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LIN28B fosters colon cancer migration, invasion, and transformation through *let-7* dependent and independent mechanisms

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Abstract

Lin28b is an RNA-binding protein that inhibits biogenesis of *let-7* microRNAs. *LIN28B* is overexpressed in diverse cancers, yet a specific role in the molecular pathogenesis of colon cancer has yet to be elucidated. We have determined that human colon tumors exhibit decreased levels of mature *let-7* isoforms and increased expression of *LIN28B*. In order to determine *LIN28B*'s mechanistic role in colon cancer, we expressed *LIN28B* in immortalized colonic epithelial cells and human colon cancer cell lines. We found that *LIN28B* promotes cell migration, invasion, and transforms immortalized colonic epithelial cells. In addition, constitutive *LIN28B* expression increases expression of intestinal stem cell markers *LGR5* and *PROM1* in the presence of *let-7* restoration. This may occur as a result of Lin28b protein binding *LGR5* and *PROM1* mRNA, suggesting that a subset of *LIN28B* functions are independent of its ability to repress *let-7*. Our findings establish a new role for *LIN28B* in human colon cancer pathogenesis, and suggest *LIN28B* post-transcriptionally regulates *LGR5* and *PROM1* through a *let-7* independent mechanism.

Keywords

LIN28B; LIN28; Let-7; colon cancer; LGR5; PROM1

Introduction

LIN28B is a homolog of *LIN28* (also called *LIN28A*) (Guo et al., 2006), which induces pluripotency in somatic cells when expressed in concert with *KLF4*, *SOX2*, and *NANOG*(Yu

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Conflict of interest

The authors declare no conflict of interest.

et al., 2007). A high degree of homology exists between *LIN28B*, *LIN28* and the heterochronic gene lin-28 in *C. elegans* (Moss and Tang, 2003). Human *LIN28* and *LIN28B* each contain a cold-shock domain and CCHC zinc fingers that confer RNA binding ability(Moss and Tang, 2003). The ability to bind RNA is critical to both Lin28 and Lin28b, as inhibition of *let-7* microRNA biogenesis is a cardinal feature of their functions.

The *let-7* family of microRNAs comprises isoforms with highly conserved sequences that exhibit functional redundancy (Pasquinelli *et al.*, 2000; Zhao *et al.*, 2010). *Let-7* biogenesis resembles that of most microRNAs in that the various isoforms are transcribed initially by RNA polymerase II as pri-microRNAs, and processed by Drosha and DGCR8 into pre-microRNAs that are subsequently exported from the nucleus (Gregory et al., 2004; Han et al., 2004; Lee et al., 2003; Lee et al., 2002). The hairpin loops of pre-microRNAs are cleaved by dicer in the cytoplasm to yield microRNA:microRNA duplexes that are disassociated to release mature *let-7* (Lee et al., 2003; Lee et al., 2002). MicroRNAs are incorporated into the RNA induced silencing complex (RISC) and bind the 3′ UTR of target transcripts to provide post-transcriptional gene regulation by mRNA sequestration or cleavage (Esquela-Kerscher and Slack, 2006).

Several established *let-7* mRNA targets have tumor promoting properties, including the canonical target *HMGA2* (Lee and Dutta, 2007; Mayr *et al.*, 2007; Park *et al.*, 2007) and the classic oncogenes *KRAS* and *c-MYC* (Akao et al., 2006; Johnson et al., 2007; Johnson et al., 2005). In addition, *let-7* microRNAs have been described as tumor suppressors and are implicated as prognostic factors in a variety of divergent cancers (Akao et al., 2006; Shell et al., 2007; Takamizawa et al., 2004). Importantly, Lin28 and Lin28b may relieve *let-7* target suppression by binding to immature *let-7* molecules and blocking further processing (Hagan et al., 2009; Heo et al., 2008; Heo et al., 2009).

In approximately two-thirds of colon cancers evaluated, we find that *let-7* microRNA levels are decreased when compared to adjacent normal colonic mucosa (unpublished observations). Interestingly, pri-*let-7* levels are maintained in colon cancer cells that display reduced mature *let-7* levels, suggesting a post-transcriptional mediated mechanism of *let-7* down-regulation. The regulator of *let-7* biogenesis *LIN28B*, also a *let-7* target (Boyerinas et al., 2008), is specifically implicated in this process because it is transactivated by c-myc (Chang et al., 2009). Nearly 70% of colorectal tumors harbor elevated levels of c-myc (Erisman et al., 1985); up-regulation occurs in the early stages of colon carcinoma as a consequence of Wnt pathway deregulation and β-catenin stabilization (Clevers, 2006; He *et al.*, 1998; Powell *et al.*, 1992; Rubinfeld *et al.*, 1993; Sikora *et al.*, 1987; Stewart *et al.*, 1986).

