LIN28B, *LET-7*, AND THE MOLECULAR PATHOGENESIS OF COLON CANCER

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Dedication

This work is dedicated to the patients represented in this study, who willfully submitted their tissues to advance the study of colon cancer and progress toward a cure, and to Charles and Sylvia who gave rise to a dynasty of Kings.

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Abstract

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Lin28b is an RNA-binding protein that inhibits biogenesis of tumor-suppressive microRNAs of the *let-7* family, and is involved in induction of pluripotency. Although *LIN28B* has been implicated in cancer, a specific role in colon tumorigenesis has not been elucidated. We have determined that colon tumors exhibit decreased levels of mature *let-7* isoforms, and that constitutive *let-7* expression inhibits migration and invasion of colon cancer cells *in vitro*. Importantly, down-regulation of *let-7a* and *let-7b* in colon tumors correlates with increased expression of *LIN28B*, suggesting tumor-promoting properties of this *let-7* inhibitor. In order to determine the role of *LIN28B* in colon cancer, we constitutively expressed *LIN28B* in immortalized human colonic epithelial cells and colon cancer cell lines via retroviral transduction. We found that constitutive *LIN28B* expression promotes migration, invasion, and soft-agar colony formation *in vitro*, as well as differentiated and metastatic phenotypes *in vivo*. Additionally, constitutive *LIN28B* expression modulates levels of several mRNA transcripts including the established *let-7* targets *IGF2BP1* and *HMGA2*. The intestinal stem cell related genes *LGR5* and *PROM1*, which are not predicted *let-7* targets, are also up-regulated with constitutive *LIN28B* expression – an effect that is not ameliorated by co-expression of *let-7*, thereby suggesting possible *let-7* independent functions of *LIN28B*. These findings are corroborated by correlation of high *LIN28B* expression in colon tumors with poor patient prognosis. In summary, this work demonstrates tumor-promoting properties of *LIN28B* in the colon, and suggests that functions of *LIN28B* may occur via *let-7* independent mechanisms. Potentially, *LIN28B* may evolve as a novel diagnostic marker or therapeutic target in colon cancer.

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Introduction

Emerging roles for *LIN28B* and *let-7* in colon cancer pathogenesis

Colorectal cancer is amongst the leading causes of cancer related deaths

Despite advances in diagnostic and therapeutic modalities, colorectal cancer remains the third most common cause of cancer-related death amongst men and women in the U.S. Nearly 140,000 new cases of colorectal cancer are diagnosed annually in the United States; the disease proves fatal to nearly 60,000 patients in the U.S. and over 600,000 individuals worldwide each year (ACS, 2010; WHO, 2010). The 5-year survival rate for patients diagnosed with early-stage, localized colorectal cancer is 91%; however, this statistic drops precipitously to 11% for individuals with metastatic disease. Early diagnosis is therefore critical, yet less than 40% of colorectal tumors are diagnosed in precancerous polyp or early stages. Due to diagnostic screening, the overall number of new colorectal cancer cases has nonetheless declined since 1985. However, incidence rates are rising amongst individuals younger than 50, where screening is generally not recommended except for those with hereditary syndromes (ACS, 2010). Accordingly, improvements in diagnostic and therapeutic modalities for colorectal cancers are needed.

The vast majority (approximately 70-80%) of colorectal tumors arise via chromosomal instability that occurs as a consequence of accumulated genetic alterations, triggered by mutations in the *APC* (adenomatous polyposis coli) tumor suppressor gene. A subset of individuals develop colorectal tumors as the result of inherited syndromes such as hereditary nonpolyposis colorectal cancer (HNPCC) or Lynch syndrome and familial adenomatous polyposis (FAP) (Rustgi, 2007). Studies of these inherited forms of colorectal cancer have offered enormous insights into the pathogenesis of sporadic colorectal tumors (Kinzler & Vogelstein, 1996; Rustgi, 2007).

Characteristics and functions of *LIN28B*

LIN28B (*C. elegans* lin-28 homolog B) is a homolog of *LIN28* (*C. elegans* lin-28 homolog A), which induces pluripotency when expressed in combination with *OCT4*, *SOX2*, and *Nanog* (Guo et al, 2006; Yu et al, 2007b). The 5,504 nucleotide open reading frame of *LIN28B* encodes a 250 amino acid, 28 kDa protein with significant sequence similarity to *LIN28*. In addition to being very similar to each other, the two proteins are conserved across species, including *C. elegans* (Guo et al, 2006; Moss & Tang, 2003). Sequence conservation occurs largely in the cold-shock and retroviral-type CCHC zinc finger domains that confer RNA binding ability, implicating *LIN28* and *LIN28B* in post-transcriptional gene regulation (Guo et al, 2006; Moss & Tang, 2003).

In *C. elegans*, lin-28 (abnormal cell LINeage family member) is a heterochronic gene that, when mutated, causes precocious development as a result of skipping larval stage two (L2) (Ambros & Horvitz, 1984). In lin-28 mutant *C. elegans*, restricted developmental processes occur one stage earlier than normal, and the molting process ceases at L3. Furthermore, mutant animals transition from larvae to adults in only three developmental stages, instead of the normal four (Ambros & Horvitz, 1984). Moreover, Lin-28 mutants are malformed and incapable of laying eggs due to abnormalities in cell division and vulval specification (Euling & Ambros, 1996a; Euling & Ambros, 1996b).

A phenotype opposite of lin-28 mutation occurs in *C. elegans* let-7 microRNA mutants. Instead of omitting stages as observed in lin-28 mutants, let-7 mutants reiterate larval fates (Reinhart et al, 2000). This aberration occurs at the L4 to adult molt (Reinhart et al, 2000), which would indicate a position for let-7 beneath lin-28 in the *C. elegans* heterochronic gene hierarchy. However, the hierarchical placement of let-7 in relation to

lin-28 in *C. elegans* remains unclear, as lin-28 contains multiple binding sites for let-7 in its 3' UTR. More recent work demonstrates *C. elegans* lin-28 as a negative regulator of let-7 biogenesis; this repressive activity is also critical to mammalian *LIN28* and *LIN28B* function.

Let-7 microRNAs are highly conserved between *C. elegans* and humans, and function by binding to the 3' UTR of target mRNAs and suppressing translation (Bartel, 2004; Lagos-Quintana et al, 2001; Pasquinelli et al, 2000; Vella & Slack, 2005). They are transcribed initially as pri-miRNAs, and are processed subsequently in the nucleus to hairpin molecules termed pre-miRNAs (Lee et al, 2003; Lee et al, 2002). The hairpin pre-miRNAs are exported from the nucleus via exportin-5, and are processed further into their ~21 nucleotide mature form by dicer (Hutvagner et al, 2001; Yi et al, 2003). Lin28 and Lin28b bind the hairpin loop of *let-7* microRNAs and recruit Zcch11, an enzyme that uridylates pre-*let-7* molecules, thereby impeding their further processing via dicer-mediated cleavage (Hagan et al, 2009; Heo et al, 2008). The ability of Lin28 and Lin28b to bind pre-*let-7* hairpin loops is dependent upon the RNA-binding activity conferred by the conserved cold-shock domain and CCHC zinc fingers (Balzer et al, 2010; Moss & Tang, 2003).

