RESEARCH COMMUNICATION

Tumor suppression by miR-26 overrides potential oncogenic activity in intestinal tumorigenesis

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Down-regulation of miR-26 family members has been implicated in the pathogenesis of multiple malignancies. In some settings, including glioma, however, miR-26-mediated repression of *PTEN* promotes tumorigenesis. To investigate the contexts in which the tumor suppressor versus oncogenic activity of miR-26 predominates in vivo, we generated miR-26a transgenic mice. Despite measurable repression of *Pten*, elevated miR-26a levels were not associated with malignancy in transgenic animals. We documented reduced miR-26 expression in human colorectal cancer and, accordingly, showed that miR-26a expression potently suppressed intestinal adenoma formation in *Apc*^{min/+} mice, a model known to be sensitive to *Pten* dosage. These studies reveal a tumor suppressor role for miR-26 in intestinal cancer that overrides putative oncogenic activity, highlighting the therapeutic potential of miR-26 delivery to this tumor type.

Supplemental material is available for this article.

Received August 13, 2014; revised version accepted October 27, 2014.

MicroRNAs (miRNAs) are a class of ~18- to 23-nucleotide (nt) noncoding RNAs that negatively regulate mRNA stability and translation. Over the last 10 years, an important role for miRNAs in cancer pathogenesis has been uncovered (Di Leva et al. 2014). Studies have revealed nearly ubiquitous dysregulation of miRNA expression in human tumors, and miRNA expression profiles have proven useful for tumor classification. Clear examples of miRNAs that act as oncogenes or tumor

suppressors through their ability to regulate key aspects of neoplastic transformation, including proliferation, apoptosis, and metastasis, have been documented.

A potential tumor suppressor role for the miR-26 family, comprising miR-26a and miR-26b, was first suggested by the discovery that expression of these miRNAs impairs MYC-mediated transformation (Chang et al. 2008). Subsequent studies demonstrated down-regulation of miR-26 across multiple tumor types, including lymphoma, hepatocellular carcinoma, breast cancer, and nasopharyngeal carcinoma (Ji et al. 2009; Kota et al. 2009; Lu et al. 2011; Zhang et al. 2011). Ectopic expression of miR-26 inhibits proliferation, induces apoptosis, and/or suppresses tumorigenicity in multiple cancer settings. The tumor suppressor activity of miR-26 is mediated through repression of direct targets that include *Cyclin D2* (*CCND2*), *Cyclin E2* (*CCNE2*), and *Enhancer of zeste homolog 2* (*EZH2*) (Sander et al. 2008; Kota et al. 2009; Lu et al. 2011; Zhang et al. 2011). In other contexts, however, including glioma, lung cancer, and T-cell lymphoblastic leukemia, miR-26 exhibits oncogenic activity through its ability to repress *PTEN* (Huse et al. 2009; Mavrakis et al. 2011; Liu et al. 2012). Further investigation of the tumor suppressor versus oncogenic activity of miR-26 in vivo is therefore needed to dissect its role in cancer pathogenesis and explore the therapeutic potential and possible adverse consequences of miR-26 delivery.

One cancer setting in which the contribution of miR-26 has remained poorly defined is colorectal cancer (CRC). While previous studies have documented anti-tumorigenic effects of miR-26 expression in CRC cell lines (Ma et al. 2011; Zhang et al. 2013), the significance of these results has remained unclear, since miRNA profiling studies have inconsistently detected miR-26 down-regulation in human colorectal tumors (Schepeler et al. 2008; Schetter et al. 2008; Motoyama et al. 2009; Ma et al. 2011; Gaedcke et al. 2012). The cellular heterogeneity of normal and neoplastic intestinal tissue can confound the detection of abnormally expressed genes within tumor epithelial cells, the relevant transformed cell type. Further analysis of miR-26 expression specifically within CRC-derived epithelial cells is therefore needed to resolve whether miR-26 is frequently down-regulated in this tumor type.