We hypothesized that *LIN28B* promotes colon tumorigenesis via suppression of *let-7*. To test this hypothesis, we constitutively expressed *LIN28B* in immortalized colonic epithelial cells and human colon cancer cell lines. We found that *LIN28B* expression increases cell migration, invasion, and soft-agar colony formation. The ability of constitutive *LIN28B* expression to promote migration and invasion is partially reversed by concomitant *let*-7 expression, suggesting these phenotypes are dependent upon Lin28b's ability to repress *let-7* biogenesis. Notably, we also found that the intestinal/colonic stem cell markers *LGR5* and

PROM1 are upregulated with constitutive *LIN28B* expression. *LGR5* and *PROM1* do not contain putative *let-7* binding sites in their 3′ UTR, and are not predicted *let-7* targets. *LGR5* and *PROM1* remain up-regulated even in the presence of *let-7* restoration in *LIN28B*-expressing cells. Mechanistically, *LGR5* and *PROM1* transcripts are enriched in Lin28b mRNA binding assays. Furthermore, Lin28b induces *LGR5* and *PROM1* 3′ UTR sequences in luciferase reporter assays. These data suggest that Lin28b modulates these genes in a *let-7* independent manner, which is a novel finding. Taken together, our data demonstrate that Lin28b promotes migration, invasion, and transformation, while up-regulating stem cell genes through mRNA binding.

Materials and Methods

Mature microRNA detection in human tumors and LIN28B-expressing cells

Total RNA was extracted from human tumors and genetically modified DLD1 and LoVo cells using the mirVana miRNA isolation kit (Ambion, Austin, TX). A Taqman® MicroRNA Assay kit (Applied Biosystems, Carlsbad, California) was employed to synthesize probe-specific cDNA for both *let-7a* and *let-7b* using TaqMan® Universal PCR Master Mix, No AmpErase UNG (Applied Biosystems, Carlsbad, CA) from 10ng of total RNA per sample. Levels of mature microRNAs were measured via qPCR for the probe-specific cDNA using proprietary primers (Applied Biosystems, Carlsbad, California) using ABI Prism 7000 Sequence Detection System (Applied Biosystems, Carlsbad, CA). PCR reactions were performed in triplicate and standardized to levels of endogenous U47. Fold change for *let-7a* and *let-7b* was determined by normalization to empty vector controls. Statistical significance of comparisons between empty vector and *LIN28B*-expressing cells was determined by applying student's t-test, with $p<0.05$ considered significant.

Biostatistical analysis of LIN28B and let-7 expression in human tumors

The distributions of *LIN28B*, *let-7a* and *let-7b* fold change were right-skewed, hence a log-transformation was used to achieve approximate normality. Pearson correlation coefficients were calculated to assess the strength of the linear association between *LIN28B* vs. *let-7a* and *let-7b*. Statistical significance of comparisons between empty vector and *LIN28B* transduced cells in migration, invasion, soft-agar assays were determined by applying student's t-test.

Generation of constitutive LIN28B-expressing cell lines

Stable *LIN28B* expression in IEC-6, DLD-1, and LoVo cells was achieved using MSCV-PIG-*LIN28B* and empty vector control plasmids (gifts from Dr. Joshua Mendell). We transfected Phoenix E (for rodent cell lines) and Phoenix A (human cell lines) cells with 30ug plasmid DNA, and monitored transfection efficiency via detection of GFP expression by light microscopy prior to virus collection. Viral containing supernatant was collected 48hrs post-transfection, filtered through a 0.45μm membrane, immediately placed in liquid nitrogen, and stored at −80° C for later use. IEC-6, DLD-1, and LoVo cells were infected by applying virus-containing media plus polybrene (4 μg/ml) to cells, then subjecting them to centrifugation at 1000g for 90 minutes. Inoculated cells were selected in puromycin, expanded, and subsequently sorted for high GFP intensity and corresponding *LIN28B*

expression. Expanded cell cultures were maintained in DMEM plus 10% FBS at 37 °C, 5% CO_2.

Let-7 expression in cell lines constitutively expressing LIN28B

We obtained a *let-7a* lentiviral expression vector (gift from Dr. Jerome Torrisani), which was developed by modifying pLenti6.2-GW (pBLOCK-iT) of the pLenti6/UbC/V5-DEST Gateway system (Invitrogen, Carsblad, CA). We transduced *LIN28B*-LoVo and *LIN28B*-DLD-1 cells using ViraPower (Invitrogen, Carsblad, CA) as per the manufacturer's protocol. GFP expression was monitored in transduced cells via light microscopy. GFP-positive cells were subsequently selected in blasticidin prior to expansion.

Intestinal crypt RNA isolation

Intestinal crypts were isolated from adult BL6 females as previously described by Flint, *et al*, 1991. RNA was isolated using TRIzol (Invitrogen, Carsblad, CA) and 1 μg total RNA use for reverse transcriptase reactions with superscript III (Invitrogen, Carlsblad, CA) and oligo dT.

Real-time quantitative PCR (rt-qPCR)

We used 3 μg isolated RNA for cDNA synthesis with random oligomers. cDNA was synthesized from 5 μg total RNA per sample using random hexamers and SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA). Synthesized cDNA was then subjected to gene expression analysis for *LIN28B*, *PROM1*, *LGR5*, *HMGA2*, and *IGF2BP1* probes, with β-actin as an endogenous control (Ambion, Austin, TX). Real-time qPCR was conducted on the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Carlsbad, CA) for each probe using TaqMan® Universal PCR Master Mix (Applied Biosystems, Carlsbad, CA) as per the manufacturer's protocol using. Fold change for each transcript was determined by normalization to empty vector controls. The statistical significance of comparisons between empty vector DLD1 and LoVo versus *LIN28B*-DLD1 and *LIN28B*-LoVo cells was evaluated by applying student's t-test, with $p<0.05$ considered significant.