In addition to binding *let-7* microRNAs and inhibiting their processing, additional roles have been suggested for Lin28 and Lin28b in mediating post-transcriptional gene regulation. First, Lin28, while predominantly cytoplasmic, shuttles to the nucleus in a manner dependent upon the cold shock domain and CCHC zinc fingers (Balzer & Moss, 2007). In addition, Lin28 localizes to P-bodies, and is found in cellular stress granules,

suggesting a possible role in mediating translational repression of mRNAs (Balzer & Moss, 2007).

Consistent with a role for Lin28 in initiating and/or modulating translation efficiency, Lin28 is associated with active polysomes in myoblasts (Polesskaya et al, 2007). Lin28 co-localizes and interacts with the translation initiation factor eIF3β in stress granules, and this association is enhanced during myoblast differentiation (Polesskaya et al, 2007). Importantly, Lin28 binds *IGF2* mRNA, driving it into polysomes and improving translational efficiency. Lin28's ability to up-regulate *IGF2* (Insulin-like growth factor 2) has been established in both skeletal myogenesis and neurogliogenesis (Balzer et al, 2010; Polesskaya et al, 2007).

In addition to binding *IGF2*, Lin28 associates with *OCT4* (octamer-binding transcription factor 4; also known as *POU5F1*) mRNA in ribonucleoproteins and polysomes (Qiu et al, 2010). Oct4 is a POU-domain containing transcription factor involved in modulating cell fate and differentiation. Increasing Oct4 levels by less than two-fold is sufficient to stimulate embryonic stem cell differentiation into primitive endoderm and mesoderm (Niwa et al, 2000). Correspondingly, reduction of Oct4 expression induces trophectoderm formation. Thus, Oct4 protein is modulated during differentiation, and is a critical determinant of cell fate (Niwa et al, 2000). Interestingly, the *OCT4* gene is common to the earliest descriptions of induced pluripotency in somatic cells. Yu *et al.* demonstrated induction of pluripotency through co-expression of *OCT4*, *SOX2*, *LIN28* and *NANOG* in fibroblasts (Yu et al, 2007b). Similarly, Takahashi *et al* described a method of reprogramming somatic cells via retroviral expression of *OCT4*, *SOX2*, *KLF4*, and *c-MYC* (Takahashi et al, 2007; Takahashi & Yamanaka, 2006).

Therefore, the ability to bind *OCT4* mRNA and influence its transcriptional efficiency is likely essential to the role of Lin28 and Lin28b in establishment and/or maintenance of pluripotency.

Cellular differentiation in the colonic epithelium

Pluripotent cells have the potential to differentiate into cellular derivatives of either endoderm, mesoderm, or ectoderm origin (De Miguel et al, 2010). During cellular differentiation, cells become more specialized while simultaneously diminishing in stem cell characteristics (Beddington & Robertson, 1989; Potten & Loeffler, 1990). Terminally differentiated cells are generally non-proliferative, and exhibit a restricted gene expression profile (Yamanaka & Blau, 2010).

Pluripotent adult stem cells in the small intestine and colon are located within deep folds of the epithelium known as crypts of Lieberkühn, and are capable of differentiating into any of the four major cell types that comprise intestinal epithelium (Barker et al, 2007; Brittan & Wright, 2002; Brittan & Wright, 2004a; Brittan & Wright, 2004b; Papailiou et al, 2010; Potten et al, 1997; Potten et al, 2009; Potten & Loeffler, 1990). The majority of the intestinal epithelium is comprised of cells from the absorptive lineage - enterocytes in the small intestine and colonocytes in the colon. These cells function mainly in absorbing micronutrients and water from the lumen, respectively (Cheng & Leblond, 1974a; Cheng & Leblond, 1974b; Cheng & Leblond, 1974c). The remaining three cell types of the intestinal epithelium are part of the secretory lineage, including Paneth, enteroendocrine, and goblet cells (Cheng & Leblond, 1974a; Cheng & Leblond, 1974b; Cheng & Leblond, 1974c); of note, Paneth cells are not found in the

colon. Paneth cells reside within intestinal crypts, secrete lysosymes, and are thought to be involved in anti-microbial functions (Ayabe et al, 2002a; Ayabe et al, 2000; Ayabe et al, 2002b), while enteroendocrine cells secrete hormones that aid in nutrient homeostasis (Drucker, 2007; Murphy & Bloom, 2006; Rindi et al, 2004) and are found in both the small intestine and colon. Goblet cells secrete mucin glycoproteins that have cytoprotective properties (Neutra et al, 1982; Phillips et al, 1984).

The terminally differentiated cells of the intestinal/colonic epithelium are shed approximately every 5-7 days and are replaced by new cell populations emerging from the crypts (Brittan & Wright, 2002; Brittan & Wright, 2004a; Brittan & Wright, 2004b; Potten et al, 2009). Accordingly, stem cells in the crypt face unique challenges compared to other adult tissues which renew with less frequency in coordinating the balance between proliferation and differentiation (Potten et al, 2009; Reya & Clevers, 2005). While some of the molecular mechanisms underlying regulation of cellular differentiation in the small intestine and colon have been elucidated, a number of critical questions remain unanswered. For example, while it is generally accepted that intestinal stem cells reside within the crypts of Lieberkühn, the actual position of stem cells within the crypts of the small intestine, i.e. between the Paneth cells at the base of the crypt (so called CBCs) or immediately superior to them at position +4, is debated (Barker et al, 2007; Brabletz et al, 2009; Potten et al, 1997; Potten et al, 2009).

In general, cellular proliferation in the intestinal epithelium is restricted to the crypt wherein the stem cells reside, while differentiation occurs as cells migrate from the crypt toward the surface (Paneth cells are a notable exception) (Barker et al, 2007; Potten et al, 1997; Potten et al, 2009; Potten & Loeffler, 1990). The process by which stem cells

give rise to transit amplifying cells of the absorptive and secretory lineages is tightly controlled via integration of Wnt, Notch, BMP, Hedgehog, and PI3K pathways, amongst others (Brabletz et al, 2009). Of these pathways, canonical Wnt (wingless-related MMTV integration site family) signaling is perhaps the most crucial, as aberrations in Wnt signaling have profound effects on intestinal pathology, and occur frequently as early events in colon cancer tumorigenesis (Clevers, 2006; Fearon & Vogelstein, 1990; Korinek et al, 1997; Morin et al, 1997; Nakamura et al, 2007; Potten et al, 2009; Reya & Clevers, 2005).

Canonical Wnt signaling: a critical pathway in the colon

In mammals, there are approximately 20 variable extracellular Wnt ligands with both overlapping and distinct functions. Canonical Wnt signaling is typified by Wnt ligands binding to a primary receptor and co-receptor combination, FRZ (frizzled) and LRP-5/6 (low density lipoprotein receptor-related protein 5) respectively. Upon receptor activation, DSH (disheveled) inhibits a complex containing axin, GSK3β , and APC; the latter two cooperate to phosphorylate β-catenin, targeting it for ubiquitin-mediated degradation via the proteasome (Hart et al, 1998; Reya & Clevers, 2005; Rubinfeld et al, 1996; Rubinfeld et al, 1993). Repression of the APC/GSK3β/axin complex by DSH results in stabilization of β-catenin, which then translocates to the nucleus and activates TCF (transcription factor) and/or LEF (lymphoid enhancer-binding factor 1) transcription factors (Behrens et al, 1996; Morin et al, 1997). Targets of Wnt signaling include proliferation factors such as cyclin D1 and c-myc, pluripotency factors including *OCT4* and *Nanog*, as well as Wnt pathway components themselves (Clevers, 2006; Cole et al,

2008; He et al, 1998; Pereira et al, 2006; Shtutman et al, 1999; Tetsu & McCormick, 1999).