In order to examine the pro- and anti-tumorigenic effects of miR-26 expression in vivo, we generated inducible miR-26a transgenic mice. Despite broad over-expression of miR-26a and a corresponding decrease in *Pten* expression, transgenic animals developed normally and did not exhibit elevated rates of malignancy. Moreover, we documented reduced miR-26 in human colorectal tumor epithelial cells and showed that enforced miR-26a expression strongly suppressed intestinal adenoma formation in *Apc*^{min/+} mice, a model in which reduced *Pten* dosage is known to accelerate tumorigenesis (Shao et al. 2007). The tumor suppressor activity of miR-26a in intestinal epithelium is mediated through the repression of anti-proliferative targets that include *Ccnd2*, *high-*

[Keywords: microRNA; miR-26; intestine; colon cancer]

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Article published online ahead of print. Article and publication date are online at <http://www.genesdev.org/cgi/doi/10.1101/gad.250951.114>.

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mobility group AT hook 1 (*Hmga1*), Myc-binding protein (*Mycbp*), SET domain containing 8 (*Setd8*), and *Ezh2*. These findings demonstrate that the tumor suppressor activity of miR-26 predominates within the intestinal epithelium, supporting the potential therapeutic efficacy of delivery of this miRNA to CRC.

Results and Discussion

A doxycycline (dox)- and Cre-inducible miR-26a transgenic mouse

To assess the oncogenic versus tumor-suppressing activity of miR-26 in vivo, we generated a transgenic mouse with dox- and Cre-regulatable miR-26a expression (Fig. 1A). The transgene consists of a dox-regulatable promoter driving expression of a lox-stop-lox (LSL) cassette, which terminates transcription, followed by a green fluorescent protein (GFP) ORF and the human pre-miR-26a-2 hairpin with ~100 base pairs (bp) of flanking sequence from the endogenous miR-26a-2 locus. The transgene, termed *LSL.eGFP.miR-26a*, was inserted downstream from the *Col1A* locus in embryonic stem cells (Beard et al. 2006). These embryonic stem cells also harbor a widely expressed reverse tetracycline transactivator transgene integrated at the *Rosa26* locus (*M2rtTA*).

LSL.eGFP.miR-26a mice were crossed to *CMV-Cre* mice (Schwenk et al. 1995) to remove the LSL cassette and broadly activate miR-26a expression. Similar to other transgenes generated using this approach (Beard et al. 2006), the strongest expression was observed in the epithelial cells of the small and large intestine, bone marrow, spleen, thymus, and liver (Fig. 1B,C; Supplemental Fig. S1). Expression of the

transgene was fully compatible with normal development and was well tolerated in adults. No overt histopathologic abnormalities or increased mortality were observed in a large cohort of transgenic animals after 1 yr of transgene expression (Supplemental Fig. S2A; data not shown).

Given the absence of increased rates of malignancy in *M2rtTA; eGFP.miR-26a* mice, we questioned whether expression of the dosage-sensitive *Pten* tumor suppressor was decreased by enforced miR-26a expression. Western blotting nevertheless confirmed that, in general, tissues with the highest miR-26a expression, such as intestinal epithelium and the liver, exhibited measureable PTEN down-regulation (Supplemental Fig. S3).

*miR-26a suppresses intestinal tumorigenesis in *Apc*^{min/+} mice*

Robust transgene expression in the intestinal epithelium and a corresponding down-regulation of PTEN within this cell type provided an opportunity to directly assess the oncogenic versus tumor suppressor activity of miR-26a in this tissue. Normally, expression of miR-26 family members increases as intestinal stem cells differentiate, as revealed by analysis of miRNA levels in sorted LGR5⁺ stem cells (Supplemental Fig. S4), suggesting that miR-26 may regulate proliferation and/or differentiation of this cell type. Nevertheless, strongly enforced expression of miR-26a had no effect on baseline intestinal histology (Supplemental Fig. S2B). *M2rtTA; eGFP.miR-26a* mice were therefore bred to the *Apc*^{min/+} model of intestinal tumorigenesis (Moser et al. 1990). Although *Pten* haploinsufficiency has previously been shown to strongly enhance tumor initiation and progression in this model (Shao et al. 2007), we observed reduced abundance of miR-26a in *Apc*^{min/+} adenomas (Fig. 2A).