Western blot analysis

Cells were lysed in RIPA buffer (5 ml 1M tris-cl PH 7.4, 30 ml 5 M NaCl, 5 ml 20% NP-40, 5 ml 10 % sodium deoxycholate, 0.5 ml 20% SDS, 50 mL ddH2O) containing protease inhibitor cocktail (Roche Diagnostics, Manheim Germany). Cellular debris was removed from lysates via centrifugation, and protein was quantitated via the BCA Protein Assay Kit (Thermo Scientific, Rockford, IL). 20 ug total protein was loaded onto 4-12% gradient gels for electroporesis using Invitrogen western blotting apparatus. Proteins were transferred to PVDF membranes as per Invitrogen protocol, blocked in 5% non-fat milk in TBS-T, and blotted with Lin28b (Abcam, Cambridge, MA), CDC34 (BD Transduction Laboratories, San Jose, California), IGF2BP1 (Cell Signaling, Boston, MA), HMGA2 (Santa Cruz, Santa Cruz, California) or β-actin (Sigma-Aldrich, St. Louis, MO) primary antibodies as per the manufacturer's protocol.

Migration and invasion assays

Invasion assay inserts (BD Biosciences, San Jose, California) were rehydrated in tissue culture incubators for 2 hours with 500μl serum-free DMEM prior to use. Cells were trypsinized, washed with PBS and resuspended in serum-free DMEM. 50,000 cells per well were plated in the upper chamber of a fluorescent transwell assay systems atop a migration insert (BD Biosciences, San Jose, California) or a rehydrated invasion assay insert in a final volume of 500μl. 750μl complete media (DMEM + 10% FBS) was added to the lower chamber as a chemoattractant. The assay system was placed in 37 °C, 5% CO_2 tissue culture incubators overnight. Assay inserts were washed in PBS, then stained with calcein AM (4 μg/ml). Fluorescence was detected at 580nm using a plate reader as per the manufacturer's protocol. Experiments were performed in triplicate and data shows mean fluorescence, with error bars depicting standard deviation.

Soft agar assays

Empty vector and *LIN28B*-expressing cells were trypsinized washed in PBS, and resuspended as a 2 ml single cell suspension containing: 50,000 cells/ml, 0.67% agarose, 10% FBS, and DMEM. The soft-agar suspensions were plated in triplicate over 3 ml of solidified 1% agarose, 10% FBS, and DMEM in 6-well plates; plated soft-agar/cell suspensions were permitted to solidify prior at room temperature prior to incubation at 37 °C, 5% CO_2. Colonies were photographed and counted at 6 weeks for IEC-6 cells, and 2 days for DLD-1 and LoVo cells. The statistical significance of comparisons between empty vector *LIN28B*-expressing colonies was determined by applying student's t-test, with $p<0.05$ considered significant.

RNA immunoprecipitation and RT-qPCR

Fifteen hours prior to collection, *LIN28B*-expressing DLD-1 and LoVo cells were incubated with 100 μM 4-thiouridine in medium. At time of collection, plates were washed once with PBS and crosslinked with 0.15 J/cm^2 of 365 nm UV light on ice. Cells were collected in NP40 lysis buffer (per (Hafner *et al.*; Keene *et al.*, 2006) supplemented with protease, phosphatase and RNase inhibitors; lysates were cleared by centrifugation and frozen at −80°C overnight. Protein A magnetic beads (Millipore, Billerica, MA) were washed in NT2 buffer (per Hafner *et al.*; Keene *et al.*, 2006) and blocked in 5% BSA, 200 μg/ml yeast tRNA for 1 hour at 4°C. Beads were washed with NT2 and incubated with secondary antibody (polyclonal Mouse anti-Rabbit IgG – Jackson ImmunoResearch, West Grove, PA) at room temperature for 1 hour. Beads were washed with NT2 and incubated with Lin28B (Cell Signalling, Danvers, MA) primary antibody or no primary antibody for 1 hour at room temperature. After thawing, lysates were precleared with Protein A magnetic beads for 1 hour, after which they were incubated with the beads-antibody complex for 1 hour at room temperature. After incubation beads were washed with high salt NT2 buffer (per Keene et al. but with 500 mM NaCl). Beads were digested with Proteinase K for 30 minutes at 55 °C and the supernatant was collected. RNA was recovered using Qiagen's RNeasy Micro Kit, cDNA was generated using oligo(dT) RT. qPCR was done using Taqman probes for *IGF2*, *LGR5*, and *PROM1* (Applied Biosystems, Carlsbad, California). Three independent experiments for *LIN28B*-LoVo and *LIN28B*-DLD-1 cells were performed, each in triplicate.

Luciferase Assays

LGR5 and *PROM1* 3′ ORF expression clones were obtained from GeneCopoeia (Rockville, MD). The 3′ UTRs of *LGR5* and *PROM1* were amplified using Platinum Taq (Invitrogen, Carsblad, CA), then subcloned into the pGL3-promoter luciferase reporter plasmid. The resulting reporter plasmids were utilized to transfect empty vector and *LIN28B*-expressing LoVo cells in 12-well plates in triplicate using Lipofectamine 2000 (Invitrogen, Carsblad, CA). 20 hours following were transfection, cells were subjected to a Dual Luciferase Reporter Assay (Promgea, Madison, WI) via passive lysis and transfer to 96-well plate for automated processing.