Wnt signaling is critical to maintaining the proliferative potential of the intestinal stem cell populations within the crypts (Kim et al, 2005; Reya & Clevers, 2005); this may occur via multiple mechanisms, but most notably via c-myc mediated repression of $p21^{CIP1/WAF1}$. An established TCF4 target, c-myc, binds the promoter of the cyclin-dependent kinase inhibitor $p21^{CIP1/WAF1}$ repressing its transcription (van de Wetering et al, 2002). Therefore, down-regulation of c-myc via withdrawal of Wnt signaling transcriptionally activates $p21^{CIP1/WAF1}$, which subsequently mediates G1 arrest and promotes cellular differentiation (Reya & Clevers, 2005; van de Wetering et al, 2002).

The molecular pathogenesis of colon cancer

Mutations in components of the canonical Wnt signaling pathway occur in the majority of colorectal cancers with inactivating mutations in the *APC* gene being the most common (Kinzler & Vogelstein, 1996). Germline mutations in the *APC* tumor suppressor gene were originally identified as the etiologic agent in Familial Adenomatous Polyposis or FAP (Ichii et al, 1993; Levy et al, 1994; Luongo et al, 1994). Later, it was determined that sporadic mutations in *APC* are the most common known genetic perturbations (70-80%) identifiable in early adenomatous polyps that may progress to cancer (Kinzler & Vogelstein, 1996; Lamlum et al, 2000; Powell et al, 1992; Rowan et al, 2000). Following truncating mutations in *APC*, activating point mutations in *KRAS* (V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog) are the most common genetic perturbation (40-50%) identified in the progression of adenomatous polyps to cancer;

tumor suppressor genes (p53 and *SMAD3/4*) are often mutated in later stage tumors (Fearon & Vogelstein, 1990; Kinzler & Vogelstein, 1996; Kressner et al, 1998).

A linear model of the molecular pathogenesis of colon cancer was developed originally by Bert Vogelstein *et al.* (Fearon & Vogelstein, 1990). Vogelstein's widely accepted model of colon cancer pathogenesis describes an accumulation of genetic alterations that correspond to progression from normal to hyperproliferative epithelium, adenomas, cancers and finally, metastasis. Truncating mutations in *APC* occur in the earliest phases, and are the paramount initial step toward hyperproliferation in colonic epithelium. Hyperproliferative foci of intestinal epithelial cells result in adenomatous polyps, and may acquire activating mutations in *KRAS*. Loss of *SMAD4* and p53 accompany progression to late stage adenomas, and accumulated mutations eventually result in colon cancer development and metastasis.

It is important to note that various pathways for colon cancer initiation exist in the colon. For example, serrated polyps (including hyperplastic polyps, admixed hyperplastic polyps/adenomas, and sessile serrated adenomas) may represent a mode of cancer development distinct from the archetypal adenoma-carcinoma sequence (Hawkins et al, 2002; He et al, 1998; Huang et al, 2004). Thus, the mechanisms involved in colon cancer pathogenesis are yet to be fully elucidated, but an unrelenting effort is being made to close this knowledge gap.

Potential roles for *LIN28B* in colon cancer pathogenesis

The c-myc transcription factor is a Wnt pathway target that is frequently overexpressed in colon cancers (Chan et al, 1987; He et al, 1998; Sikora et al, 1987;

Stewart et al, 1986). C-myc transcriptionally activates *LIN28B*, and this activity is a necessary mediator for at least a subset of c-myc functions (Chang et al, 2009); it remains to be determined whether Lin28b mediates a portion of c-myc-induced activities that promote tumorigenesis in the colon. Additionally, since *KRAS* is an established *let-7* target, *LIN28B* overexpression may up-regulate *KRAS* activity via repression of *let-7* biogenesis (Jeong et al, 2009; Johnson et al, 2005).

To date, despite divergences in amino acid sequences, molecular or biochemical functions unique to either Lin28 or Lin28b have not been determined. However, while overexpression of both *LIN28* and *LIN28B* occurs in multiple cancers, *LIN28B* overexpression has been demonstrated in specific cancers more frequently. For example, *LIN28B* is overexpressed in hepatocellular carcinomas (Guo et al, 2006). More interestingly, a broad analysis of *LIN28* and *LIN28B* expression in various tumors and cell lines conducted by Viswanathan *et al*. pointed to *LIN28B* as perhaps the more relevant homolog in tumorigenesis. Viswanathan *et al*. implicated a role for *LIN28B* in Wilms' tumors where the c-myc binding site on *LIN28B*'s promoter is frequently demethylated and chronic myeloid leukemia where expression of *LIN28B* occurs more commonly in blast crisis. In addition, Viswanathan *et al*. demonstrated *LIN28B* overexpression in non-small cell lung cancer, breast cancer, melanoma, while aberrations in *LIN28* expression were not as readily identifiable (Viswanathan et al, 2009).

We have hypothesized that *LIN28B* promotes tumorigenesis via repression of *let-7* in the colon. In formulating this hypothesis, we first demonstrated decreased expression of *let-7* in colon tumors, and identified potential tumor-suppressive functions of *let-7* in

colon cancer cells *in vitro*. Our initial findings, which indicate that *let-7* microRNAs repress migration and invasion of colon cancer cells *in vitro*, will be discussed in Chapter 1. As the role of *let-7* was elucidated further, mounting evidence pointed to a significant role of Lin28b in colon tumorigenesis, in part, due to its ability to post-transcriptionally regulate *let-7*. Accordingly, we constitutively expressed *LIN28B* in immortalized epithelial and colon cancer cells *in vitro*, and found that Lin28b promotes migration, invasion, and transformation in colon cancer cells, as described in Chapter 2. Interestingly, our *in vitro* findings suggested *let-7* independent functions of *LIN28B*, which we further evaluated *in vivo*. Via constitutive expression of *LIN28B* in xenograft tumors, we have identified potential functions of *LIN28B* in modulating differentiation programs and promoting metastasis in colon cancers. These *in vivo* findings will be detailed in Chapter 3, followed by an in-depth discussion of the broader implications of this work, and potential future directions.

Specific Aims

Hypothesis: *LIN28B* promotes tumorigenesis via suppression of *let-7* in the colon

The overarching hypothesis of this thesis is that ***LIN28B promotes tumorigenesis via suppression of let-7 in the colon*.** This hypothesis has been pursued by the following inter-related specific aims:

Specific Aim #1: To demonstrate tumor-suppressive functions of *let-7* in colon cancer.

The *let-7a-3-b* microRNA cluster is transcribed from a region on chromosome 22q13.31 that undergoes LOH in colon cancer. We measured mature microRNA levels in colon tumors compared to normal colonic epithelium and found that *let-7a* and *let-7b* are reduced in about two-thirds of tumors. Given that *let-7* is reduced in a subset of colorectal cancers, *we hypothesized that re-introduction of* let-7 *in colon tumors suppresses tumorigenesis*. We tested this hypothesis by constitutively expressing the bicistronic *let-7a-3-b* cluster in multiple colon cancer cell lines via retroviral transduction. Subsequently, we evaluated colony formation in soft agar, and assessed changes in migration and invasion via *in vitro* Boyden chambers assays.