Apc^{min/+}; *M2rtTA; eGFP.miR-26a* mice and *Apc*^{min/+}; *M2rtTA* controls were given dox at 28 d of age and sacrificed at 150 d. Enforced miR-26a expression dramatically reduced tumor number and size (Fig. 2B,C). Interestingly, GFP immunohistochemistry revealed that the small number of adenomas that arose in *Apc*^{min/+}; *M2rtTA; eGFP.miR-26a* mice uniformly lacked expression of the transgene (Fig. 2D,E). Costaining of E-cadherin confirmed that GFP-negative cells were of epithelial origin in adenomas (Fig. 2E). Thus, adenoma formation in *Apc*^{min/+}; *M2rtTA; eGFP.miR-26a* mice confers strong selection for silencing or deletion of the transgene. These results indicate that despite resulting in measureable repression of PTEN in the intestinal epithelium, enforced miR-26a expression strongly suppresses tumorigenesis in this setting.

Reduced expression of miR-26 in human CRC

Given the reduced expression of miR-26a in *Apc*^{min/+} intestinal adenomas and its dramatic anti-tumorigenic activity in this model, we sought to determine whether down-regulation of miR-26 family members is a common feature of human CRC. While previous studies of CRC have inconsistently detected miR-26 down-regulation (Schepeler et al. 2008; Schetter et al. 2008; Motoyama et al. 2009; Ma et al. 2011; Gaedcke et al. 2012), the heterogeneous cellular composition of colorectal tumors can confound these measurements. We therefore obtained biopsies of human CRCs and paired

Figure 1. A dox- and Cre-inducible miR-26a transgenic mouse. (A) Schematic of the *LSL.eGFP.miR-26a* transgene. (TRE) Tetracycline-responsive element; (pA) poly(A) signal. (B) Quantitative RT-PCR measurement of miR-26a expression in the indicated tissues of *M2rtTA* and *M2rtTA; eGFP.miR-26a* mice normalized to U6 after 2 wk of dox treatment. (C) miR-26a expression in purified small intestinal epithelium of dox-treated transgenic or control mice. For B and C, average values from three independent mice of each genotype are shown. Error bars for this and all subsequent figures represent standard deviations.

Figure 2. miR-26a suppresses tumorigenesis in $Apc^{min/+}$ mice. (A) miR-26a expression normalized to U6 in adenomas and paired normal tissue from $Apc^{min/+}$ mice. $n = 10$ samples per condition. P -values for A and B were calculated by two-tailed t -test. (B) Quantification of total tumor number (left) and the fraction of tumors that were <1 mm in size (right) in mice of the indicated genotypes. The ends of the boxes represent the 25th and 75th percentiles, the bars indicate the 10th and 90th percentiles, and horizontal lines within the boxes represent the median. $n = 21$ $Apc^{min/+}; M2rtTA$ animals and 15 $Apc^{min/+}; M2rtTA; eGFP.miR-26a$ animals. (C) Representative segments of small intestine from mice of the indicated genotypes. (D) H&E and anti-GFP staining of representative adenomas from mice of the indicated genotypes. (E) Magnified image of adenoma from the $Apc^{min/+}; M2rtTA; eGFP.miR-26a$ mouse in D illustrating GFP-negative staining of E-cadherin-positive adenoma cells.