Results

Reduced levels of mature *let-7a* and *let-7b* microRNAs and increased *LIN28B* expression in colon tumors

We measured levels of mature *let-7* microRNA isoforms in four samples of human normal colonic epithelia via qPCR. We found that *let-7a* and *let-7b* are the predominant *let-7* isoforms present in the normal colon (Figure 1). Subsequently, we interrogated 22 human colon adenocarcinomas paired with adjacent normal colonic mucosa by measuring levels of mature *let-7a* and *let-7b*. Of the 22 pairs assayed, 10 tumors displayed reduced levels (greater than 60% reduction) of *let-7a* and *let-7b* compared to their normal colonic epithelium counterpart (Figure 2a; Table 1). Initially, we surmised this variation in levels of mature *let-7* was attributable to differences in expression levels. Yet, qPCR for the *let-7a-3-b* cluster pri-microRNA sequence revealed similar expression in tumors (data not shown). Alternatively, post-transcriptional regulatory mechanisms may account for *let-7a* and *let-7b* down-regulation in tumors. Since *LIN28B* may be transactivated by c-myc, which is frequently up-regulated in colon cancer, we hypothesized that *LIN28B* overexpression occurs in colon tumors resulting in inhibition of mature *let-7* biogenesis. Consequently, we examined *LIN28B* expression in colon tumors, and found that *LIN28B* transcript levels are increased in a subset of tumors when compared to adjacent normal colonic epithelium (Figure 2b; Table 1). Furthermore, *LIN28B* expression negatively correlates with levels of mature *let-7a* ($r=-0.47$, $p=0.0297$) and *let-7b* ($r=-0.41$, $p=0.0637$) in colon tumors.

LIN28B transforms immortalized colonic epithelial cells and promotes tumorigenesis

To assess the potential oncogenic functions of Lin28b in the colon, we constitutively expressed human *LIN28B* in immortalized intestinal epithelial (IEC-6) via retroviral transduction (Figure 3a). Human Lin28b expression in IEC-6 cells reduces levels of mature *let*-7 isoforms (Figure 3b). However, cellular proliferation rates were not affected (data not shown). We further examined cell behavior using *in vitro* transwell migration and invasion assays, and found that Lin28b promoted both invasion and migration of IEC-6 cells in culture (Figure 3c). Additionally, IEC-6 cells, which do not form colonies in soft agar, do so in the presence of constitutive *LIN28B* expression (Figure 3d). These observations extend to human colon cancer cell lines as well. Constitutive expression of *LIN28B* in DLD-1 (data not shown) and LoVo colon cancer cells reduces mature *let-7a* and *let-7b* levels (Figure 4a-b). Furthermore, Lin28b increases migration, invasion, (Figure 4c) and soft agar colony formation in colon cancer cell lines (Figure 4d).

Let-7 expression partially abrogates phenotype induced by *LIN28B* constitutive expression

We sought to determine whether the increase in migration and invasion induced by constitutive *LIN28B* expression in colon cancer cell lines was *let-7* dependent. To that end, we co-expressed a decoy *let-7a* hairpin molecule in cells constitutively expressing *LIN28B*. The loop structure of this miRNA is derived from mir-30, thereby eliminating the Lin28b binding site and allowing these *let-7* precursors to evade Lin28b-mediated inhibition. Co-expression of the decoy hairpin molecule increases mature *let-7a* levels by more than 100% in cells that constitutively express *LIN28B* (Figure 5a). We subjected cell lines co-expressing *LIN28B* and *let-7* to Boyden chamber migration and invasion assays and found that *let-7* restoration reduces cell migration and invasion of colon cancer cells in the presence of *LIN28B* overexpression (Figure 5b-c). Notably, co-expression of *let-7* in cells that constitutively express *LIN28B* does not reduce cell migration and invasion to the level of empty vector controls (Figure 5b-c), which may suggest that the ability of Lin28b to promote migration and invasion may be only partially dependent on *let-7* repression.

LGR5 and *PROM1* are upregulated with *LIN28B* overexpression and remain upregulated following let-7 restoration

In order to elucidate further the molecular mechanisms of *LIN28B* functions in colon tumorigenesis, we assessed gene expression in immortalized colonic epithelial and colon cancer cells transduced with a retroviral vector to attain constitutive *LIN28B* expression. As expected, we found increased expression of known *let-7* targets, including *IGF2BP1*, *CDC34*, and *HMGA2* (Boyerinas *et al.*, 2008; Johnson *et al.*, 2007; Lee and Dutta, 2007) (Figure 6a).

Intriguingly, constitutive *LIN28B* expression also up-regulates the intestinal/colonic epithelial stem cell markers *LGR5* and *PROM1*. This finding is particularly intriguing in light of the fact that *LIN28B* is also expressed in the intestinal crypts (Supplementary Figure 1). Thus, the ability of *LIN28B* to increase *LGR5* and *PROM1* expression may indicate a possible role for *LIN28B* in stem cell function. Accordingly, we sought to elucidate the mechanisms whereby *LIN28B* up-regulates *LGR5* and *PROM1* transcripts.

Interestingly, *LGR5* and *PROM1* lack conventional *let-7* binding sites in their 3′ UTR sequences, and are not predicted by Target Scan and miRanda algorithms to be *let-7* targets. In order to determine whether regulation of these genes by Lin28b was *let-7* dependent, we restored mature *let-7* levels in cells expressing constitutive *LIN28B* via co-expression of decoy *let-7* hairpin molecules, then measured *LGR5* and *PROM1* transcript levels. While co-expression of *let-7* in *LIN28B* cells resulted in decreased levels of established *let-7* targets, *HMGA*2 and *IGF2BP1* (Figure 6b), *LGR5* and *PROM1* remained elevated following *let-7* restoration (Figure 6c), suggesting regulation of these genes by *LIN28B* may occur via a *let-7* independent mechanism.