Specific Aim #2: To determine the role of *LIN28B* in promoting colon cancer *in vitro*.

Several lines of evidence suggest that *let-7* microRNAs are primarily regulated through post-transcriptional inhibition of processing. The *let-7* inhibitor *LIN28B* is overexpressed in various cancers, including colorectal adenocarcinomas. *We hypothesized that LIN28B promotes tumorigenesis in the colon in a* let-7 *dependent manner*. To test this hypothesis, we expressed *LIN28B* in immortalized colonic epithelial and colon cancer cell lines. We

then assayed cell migration, invasion, and soft-agar colony formation. In addition, we evaluated gene expression in *LIN28B*-expressing via microarray analysis.

Specific Aim #3: To evaluate the role of *LIN28B* in colon tumorigenesis *in vivo.*

Previous work suggests *LIN28B* is a potential therapeutic and/or diagnostic target, necessitating additional analysis of *LIN28B* functions *in vivo*. In order to elucidate further *in vivo* functions of *LIN28B* in tumorigenesis, we xenografted colon cancer cells constitutively expressing *LIN28B* into nude mice, where we analyzed tumor growth and overall architecture. Additionally, we conducted a microarray analysis in order to expose novel mechanisms whereby *LIN28B* may promote tumor formation and/or progression. Finally, we sought to determine whether *LIN28B* overexpression correlates with reduced survival of patients diagnosed with colorectal adenocarcinomas. To this end, we measured Lin28b levels in tumors and normal mucosa via immunostaining.

Chapter 1

Reintroduction of *let-7* suppresses migration and invasion in colon cancer cell lines

Guide to Chapter 1 Tables and Figures

Background and Significance

The vast majority of colorectal tumors arise via chromosomal instability, acquiring gross chromosomal aberrations that frequently result in deletion of tumor suppressor genes (Lengauer et al, 1997). Our lab previously identified a minimal region of deletion on chromosome 22q13.31 in nearly 30% of colorectal tumors (Castells et al, 1999). Allelic loss on chromosome 22q is also found in tumors of the breast, ovary, oral cavity, endocrine pancreas, and brain (Allione et al, 1998; Bryan et al, 1996; Castells et al, 2000; Chung et al, 1998; Miyakawa et al, 1998; Rey et al, 1993). Of note, we found that two microRNAs of the *let-7* family, *let-7a-3* and *let-7b*, are transcribed as a bicistronic cluster from their locus on chromosome 22q13.31 (Griffiths-Jones, 2004; Griffiths-Jones et al, 2006; Griffiths-Jones et al, 2008; mirBase, 2010).

MicroRNAs are 20-22 nucleotide molecules that inhibit translation by complimentary binding to the 3' UTR of target mRNA transcripts (Bartel, 2004; Lagos-Quintana et al, 2001). A large number of genes bear putative microRNA binding sites, and it is postulated that microRNAs may regulate up to one-third of the genome (Esquela-Kerscher & Slack, 2006; Lewis et al, 2005). MicroRNAs are frequently found clustered as unique polII transcriptional units or are encoded within the introns of protein-coding genes (Lagos-Quintana et al, 2001). They are transcribed initially as pri-miRNAs, and are subsequently processed in the nucleus to hairpin molecules termed pre-miRNAs (Lee et al, 2003; Lee et al, 2002). The hairpin pre-miRNAs are exported from the nucleus via exportin-5, and are further processed into their ~21 nucleotide mature form by dicer (Hutvagner et al, 2001; Yi et al, 2003).

One of the most highly conserved families of microRNAs is that of *let-7* (Pasquinelli et al, 2000). There are twelve *let-7* mammalian orthologs of the four *let-7* family members described in *C. elegans* (Vella & Slack, 2005). *Let-7* microRNAs are relatively homogenous, and a single family member may possess multiple isoforms in the genome. Accordingly, *let-7a-3* is one of three *let-7a* isoforms that exists in mammals, and the mature sequence of *let-7a* differs from *let-7b* by only two nucleotides (mirBase, 2010).

A number of studies suggest that *let-7* microRNAs coordinate distinctive cellular processes, including proliferation (Johnson et al, 2007; Peng et al, 2008), and differentiation (Johnson et al, 2007; Shell et al, 2007; Tsonis et al, 2007; Yu et al, 2007a). Intriguingly, a bioinformatics study suggested that *let-7* suppresses a number of genes which are down-regulated toward the completion of embryogenesis, but then re-expressed in tumors (Boyerinas et al, 2008). A number of *let-7* targets demonstrate an ability to participate in tumorigenesis. For example, the canonical *let-7* target *HMGA2* (High-mobility group AT-hook 2) is known to drive epithelial-mesenchymal transition, and to promote invasion and metastasis (Fabjani et al, 2005; Langelotz et al, 2003; Motoyama et al, 2008; Park et al, 2007; Thuault et al, 2006). Additional *let-7* targets include the cell cycle regulatory protein, *CDC34* (cell division cycle 34 homolog), and the oncofetal gene *IGF2BP1* (Insulin-like growth factor 2 mRNA-binding protein 1) (Boyerinas et al, 2008; Legesse-Miller et al, 2009).

Expression of *let-7* isoforms is reduced in a number of human malignancies, and *let-7* microRNAs exhibit tumor suppressive functions in a variety of cancers, most notably non-small cell lung and breast cancers (Akao et al, 2006; Esquela-Kerscher &

Slack, 2006; Kumar et al, 2008; Shell et al, 2007; Takamizawa et al, 2004; Viswanathan et al, 2008; Yu et al, 2007a). Moreover, reduced *let-7* expression correlates particularly well with poorly-differentiated tumors, and thus serves as an excellent prognostic marker (Shell et al, 2007; Takamizawa et al, 2004; Yu et al, 2007a).

Let-7a-3 and *let-7b* are clustered within an intron of an unnamed gene and transcribed together from a locus on 22q13.31. Since *let-7* isoforms exhibit tumor-suppressive functions in other cancers, we hypothesized that *let-7a-3* and *let-7b* suppress tumorigenesis within the colon. To test this hypothesis, we first assayed levels of mature *let-7* in paired normal colon and colon cancers. We found that both *let-7a-3* and *let-7b* are reduced in 32 of 40 colon tumors we assayed (approximately two-thirds). In order to further elucidate the role of *let-7a-3* and *let-7b* in colon tumorigenesis, we expressed a pri-microRNA derived from the genomic sequence encoding the two isoforms in colon cancer cell lines via retroviral transduction. We found that overexpression of *let-7a* and *let-7b* in colon cancer cells repressed cellular migration and invasion *in vitro*. In long-term assays, we found the increase in mature *let-7* to be ameliorated in cells transduced with retrovirus, likely as a consequence of endogenous post-transcriptional regulatory mechanisms.

Experimental Procedures

Human tissue samples. Human colorectal cancer and adjacent normal colon tissues were obtained from a collaborator (Dr. Naomoto) from the Department of Surgery, Okayama University Medical School, Japan under Institutional Review Board approval. Normal and tumor colon samples were stored initially in RNAlater at -80^{o} C. Tumor tissue were homogenized mechanically on ice and fine particulates were collected via centrifugation at 4^{o} C. RNA was isolated from homogenized tissues using a mirVana RNA isolation kit (Ambion, Austin, TX) and evaluated for integrity using a Bioanalyzer (Agilent, Santa Clara, CA).