normal colons and measured miR-26 levels in the unfractionated tissue as well as in purified epithelium. While neither miR-26a nor miR-26b were detectably reduced in unfractionated tumor tissue (Fig. 3A), clearly lower levels of these miRNAs were observed when comparing purified tumor epithelial cells with paired normal colonic epithelial cells (Fig. 3B). Given our finding that miR-26 expression increases as intestinal epithelial cells differentiate (Supplemental Fig. S4), it is possible that reduced miR-26 levels in tumors is a secondary consequence of impaired differentiation of tumor epithelial cells. Nevertheless, taken together with our functional data from $Apc^{min/+}$ mice and previous studies that have demonstrated anti-tumorigenic effects of miR-26 expression in human CRC cell lines (Ma et al. 2011; Zhang et al. 2013), these data strongly suggest a tumor suppressor role for miR-26 in human colon cancer.

miR-26a expression results in a cell-autonomous decrease in intestinal epithelial migration and proliferation

The uniform loss of transgene expression in adenomas of $Apc^{min/+}; M2rtTA; eGFP.miR-26a$ mice precluded the use of tumor tissue from these animals to investigate the mechanism of tumor suppression. We therefore more closely examined the intestinal epithelium of transgenic animals to determine whether a relevant phenotype was detectable in preneoplastic tissue. Although expression of miR-26a does not result in any overt histologic abnormality in the intestine (Supplemental Fig. S2B), bromodeoxyuridine (BrdU) pulse-chase assays revealed a dramatic decrease in epithelial turnover in transgenic mice, indicative of an epithelial migration defect (Fig. 4A,B; Supplemental Fig. S5). This effect was accompanied by a decrease in phosphorylated histone 3 (p-H3)-positive mitotic cells within intestinal crypts, demonstrating decreased proliferation (Fig. 4C,D). Reduced migration and proliferation was not associated with precocious or impaired differentiation of epithelial cells (Supplemental Fig. S6; data not shown). Notably, there is precedent for a lack of global changes in intestinal morphology in the setting of altered crypt proliferation, as we observed (Haigis et al. 2006).

To assess whether miR-26a inhibited proliferation and migration in a cell-autonomous manner, the Cre-regulatable transgene ($LSL.eGFP.miR-26a$) was activated specifically in intestinal epithelium by crossing to *Villin-Cre* mice (Supplemental Fig. S7; Madison et al. 2002). Similar to the effect of broad miR-26a expression, epithelial-restricted miR-26a expression was sufficient to significantly delay transit of BrdU⁺ cells (Fig. 4E,F). These data document potent cell-autonomous anti-proliferative and anti-migratory effects of enforced miR-26a expression within the intestinal epithelium.

miR-26a represses expression of proproliferative transcripts, including *Ccnd2*, *Hmga1*, *Mycbp*, *Setd8*, and *Ezh2*

In order to identify the targets of miR-26a that mediate its anti-proliferative effects, microarrays were used to assay gene expression in purified epithelial cells from dox-treated $M2rtTA$ or $M2rtTA; eGFP.miR-26a$ mice. One-hundred-sixty-six transcripts were down-regulated and 132 transcripts were up-regulated by ≥ 1.5 -fold in miR-26a transgenic animals (Supplemental Table S1). Sylamer analysis (van Dongen et al. 2008) revealed dramatic enrichment of hexamers that are complementary to the miR-26 seed sequence within the 3' untranslated regions (UTRs) of down-regulated genes (Supplemental Fig. S8). Furthermore, gene set enrichment analysis (GSEA) (Subramanian et al. 2005) demonstrated significant down-regulation of miR-26 targets predicted by TargetScan (Supplemental Fig. S9A; Grimson

Figure 3. miR-26a is down-regulated in human CRC cells. (A,B) miR-26a and miR-26b expression in unfractionated colorectal tumors relative to paired normal tissue (A) or purified epithelial cells from tumors relative to paired normal colonic epithelial cells (B). $n = 10$ paired samples for each analysis. P -values were calculated by one-sample t -test. Box plots are defined as in Figure 2B.

et al. 2007). These findings demonstrate strong engagement of canonical miR-26a targets in the intestinal epithelium of transgenic animals.