LGR5 and *PROM1* mRNAs associate with Lin28b protein

The cold-shock domain and dual zinc fingers present in Lin28b confer RNA binding ability, and thus permit Lin28b to bind and inhibit *let-7* microRNAs. However, the RNA-binding activity of Lin28b may also be involved in regulation of additional mRNA transcripts. A

similar phenomenon has been described for the Lin28b homolog Lin28, which binds to and stabilizes *IGF2* mRNA during differentiation of myoblasts (Polesskaya *et al.*, 2007). In order to determine whether Lin28b associates with *LGR5* and *PROM1* mRNA transcripts, we first examined RNA-protein interactions of Lin28b in colon cancer cell lines through RNA immunoprecipitation followed by quantitative RT-qPCR. *IGF2* transcripts are enriched in Lin28b immunoprecipitates, suggesting Lin28b binds *IGF2* (Figure 7a), as shown previously for Lin28. We also observed greater than ten-fold enrichment of *LGR5*, and greater than five-fold enrichment of *PROM1* in RNA pools that co-immunoprecipitate with Lin28b (Figure 7a). We did not observe enrichment for *GAPDH* (Figure 7a), *TBP*, or *HPRT1* (data not shown) mRNA in Lin28b immunoprecipitates, indicating that the interaction of Lin28b with *LGR5* and *PROM1* transcripts is specific. In order to further evaluate the specificity of these protein-mRNA interactions, we subcloned the 3′ UTRs of both *LGR5* and *PROM1* downstream of luciferase coding regions. The resulting plasmids were transfected into empty vector and *LIN28B*-expressing colon cancer cells. We observed increased luciferase activity with the addition of *LGR5* and *PROM1* 3′ UTRs to luciferase mRNA in cells that constitutively express *LIN28B* (Figure 7b), suggesting Lin28b enhances translation of transcripts containing these 3′ UTRs. Taken together, these data suggest *LGR5* and *PROM1* transcripts may be regulated directly in cells by Lin28b through its intrinsic RNA-binding activity, implicating a novel *let-7* independent mechanism of Lin28b function in mRNA regulation.

Discussion

We found the major *let-7* isoforms expressed in normal colonic epithelium (*let-7a* and *let-7b*) are down-regulated in approximately two-thirds of colon tumors. In a subset of these tumors, we found concomitant upregulation of the post-transcriptional inhibitor of *let-7* processing, namely *LIN28B*. In order to determine the role of *LIN28B* in colon cancer, we constitutively expressed *LIN28B* in immortalized rat colonic epithelial cells and transformed human colon cancer cell lines. We found that constitutive *LIN28B* expression fosters cell migration, invasion, and cellular transformation, demonstrating a previously unrecognized role for *LIN28B* in colon tumorigenesis.

Furthermore, we found that constitutive *LIN28B* expression in immortalized colonic epithelial and colon cancer cells results in decreased levels of mature *let-7* isoforms, thereby relieving suppression of *let-7* targets. Interestingly, expression of *LGR5* and *PROM1* – intestinal/colonic stem cell markers that are not predicted *let-7* targets – is also increased in the presence of Lin28b. *LGR5* and *PROM1* expression remains upregulated following restoration of *let-7* in cells constitutively expressing *LIN28B*, and Lin28b protein is capable of binding *LGR5* and *PROM1* transcripts. Taken together, these results suggest that a subset of Lin28b functions may occur via *let-7* independent mechanisms.

We have demonstrated increased *LIN28B* expression in colon tumors, which likely occurs as a result of increased *LIN28B* transcriptional activity mediated by c-myc. Since c-myc is a transcriptional target of canonical Wnt signaling, it is possible that *LIN28B* is up-regulated in colon tumors as a consequence of *APC* mutation (or other changes that deregulate Wnt signaling), which occurs in the vast majority of colon tumors (Pino and Chung, 2010).

Alternatively, up-regulation of *LIN28B* in colon tumors may occur as a result of increased mRNA stabilization. Interestingly, this may occur as a result of decreased *let-7* in colon tumors, since *LIN28B* is also a predicted *let-7* target. The ability of *let-7* and *LIN28B* to regulate one another other likely represents a feedback loop that allows the cell to tightly regulate levels of each, further highlighting their importance in cellular processes.

We have shown that Lin28b does not function exclusively via repression of *let-7* biogenesis. Interestingly, recent evidence demonstrates that Lin28 also does not function exclusively through inhibition of *let-7* processing, but blocks gliogenesis in favor of neurogenesis in undifferentiated cells by stabilizing *IGF2* mRNA (Balzer *et al.*, 2010). Considering the high degree of homology between Lin28 and Lin28b, and the RNA-binding activity inherent to both, it is possible that both Lin28 and Lin28b modulate expression of a number of genes in addition to *IGF2*, *LGR5*, and *PROM1*, independent of their ability to inhibit *let-7* biogenesis. As both Lin28 and Lin28b are implicated in multiple processes, including tumorigenesis, pluripotency, and cell fate decision, it becomes increasingly important to fully elucidate the mechanisms by which these homologs function as pursued in this study.