Mature microRNA detection in human tumors and transduced cells. Total RNA was extracted from human tissues, transfected 293T, or transduced LIM 2405 and LoVo cells using the mirVana miRNA isolation kit (Ambion, Austin, TX). Taqman® MicroRNA Assay kits for both *let-7a* and *let-7b* (Applied Biosystems, Carlsbad, CA) were employed to synthesize probe-specific cDNA from 10ng of total RNA per sample. Levels of mature microRNAs were measured via qPCR on the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Carlsbad, CA). Probe-specific cDNA was amplified using proprietary primers (Applied Biosystems, Carlsbad, California) and TaqMan® Universal PCR Master Mix, No AmpErase UNG (Applied Biosystems, Carlsbad, CA). PCR amplifications were performed in triplicate and normalized 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 *let-7a-3-b* was determined by applying student's t-test, with $p<0.05$ considered significant.

Detection of *let-7a3-b* pri-microRNA. Total RNA was extracted from human tissues, transfected 293T, or transduced LIM 2405 and LoVo cells using the mirVana miRNA isolation kit as described above. cDNA was synthesized from 5 μg total RNA per sample using random hexamers and SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA). Detection of *let-7a3-b* pri-microRNA intronic sequence was achieved using primers specific to the intronic sequence between *let-7a-3* and *let-7b* which bear the following sequences: ggggccgcctacactgagaag and ctggggcacgtgctgggaacct. Fold change for *let-7a-3-b* were determined by using Image J analysis to quantify individual band intensities, followed by normalization to empty vector controls.

Biostatistical analysis of *let-7* expression in human tissues. The distributions of *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 *let-7a* and *let-7b*, *let-7* expression and tumor stage, and *let-7* expression and survival. 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.

Retroviral expression vector construction. A ~1.1 kb sequence containing *let-7a3-b* cluster pri-microRNA was amplified from chromosome 22q31.31 in normal human

colonic epithelial cells using primers with the aforementioned sequences. The ~1.1kb clone was subcloned into a self-contained tetracycline-inducible vector designated pBSTR1 (Paulus et al, 1996) via restriction digest with *SnaBI* and *EcoRI* enzymes (New England Biolabs, Ipswich, MA) followed by blunt end ligation with T4 DNA ligase (Invitrogen, Carsblad, CA). Proper orientation of insert and absence of mutations was confirmed via sequencing at The University of Pennsylvania's DNA sequencing facility.

Virus packaging and transient expression of *let-7a-3-b* in 293T cells. 30μg of the pBSTR1-let-7a-3-b plasmid construct was transfected into 293T cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA), as per the protocol. Transfection efficiency was determined by detection of GFP protein expression via light microscopy. Supernatant containing viral particles was collected every 24 hours, filtered through a 0.45μm membrane, and immediately placed in liquid nitrogen. Viral supernatants were stored at -80^{o} C prior to use. 293T cells utilized in viral packing were harvested by scraping in PBS over ice and stored at -80^{o} C for subsequent RNA and protein isolation.

Western blot analysis. Cells were lysed in RIPA buffer 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 μg total protein was loaded onto 4-12% gradient gels for electrophoresis in NuPAGE® Running Buffer (Invitrogen, Carlsbad, CA) using an Invitrogen (Carlsbad, CA) western blotting apparatus. Proteins were transferred in NuPAGE® Transfer Buffer to PVDF membranes per Invitrogen protocol,

at 20 V overnight. The following day, membranes were blocked in 5% non-fat milk in TBS-T, and blotted with Lin28b (1:100) (Abcam, Cambridge, MA) or β-actin (1:1000) (Sigma-Aldrich, St. Louis, MO) primary antibodies as per the manufacturer's protocol. Lin28b and β-actin were detected using secondary rabbit (1:1000) and mouse (1:1000) antibodies respectively. Visualization of detected proteins on film using Amersham ECL Plus™ Western Blotting Detection Reagents (GE Healthcare, Fairfield, CT).

Generation of colon cancer cell lines constitutively expressing *let-7a-3-b*. Inducible expression of *let-7a-3* and *let-7b* in colon cancer cell lines was achieved by transducing LIM2405 and LoVo cells. Prior to transduction, viral-containing media was decanted from 293T cells transfected with pBSTR1-let-7a-3-b plasmid. Viral supernatant plus polybrene (4 μg/ml) was applied to cells, which were then subjected to centrifugation at 1000g for 90 minutes. Inoculated cells were selected in puromycin, and changes in mature *let-7a* and *let-7b* were detected using the mature microRNA detection strategy described above. Expanded cell cultures were maintained in DMEM plus 10% FBS at 37^{o} C, 5% CO_2.

Boyden chamber 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 fluorescent transwell assay systems atop a migration insert (BD Biosciences, San Jose, California) or a rehydrated invasion assay insert (BD Biosciences, San Jose,

California) 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 incubated at 37^{o} C, 5% CO_2 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.

Results

Colon tumors exhibit reduced mature *let-7a* and *let-7b* levels

We compared mature *let-7a* and *let-7b* microRNA levels in normal colonic epithelium versus adjacent colonic tumors. RNA was harvested from tissues and utilized to generate the probe-specific cDNA necessary to quantitate the mature *let-7* isoforms via qPCR. In approximately two-thirds of the tumors assayed, we found a greater than 60% reduction in *let-7* levels when compared to adjacent normal colonic epithelium (Table 1; Figure 1). Importantly, *let-7a* and *let-7b* are the primary *let-7* isoforms expressed in the normal colon, thus simultaneous reduction in their expression could have potential broad consequences in colon cancer. Though mature *let-7a* and *let-7b* levels correlate with one another (Pearson correlation coefficient, $r = 0.84$; $p<0.0001$), they do not statistically correlate with tumor stage or probability of survival (data not shown).

Constitutive *let-7a-3-b* expression inhibits cell migration and invasion

In order to elucidate the functions of *let-7a-3* and *let-7b* in colon tumors, we subcloned *let-7a-3-b* pri-microRNA into a self-contained, tetracycline-inducible, retroviral expression vector. We then transiently transfected 293T cells with *let-7a-3-b* expression and measured mature *let-7a* and *let-7b* in transfected cells. Constitutive *let-7a-3-b* pri-microRNA expression increases levels of both *let-7a* and *let-7b* mature microRNAs by more than 8 fold (Figure 2a). Furthermore, increases in *let-7a* and *let-7b* decreases protein levels of known *let-7* targets, including *HMGA2*, *CDC34*, and

IGF2BP1 (Figure 2b); this confirms processing of exogenously expressed *let-7a-3-b* into functionally mature *let-7a* and *let-7b* microRNAs.

We then transduced LIM 2405 and LoVo colon cancer cell lines using retrovirus-containing media generated from transfected 293T cells. LIM 2405 and LoVo cells were selected because they display the lowest levels of *let-7a* and *let-7b* compared to several other colon cancer cell lines tested (data not shown). We confirmed a more than two-fold increase in mature *let-7a* and *let-7b* levels via RT-qPCR with constitutive expression of *let-7a-3-b* in LIM 2405 and LoVo cells (Figure 3a). Subsequently, we subjected LIM 2405 and LoVo cells transduced with *let-7a-3-b* to migration and invasion assays. We found that colon cancer cells with increased *let-7a* and *let-7b* exhibited reduced migration and invasion compared to the cell lines with empty vector controls (Figure 3b-c).