GSEA analysis also revealed broad repression of pro-proliferative transcripts in miR-26a-expressing epithelial cells. Thirteen of 15 of the top gene ontology biological process gene sets that were repressed in transgenic animals are related to cell cycle control, mitosis, or DNA replication (Fig. 5A). To identify direct miR-26a targets that contribute to the anti-proliferative activity of this miRNA, we compiled a list of genes that are predicted to be miR-26 targets by TargetScan and were repressed by ≥ 1.5 -fold by miR-26a (Supplemental Table S1). On the basis of their previously documented roles in proliferation of colorectal or other types of cancer cells or involvement in Wnt signaling, a key driver of proliferation in this tissue, we selected *Ccnd2*, *Hmga1*, *Mycbp*, and *Setd8* for additional study. *Ccnd2*, an established miR-26a target (Kota et al. 2009), is commonly overexpressed in colorectal tumors (Mermelshtein et al. 2005) and is essential for tumorigenesis in *Apc*^{min/+} mice (Myant and Sansom 2011a). *Hmga1* is an oncogenic chromatin remodeling factor that is broadly overexpressed in human malignancies, including CRC. Transgenic overexpression of *Hmga1* drives intestinal epithelial proliferation and polyp formation, whereas knockdown in colon cancer cell lines reduces tumor-forming ability (Belton et al. 2012). Although not previously studied in the context of the intestinal epithelium, *Mycbp* is believed to act as a coactivator of MYC-mediated transcription, and its depletion impairs proliferation of breast cancer cells (Taira et al. 1998; Xiong et al. 2010). *MYC* is a critical Wnt/ β -catenin target gene that is essential for intestinal homeostasis and tumorigenesis (Myant and Sansom 2011b). Moreover, *MYCBP* itself has been reported to be a Wnt/ β -catenin target, further highlighting its potential importance in intestinal epithelial proliferation (Jung and Kim 2005). *Setd8* is a critical cofactor for Wnt target gene activation (Li et al. 2011) and, accordingly, GSEA analysis revealed significant down-regulation of β -catenin targets in miR-26a transgenic epithelium (Supplemental Fig. S9B). Last, although microarray analysis showed a <1.5 -fold decrease in expression, we included *Ezh2* in validation studies because GSEA analysis showed significant repression of *EZH2* target gene sets in transgenic mice (Supplemental Fig. S9C). *EZH2* is a verified miR-26 target that is overexpressed in CRCs, and its depletion impairs proliferation of human CRC cells (Fluge et al. 2009; Fussbroich et al. 2011).

Quantitative PCR confirmed significant down-regulation of *Ccnd2*, *Hmga1*, *Mycbp*, *Setd8*, and *Ezh2* in miR-

26a-expressing intestinal epithelium (Fig. 5B). Consistent with its effects on PTEN protein abundance (Supplemental Fig. S3), miR-26a also strongly repressed *Pten* transcript levels in epithelial cells. Multiple studies have previously validated direct targeting of *Ccnd2* and *Ezh2* by miR-26 (Sander et al. 2008; Kota et al. 2009; Lu et al. 2011). Both *Hmga1* and *Mycbp* have a single highly conserved predicted miR-26-binding site in their 3' UTRs, whereas *Setd8* has two conserved predicted sites (Fig. 5C,E,G; Supplemental Fig. S10A). Consistent with a recent report (Lin et al. 2013), we documented that the human and mouse *HMGA1* miR-26-binding sites, but not mutated versions, conferred miR-26a-mediated repression when placed in the 3' UTR of a luciferase reporter transcript (Fig. 5D; Supplemental Fig. S11A). Similar experiments validated the functionality of the *MYCBP* and one of the *SETD8* miR-26-binding sites (Fig. 5F,H; Supplemental Figs. S10B,C, S11B,C).