Mutations that specifically ameliorate the ability of Lin28b to inhibit *let-7* would be useful in further elucidating the mechanisms of Lin28b's functions. One might consider introducing point mutations into the cold-shock domain of Lin28b; this approach has been utilized previously for evaluating *let-7* independent functions of Lin28 (Balzer *et al.*, 2010). However, cold-shock domain mutations may disrupt all RNA-binding activities of *LIN28B*, for example, *LGR5* and *PROM1* binding, thereby precluding assessment of specific *let-7* independent functions. Thus, determining the specific domains and/or amino acid residues of *LIN28B* essential for repression of *let-7* biogenesis is critical.

Our finding that *LIN28B* up-regulates *LGR5* and *PROM1* points to a potential specific function of *LIN28B* in intestinal and colonic epithelial stem cells. Within the intestine, expression of the cell surface protein *PROM1* is restricted to the crypts and adjacent epithelial cells (Snippert *et al.*, 2009), while expression of the orphan receptor *LGR5* occurs exclusively in cycling columnar cells within the crypt base (Barker *et al.*, 2007). Since co-expression of *LGR5* and *PROM1* marks intestinal and colonic epithelial stem cells, up-regulation of these factors by Lin28b suggests a possible role for Lin28b in establishment and/or maintenance of intestinal stem cells.

Interestingly, adenomas may arise in the colon from *PROM1*$^{+}$ crypt cells (Zhu *et al.*, 2009), and overexpression of *LGR5* in colorectal adenocarcinomas correlates with late-stage tumorigenesis, invasion, and metastasis (Uchida *et al.*, 2010). We have demonstrated that constitutive *LIN28B* expression promotes both tumorigenesis as well as induction of *LGR5* and *PROM1* in colonic epithelial cells. *LGR5* and *PROM1* up-regulation occurring in the context of tumorigenesis fostered by *LIN28B* overexpression may support the emerging premise of stem cells in sustaining tumorigenesis. Targeting stem cell-like tumor cells within colon cancers is potentially an effective therapeutic strategy, and overexpression of *LIN28B* may serve as an indicator of stem cell-like tumor cells that could be targeted. This underscores the importance of fully elucidating the role of *LIN28B* in both tumorigenesis

and pluripotency within the colon, as well as in other tissues where *LGR5* and *PROM1* mark stem cell populations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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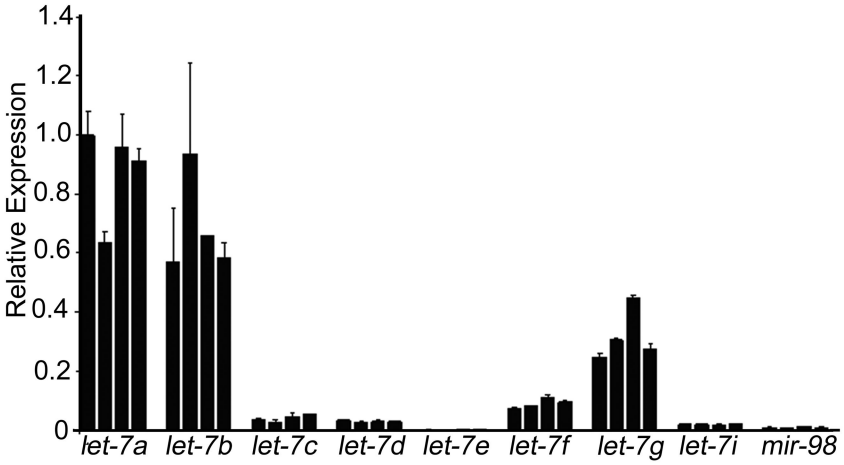

Figure 1. Expression of let-7 isoforms in isolated human colonic epithelia

Levels of mature *let-7* microRNA family members were measured in four isolated normal human colonic epithelia via qPCR. Levels of mature microRNAs are depicted relative to *let-7a*. *let-7a* and *let-7b* account for the vast majority of *let-7* microRNAs present in human colonic epithelia.

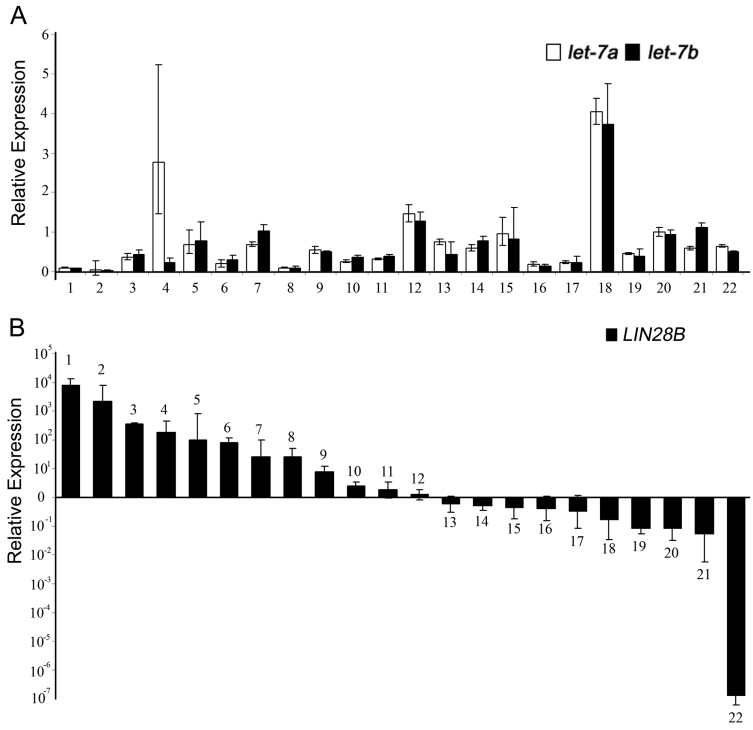

Figure 2. Mature *let-7a* and *let-7b* levels and *LIN28B* expression in human colon tumors

LIN28B mRNA and mature *let-7a* and *let-7b* microRNAs measured in human colon tumors via qPCR. *LIN28B* expression inversely correlates with levels of mature *let-7a* (r=−0.47, p=0.0297) and *let-7b* (r=−0.41, p=0.0637) mature levels in colon tumors. Data summarized in Table 1.