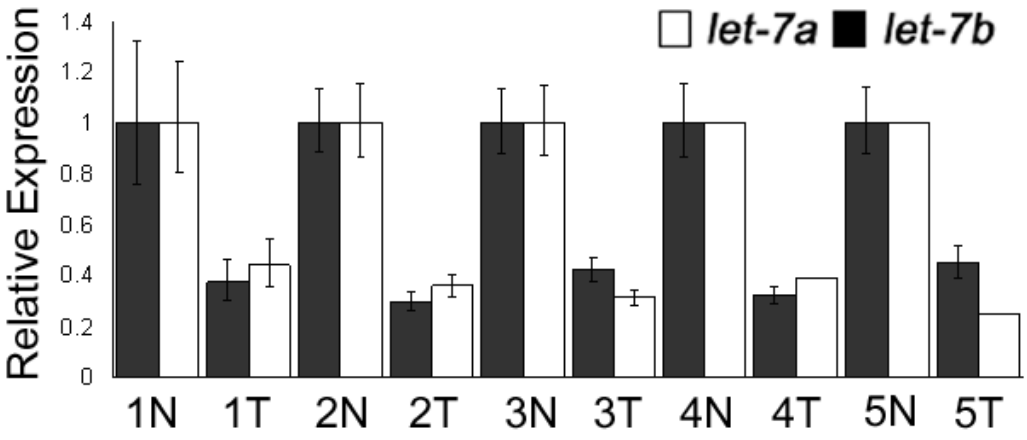
Processing of *let-7a-3-b* pri-microRNA into mature *let-7a* and *let-7b* declines over time

Next, we sought to determine the effects of *let-7a* and *let-7b* overexpression on cellular proliferation in LIM 2405 and LoVo cells. Contrary to our hypothesis, we did not observe significant differences in cellular proliferation between empty vector and *let-7a-3b*-expressing cells on WST (water-soluble tetrazolium salt) proliferation assays (data not shown). This prompted re-evaluation of *let-7* expression in LIM 2405 and LoVo cells transduced with *let-7a-3-b* retrovirus. We measured mature *let-7a* and *let-7b* levels in *let-7a-3-b* transduced cells that had undergone 6 passages. Surprisingly, we observed reduced levels of *let-7a* and *let-7b* mature microRNAs in *let-7a-3-b*-expressing cells compared to empty vector controls (Figure 4). The change in mature *let-7a* and *let-7b*

levels is perhaps attributable to decreased post-transcriptional *let-7* processing, suggesting activation of *let-7* inhibitors, namely *LIN28B*.

Chapter 1 Tables & Figures

Figure 1



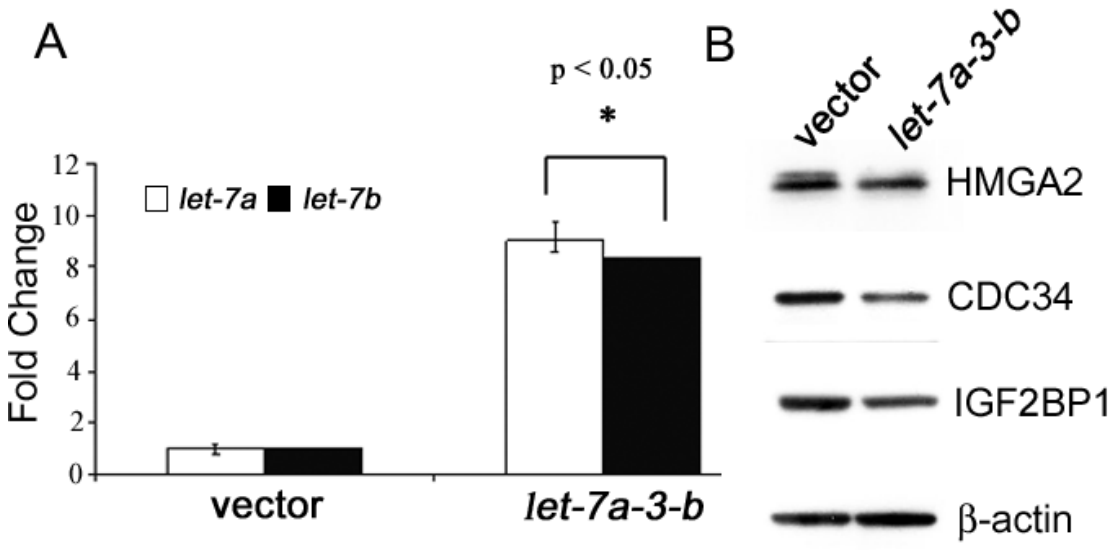
Reduction of mature let-7a and let-7b levels in colon tumors compared to adjacent normal colon. Representative analysis of 5 tumors (T) matched with adjacent normal colonic epithelia (N). RNA was isolated from normal and tumor epithelia and pairs assayed for mature *let-7a* and *let-7b* via quantitative real-time PCR (RT-qPCR). Each pair is normalized to mature *let-7a* and *let-7b* levels in normal colonic epithelium (N). Mature *let-7a* and *let-7b* levels correlate with each other (Pearson correlation coefficient, $r = 0.84$; $p<0.0001$).

Table 1

Fold Expression	Tumors
>1.2	3
1.2-0.6	9
< 0.6	20
Total	32

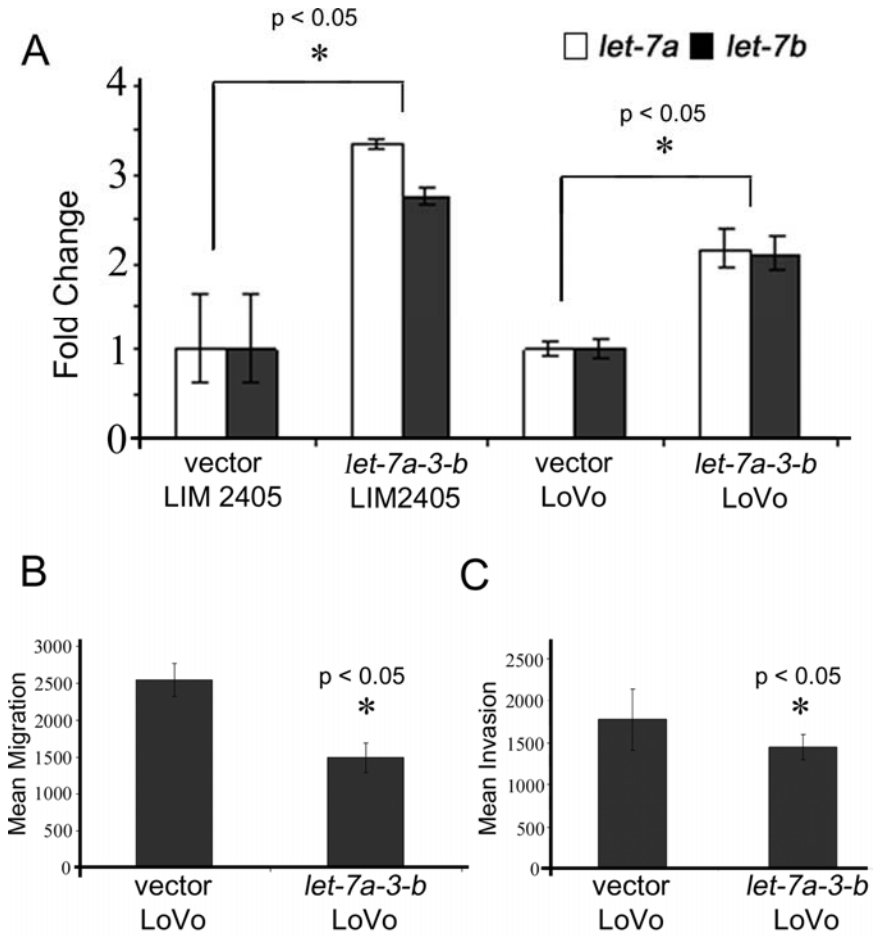
Mature *let-7a* and *let-7b* levels are reduced in colon tumors. Summary of qPCR data for 32 pairs of tumors matched with adjacent normal colonic epithelia. RNA was isolated from tissues and quantitative real-time RT-PCR was performed to determine levels of mature *let-7a* and *let-7b* microRNAs; *let-7a* and *let-7b* levels in tumors were normalized to normal colonic epithelia counterparts. Mature *let-7a* and *let-7b* levels correlate with each other (Pearson correlation coefficient, $r = 0.84$; $p<0.0001$), and are concomitantly reduced in 20 of 32 (nearly two-thirds) of colon tumors as compared to normal colon.

