were curated from the literature (Senemaa et al. 2003; Kaelin 2008) plus the IPA knowledgebase. The FIH-sensitive and FIH1-insensitive gene sets were curated from Dayan et al. (2006). Notch signaling pathway genes were retrieved from MSigdb [Liberzon et al. 2011].

MicroRNA target gene analyses

The microRNA family and sequences were retrieved from TargetScan 6.2 [Lewis et al. 2005] and miRBase 19 [Ambros et al. 2003]. Cross-reference between TargetScan and miRBase was performed using custom scripts. Predicted microRNA target gene sets were retrieved from TargetScan 6.2. MicroRNA target gene differential expression analyses were carried out using customized scripts to intersect mRNA-seq data, microRNA expression data, and TargetScan prediction. For each microRNA, its target gene set included all annotated genes that had the particular seed sequence at the 3′ UTR [TargetScan].

Accession

Illumina sequencing data have been deposited to Gene Expression Omnibus [accession no. GSE57043] and NCBI Sequence Read Archive [accession no. PRJNA244460].

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References


MicroRNA depletion suppresses tumor angiogenesis