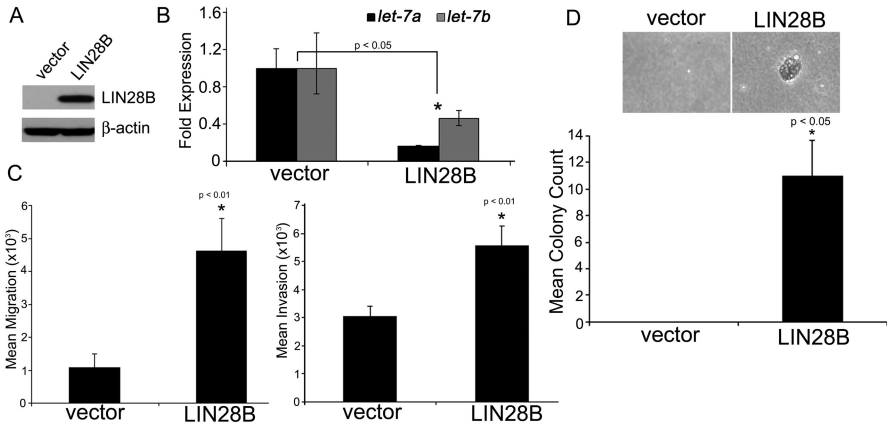

Figure 3. LIN28B constitutive expression promotes invasion, migration, and soft-agar colony formation in IEC-6 cells

(A) Western blot for Lin28b in IEC-6 cells transduced with MSCV-PIG-LIN28B retrovirus. (B) Decreased levels of mature *let-7a* and *let-7b* in IEC-6 cells following *LIN28B* constitutive expression. (C) Lin28b increases migration and invasion in IEC-6 cells. (D) *LIN28B* expression confers the ability of IEC-6 cells to form colonies in soft-agar.

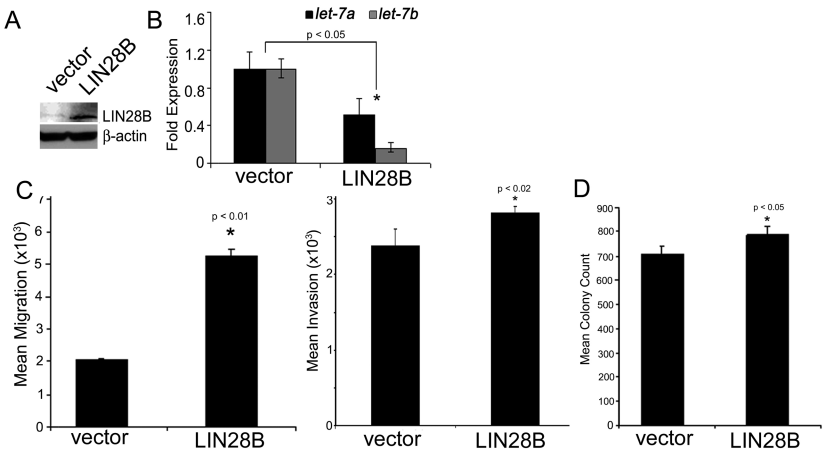

Figure 4. LIN28B constitutive expression promotes invasion, migration, and soft-agar colony formation in colon cancer cells

(A) Western blot for Lin28b in LoVo colon cancer cells transduced with MSCV-PIG-LIN28B retrovirus. (B) Decreased levels of mature *let-7a* and *let-7b* in LoVo cells following *LIN28B* constitutive expression. (C) *LIN28B* overexpression in LoVo cells increases migration and invasion. (D) *LIN28B* expression in colon cancer cells increases soft-agar colony formation.

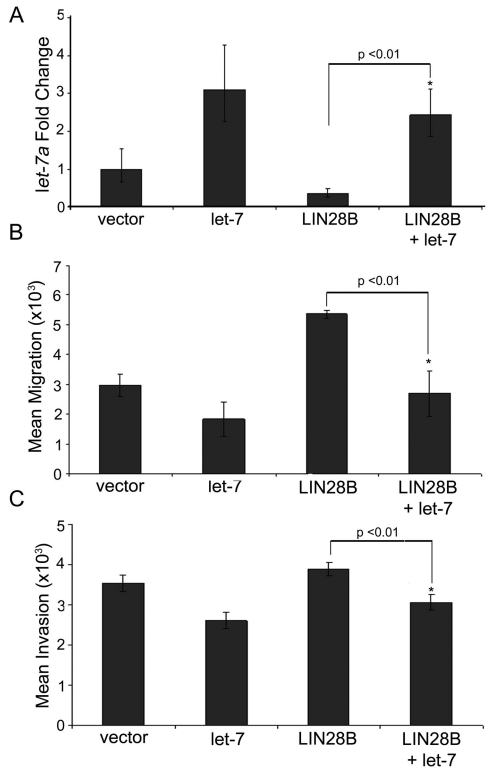

Figure 5. let-7 restoration abrogates increased migration and invasion phenotype of colon cancer cells constitutively expressing LIN28B

(A) qPCR for mature *let-7a* in *LIN28B*-LoVo cells transiently transfected with Lin28b-resistant *let-7a* hairpin precursors. Mature *let-7a* levels are restored with decoy hairpin expression in cells that constitutively express *LIN28B*. (B) Restoration of *let-7a* and *let-7b* reduces *in vitro* migration of *LIN28B*-LoVo cells. (C) Restoration of *let-7a* and *let-7b* reduces *in vitro* invasion of *LIN28B*-LoVo cells.