Figure 2



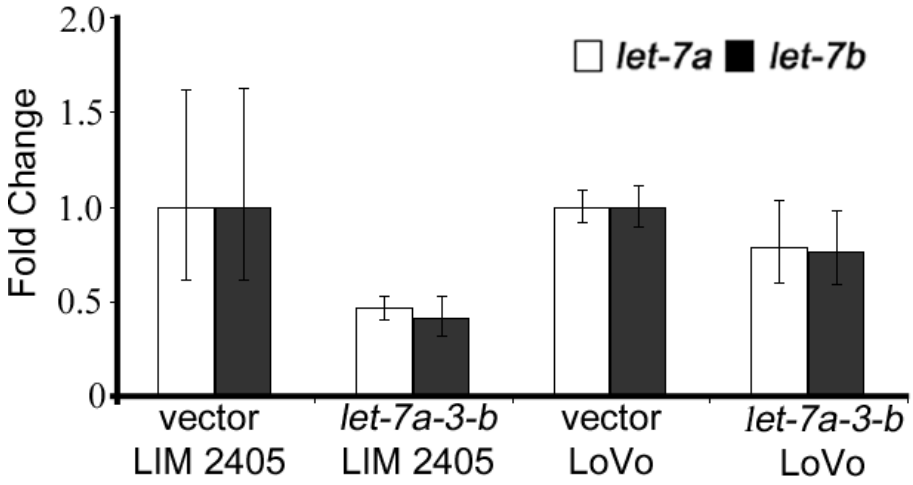
Constitutive *let-7a-3-b* pri-microRNA expression increases levels of mature *let-7a* and *let-7b* microRNAs and represses *let-7* targets. (A) *Increased levels of mature let-7a and let-7b in transfected 293T cells*. RNA was isolated from 293T cells transfected with *let-7a-3-b*, RT-qPCR was performed to determine mature *let-7a* and *let-7b* levels. Constitutive expression of *let-7a-3-b* pri-microRNA increases cellular levels of mature *let-7a* and *let-7b* isoforms. (B) ***let-7a-3-b* expression represses *let-7* targets.** Western blot was performed to assess protein levels of *let-7* targets in transfected 293T cells. Increased mature *let-7a* and *let-7b* levels reduce expression of *let-7* targets.

Figure 3



Reintroduction of *let-7* suppresses migration and invasion in colon cancer cell lines. (A) *Increased levels of mature* let-7a *and* let-7b *in transduced LIM 2405 and LoVo cells***.** RNA was isolated from LIM 2405 and LoVo cells transduced with *let-7a-3-b*, and mature *let-7a* and *let-7b* levels detected via RT-qPCR. *let-7a-3-b* expression increases levels of mature *let-7a* and *let-7b* isoforms by more than two-fold. (B & C) *Reduced cell migration and invasion with increased* let-7. *Let-7a-3-b* expressing LoVo cells were subjected to *in vitro* Boyden chamber migration assays. Increased mature *let-7a* and *let-7b* reduces migration and invasion of LoVo cells.

Figure 4



Processing of *let-7a-3-b* into mature *let-7a* and *let-7b* declines with serial passage. RNA was isolated from passaged LIM 2405 and LoVo cells transduced with *let-7a-3-b*; mature *let-7a* and *let-7b* was detected via RT-qPCR. Though mature *let-7a* and *let-7b* levels are initially increased, they are reduced as compared to empty vector controls after passaging.

Chapter 1 Summary

We have demonstrated reduced mature *let-7a-3* and *let-7b* levels in approximately two-thirds of colon tumors evaluated when compared to adjacent normal colonic epithelium. In addition, we enhanced mature *let-7a* and *let-7b* levels in colon cancer cell lines via constitutive expression of a pri-microRNA, and observed repression of *let-7* targets, apart from suppressed cellular migration and invasion. Interestingly, we observed reduction of mature *let-7* levels with passaging of cells transduced to constitutively express *let-7a* and *let-7b* pri-microRNA. This is perhaps due to post-transcriptional regulation. The best characterized mechanisms of post-transcriptional regulation of *let-7* involve the RNA-binding proteins Lin28 and Lin28b. Lin28b has been frequently implicated in cancer, and is over-expressed in colon tumors. The data presented in the following chapter reveals oncogenic properties of Lin28b, which are, in part, attributable to Lin28b's ability to repress *let-7* biogenesis.

Chapter 2

LIN28B promotes transformation, migration and invasion in colon cancer cells

Guide to Chapter 2 Tables and Figures

Background and Significance

Currently, Lin28 and Lin28b are the best characterized post-transcriptional repressors of *let-7*. Both proteins are capable of inhibiting *let-7* biogenesis at the pri-microRNA and pre-microRNA stages. Both Lin28 and Lin28b may impede processing of *let-7* precursors by mediating their terminal uridylation via recruitment of the enzyme Zcch11. The addition of uridine nucleotides to *let-7* pre-microRNAs disrupts dicer-mediated cleavage, thereby blocking the formation of mature *let-7* microRNAs (Hagan et al, 2009; Heo et al, 2008; Heo et al, 2009).

Several established *let-7* targets are oncogenic, including the canonical target *HMGA2* (Lee & 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, tumor-suppressive functions have been attributed to *let*-7 isoforms, which are frequently down-regulated in diverse cancers, and serve also as prognostic factors (Akao et al, 2006; Shell et al, 2007; Takamizawa et al, 2004). Given the tumor-suppressive properties of *let-7* microRNAs, tumor-promoting properties would be predicted for their inhibitors, namely *LIN28* and *LIN28B*. Multiple lines of evidence suggest a role for *LIN28B* in tumorigenesis. For example, *LIN28B* has been implicated in hepatocellular carcinoma (Guo et al, 2006). A recent study reveals *LIN28B* induces transformation and epithelial-mesenchymal transition, thereby promoting migration and invasion in hepatocellular carcinoma cells (Wang et al, 2010). Moreover, a broad analysis of various cancers reveals frequent *LIN28B* overexpression (Viswanathan et al, 2009). More specifically, *LIN28B* may be involved in colon carcinogenesis since it is a direct target of

c-myc (Chang et al, 2009). Nearly 70% of colorectal tumors exhibit elevated c-myc levels; upregulation occurs in the early stages of colon carcinoma as a consequence of Wnt pathway deregulation and β-catenin stabilization (Erisman et al, 1985; He et al, 1998; Sikora et al, 1987; Stewart et al, 1986).