The tumor suppressor activity of miR-26 predominates in intestinal cancer

The inducible miR-26a transgenic mouse described here provides a valuable model for the evaluation of miR-26 functions in various tissue contexts in vivo. Given the previous findings of both pro- and anti-tumorigenic activity of miR-26, it has remained unclear whether delivery of this miRNA would be a safe and efficacious therapeutic approach for specific cancers. We now show that broad overexpression of miR-26a for prolonged periods is well tolerated and does not result in any overt malignancy. Nevertheless, as reported (Huse et al. 2009), we confirmed that miR-26a represses the dosage-sensitive tumor suppressor *Pten*. The intestinal epithelium is a site of particularly high expression of miR-26a in *M2rtTA; eGFP.miR-26a* mice, exhibits a corresponding

Figure 4. miR-26a cell-autonomously inhibits intestinal epithelial proliferation. (A) Distribution of BrdU⁺ cells in small intestines of dox-treated mice of the indicated genotypes 72 h after BrdU administration. (B) Quantification of the fraction of BrdU⁺ cells in the upper third of villi. For all panels, 30 villi per mouse and three mice per genotype were quantified, and P -values were calculated using a nested ANOVA. Box plots are defined as in Figure 2B. (C,D) Representative p-H3 staining (C) and quantification (D). (E,F) BrdU staining (E) and quantification (F) as described in A and B.

Figure 5. miR-26a represses proliferative targets in intestinal epithelium. (A) The top 15 gene ontology biological process terms enriched among miR-26a-repressed transcripts identified by GSEA. (B) Expression of the indicated transcripts, normalized to 18S rRNA, in purified intestinal epithelium from dox-treated mice of the indicated genotypes. Means and standard deviations from $n = 3$ –5 animals per genotype shown. (C,E,G) Schematic representation of the miR-26-binding sites in the 3' UTRs of *HMGA1* (C), *MYCBP* (E), and *SETD8* (G). Mutations introduced into reporter constructs are shown below each alignment and are highlighted in red. (D,F,H) Relative firefly luciferase activity of reporter constructs containing the indicated miR-26-binding site or its mutated version following transfection into HCT116 cells with control or miR-26a mimic. $n = 3$ replicates per condition. (*) $P < 0.05$; (**) $P < 0.01$ (two-tailed t -test).

strong down-regulation of PTEN, and has been shown to be prone to spontaneous tumor development in the setting of *Pten* haploinsufficiency (Di Cristofano et al. 1998). The absence of any baseline intestinal pathology in transgenic animals and the strong suppression of tumorigenesis in *Apc*^{min/+} mice demonstrate that the tumor suppressor activity of miR-26a overrides the potential oncogenic consequences of *Pten* repression in this tissue. Importantly, the balance between miR-26-mediated tumor suppression versus oncogenic activity may differ in distinct tissue or tumor contexts, likely explaining why expression of miR-26 accelerates tumorigenesis in settings such as PDGF-driven gliomagenesis in mice (Huse et al. 2009). Combining the *LSL.eGFP.miR-26a* mouse with various tissue-specific tTA or Cre driver lines and tumor models offers a flexible experimental approach for elucidating the effects of miR-26 expression in different cancer settings in vivo.

miR-26 represses a broad proliferative gene expression program in intestinal epithelium, likely accounting

for its potent anti-proliferative effects and tumor suppressor activity in this tissue. In particular, the miR-26 targets *Ccnd2*, *Hmga1*, and *Ezh2* are all known to be important for intestinal tumorigenesis (Fussbroich et al. 2011; Myant and Sansom 2011a; Belton et al. 2012). Our finding that *Mycblp*, a Wnt/ β -catenin target gene demonstrated to be important for cell proliferation in other cell types (Jung and Kim 2005; Xiong et al. 2010), is directly repressed by miR-26 suggests that it may also participate in intestinal neoplasia. Likewise, impairment of Wnt signaling through down-regulation of *Setd8* (Li et al. 2011), another newly identified miR-26 target, would be expected to potentially inhibit intestinal epithelial migration, proliferation, and neoplastic transformation. In aggregate, the predicted result of coordinated depletion of these targets could plausibly overcome the protumorigenic consequences of *Pten* down-regulation in this tissue.