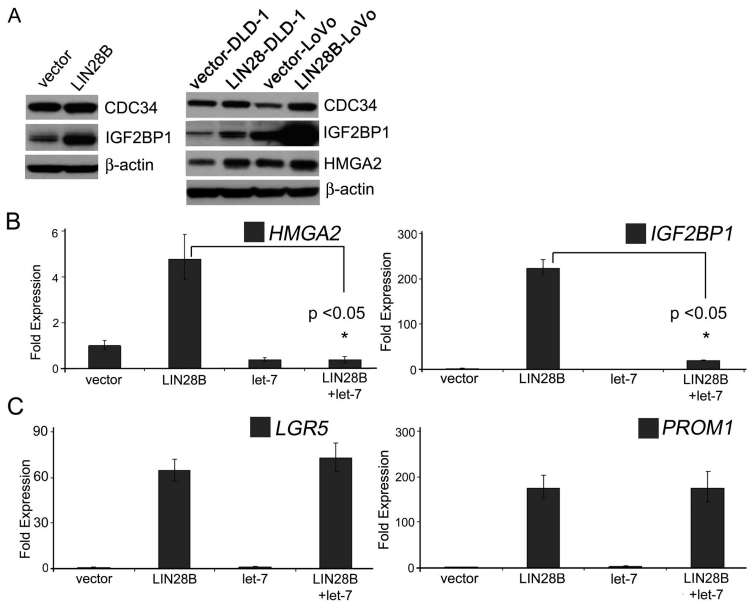

Figure 6. let-7 dependent and independent gene regulation in cells constitutively expressing LIN28B

(A) Western blot for *let-7* targets *CDC34*, *IGF2BP1* and *HMGA2* in IEC-6, DLD-1, and LoVo cells that constitutively express *LIN28B*. (B) Increased mature *let-7* levels in *LIN28B*-LoVo cells restores repression of *let-7* targets. (C) Up-regulation of *LGR5* and *PROM1* in *LIN28B*-expressing cells is maintained following *let-7* restoration.

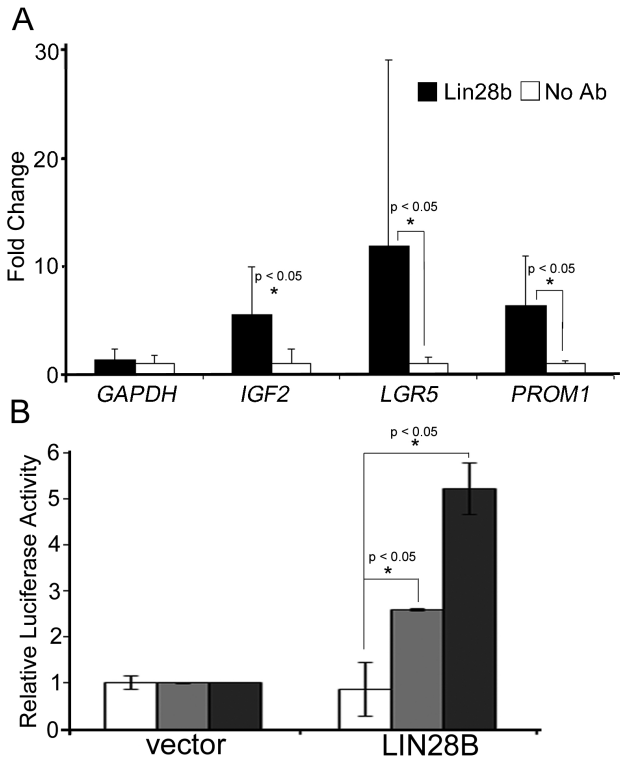

Figure 7. *LGR5* and *PROM1* mRNAs interact with Lin28b protein

(A) RNAs co-immunoprecipitating with Lin28b in colon cancer cells were reverse transcribed with an oligo-dT primer and qPCR performed for *GAPDH*, *IGF2*, *LGR5*, and *PROM1* mRNAs. Fold change is relative to mRNA levels immunoprecipitated in the absence of Lin28b antibody, following normalization to *ACTB* (β-actin) mRNA levels. Error bars depict standard deviation from the mean. (B) Luciferase assays were performed to assess association of Lin28b protein with *LGR5* and *PROM1* 3′ UTR sequences in colon cancer cells. Luciferase activity is enhanced by *LGR5* and *PROM1* 3′ UTRs in the presence of increased *LIN28B* expression. Error bars depict standard deviation from mean.

Table 1

Decreased levels of mature *let-7a* and *let-7b* isoforms and overexpression of *LIN28B* in colon tumors compared to normal colonic epithelium.

Expression Ratio	**mature *let-7***	**Fold Expression**	***LIN28B***
>0.6	10	>2	10
<0.6	12	0-2	12
Total	22	Total	22

LIN28B overexpression correlates with *let-7* down-regulation in colon tumors

Fisher's exact test p-value = 0.09919