In addition to establishing the tumor suppressor activity of miR-26 in the mouse intestine, our analysis of human CRC specimens has clarified previous work that has inconsistently detected reduced expression of this miRNA family in human colorectal tumors (Ma et al. 2011; Gaedcke et al. 2012; Zheng et al. 2013). Through the analysis of purified CRC epithelial cells, the relevant transformed cell type in this malignancy, we documented a clear reduction of miR-26a and miR-26b in these cancer cells. These findings are consistent with a tumor suppressor role for miR-26 in human CRC. Given that broad miR-26a expression was well tolerated in mice and potentially suppressed intestinal tumorigenesis, these findings suggest that miR-26 delivery could be an efficacious and nontoxic therapeutic strategy for colon cancer.

Materials and methods

Mouse strains

CMV-Cre (Schwenk et al. 1995), *Apc*^{min/+} (Moser et al. 1990), *Villin-Cre* (Madison et al. 2002), and *Lgr5*^{+/eGFP} (Barker et al. 2007) mice were obtained from Jackson Laboratory. Strains used in this study were backcrossed to C57BL/6 mice for at least 10 generations. Dox (2 mg/mL, supplemented with 10 mg/mL sucrose) was administered in the drinking water to induce transgene expression. All experiments were approved by the Institutional Animal Care and Use Committees of the Johns Hopkins University School of Medicine and The University of Texas Southwestern Medical Center.

Apc^{min/+} adenoma quantification

Apc^{min/+}; *M2rtTA* and *Apc*^{min/+}; *M2rtTA*; *eGFP.miR-26a* mice were given dox at 28 d of age. At 150 d of age, animals were sacrificed, and intestines were fixed overnight in 10% neutral buffered formalin. A dissecting microscope with a reticle was used to quantify adenoma number and size in a genotype-blind manner.

Isolation of human and mouse intestinal epithelial cells

Mouse and human intestinal epithelial isolation and sorting of LGR5⁺ cells from *Lgr5*^{+/eGFP} mice were carried out as previously described (Chivukula et al. 2014). Tissue from colorectal tumors and paired normal colons was obtained through the University of Texas Southwestern Tissue Resource.

Microarray data have been deposited in the Gene Expression Omnibus repository under accession number GSE62831.

Acknowledgments

We thank Chip Hawkins in the Johns Hopkins Transgenic Core for assistance with generation of *LSL.eGFP.miR-26a* mice, John Shelton in the University of Texas Southwestern Molecular Pathology Core for assistance with histopathology, Cheryl Lewis in the University of Texas Southwestern Tissue Resource Core for assistance with human tissue

specimens, Quan-Zhen Li in the University of Texas Southwestern Microarray Core for assistance with gene expression profiling, and Jeanetta Marshall-Wynn for mouse colony maintenance. Kathryn O'Donnell, Tsung-Cheng Chang, and Kenneth Chen provided valuable technical assistance and advice. This work was supported by grants from Cancer Prevention Research Institute of Texas (CPRIT) (R1008 to J.T.M.) and the National Institutes of Health (R01CA120185 and P01CA134292 to J.T.M., 5P30CA142543 to the University of Texas Southwestern Simmons Cancer Center, and 5T32GM007309 to L.R.Z. and R.R.C.). J.T.M. is a CPRIT Scholar in Cancer Research.

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Genes Dev. 2014, **28**: originally published online November 13, 2014
Access the most recent version at doi:[10.1101/gad.250951.114](https://doi.org/10.1101/gad.250951.114)

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