LIN28B may promote tumorigenesis in the colon via derepression of multiple oncogenic *let-7* targets. Thus, potential functions of *LIN28B* in colorectal tumorigenesis may be deduced based upon the tumorigenic properties of known *let-7* targets. Although there are several *let-7* microRNAs, and each isoform is complementary to multiple target mRNA 3' UTRs, some targets are unique in that their *let-7* binding sites exhibit perfect, or near perfect sequence complementarity to *let-7*. Two such targets include *HMGA2* (the first to be characterized, and thus canonical *let-7* target) and *IGF2BP1* (Boyerinas et al, 2008; Lee & Dutta, 2007; Mayr et al, 2007; Park et al, 2007); both of these targets have described roles in cancer progression (Berlingieri et al, 1995; Boyerinas et al, 2008; Fedele et al, 1998).

HMGA2 (High mobility group A2; also known as *HMGI-C*) is a transcription factor belonging to a family of *HMGA* proteins. *HMGA2* and other *HMGA* family members are overexpressed in a number of cancers, most notably ovarian cancer where a role for *HMGA2* is well described (Abe et al, 2003; Finelli et al, 2002; Park et al, 2007; Pierantoni et al, 2005). *HMGA2* promotes anchorage-independent proliferation and induces neoplastic transformation of NIH3T3 fibroblasts (Berlingieri et al, 1995; Fedele et al, 2002; Fedele et al, 1998).

IGF2BP1 (Insulin-like growth factor 2 mRNA-binding protein 1) is a member of the IGF-II mRNA-binding protein (IMP) family; these proteins bind the 5' UTR of *IGF2*

(insulin-like growth factor 2) transcripts, thus stabilizing *IGF2* mRNA and regulating its translation (Nielsen et al, 1999). *IGF2BP1* promotes cellular motility, a property which is perhaps critical to its role in embryogenesis and ability to promote tumorigenesis (Boyerinas et al, 2008).

Based upon the work presented in Chapter 2, we surmised that a post-transcriptional regulator of *let-7* is involved in the molecular pathogenesis of colon cancer. We hypothesized the known *let-7* inhibitor *LIN28B* promotes oncogenesis via suppression of *let-7* in the colon. Given that key *let-7* targets *HMGA2* and *IGF2BP1* function in promoting anchorage-independent growth and cellular motility, we further hypothesized that *LIN28B* promotes cellular migration, invasion, and transformation of colonic epithelial cells via inhibition of *let-7*.

In order to elucidate the role of *LIN28B* in colon tumorigenesis, we first evaluated *LIN28B* expression and *let-7a* and *let*-7b levels in tumors paired with adjacent normal colonic epithelium. We found a correlation between increased *LIN28B* expression and decreased *let-7a* and *let-7b* mature microRNAs. Subsequently, we constitutively expressed *LIN28B* in immortalized colonic epithelial and human colon cancer cell lines. We found that *LIN28B* expression increases cell migration, invasion, and soft-agar colony formation. However, these phenotypes are only reversed partially by concomitant *let*-7 expression, thereby suggesting potential *let-7* independent functions of *LIN28B*. To pursue this possibility, we conducted a microarray analysis that revealed several genes up-regulated with *LIN28B* constitutive expression in colon cancer cells. These genes include the intestinal stem cell markers *LGR5* and *PROM1* - both remain elevated

following *let-7* restoration, and thus may be regulated by *LIN28B* in an indirect or *let-7* independent mechanism. These findings implicate *LIN28B* as a potential diagnostic marker and/or therapeutic target in colon cancer.

Experimental Procedures

Human tissue samples. Human colorectal cancer and adjacent normal colon tissues were obtained from a collaborator (Dr. Naomoto) from the Department of Surgery, Okayama University Medical School, Japan under Institutional Review Board approval. Normal and tumor colon samples were stored in RNAlater (Qiagen, Valencia, CA), at -80^{o} C for later use. Tumor tissue was homogenized mechanically on ice and fine particulates were collected via centrifugation at 4^{o} C. RNA was isolated from homogenized tissues using a mirVana RNA isolation kit RNA was isolated using mirVana kit (Ambion, Austin, TX) and evaluated for integrity using a Bioanalyzer (Agilent, Santa Clara, CA).

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, where $p<0.05$ is considered statistically significant. Pearson correlation coefficients were calculated to assess the strength of the linear association between *let-7a* and *let-7b*, *let-7* expression and tumor stage, and *let-7* expression and survival.

Mature MicroRNA detection. 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 *let-7a-3-b* was determined by applying student's t-test, with $p<0.05$ considered significant.

Detection of *let-7a3-b* pri-microRNA expression. Total RNA was extracted from human tissues, transfected 293T, or transduced LIM 2405 and LoVo cells using the mirVana miRNA isolation kit as described above. cDNA was synthesized from 5 μg total RNA per sample using random hexamers and SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA). Detection of *let-7a3-b* pri-microRNA intronic sequence was achieved using PCR primers specific to the intronic sequence between *let-7a-3* and *let-7b* with the following sequences: ggggccgcctacactgagaag and ctggggcacgtgctgggaacct (in triplicate). Fold change for *let-7a-3-b* was determined by using Image J analysis to

quantify individual band intensities, followed by normalization to empty vector controls. Statistical significance of comparisons between empty vector and *let-7a-3-b* was determined by applying student's t-test, with $p<0.05$ considered significant.

mRNA expression analysis via 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*, *DKK1*, *CCND2*, and *KIT* 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. 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.

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^{o} 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 ^{o}C, 5% CO_2.

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 μg total protein was loaded onto 4-12% gradient gels for electrophoresis 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 fluorescent transwell assay systems atop a migration insert (BD Biosciences, San Jose, California) or a rehydrated invasion assay insert (BD Biosciences, San Jose, California) 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 incubated at 37^{o} C, 5% CO_2 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.

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 ^{o}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.

***Let-7* expression in cell lines constitutively expressing *LIN28B*.** We obtained a *let-7a* lentiviral expression vector (gift from 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 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.

Microarray analysis. We isolated RNA from *LIN28B*-DLD-1 and *LIN28B*-LoVo cells using a mirVana kit (Ambion, Austin, TX). Isolated RNA was submitted to The University of Pennsylvania Microarray Facility where quality control tests of the submitted total RNA samples were performed via Agilent Bioanalyzer and Nanodrop spectrophotometry. Subsequently, 100ng of total RNA was converted to first-strand cDNA using reverse transcriptase primed by a poly(T) oligomer that incorporated a synthetic RNA sequence. Second-strand cDNA synthesis was followed by ribo-SPIA (Single Primer Isothermal Amplification, NuGEN Technologies Inc. San Carlo, CA) for linear amplification of each transcript. The resulting cDNA was fragmented, assessed by Bioanalyzer, and biotinylated. cDNA yields were added to Affymetrix hybridization cocktails, heated at 99ºC for 2 min and hybridized for 16 h at 45ºC to Affymetrix Human Gene 1.0 ST Array GeneChips (Affymetrix Inc., Santa Clara CA). The microarrays were then washed at low (6X SSPE) and high (100mM MES, 0.1M NaCl) stringency and stained with streptavidin-phycoerythrin. Fluorescence was amplified by adding biotinylated anti-streptavidin and an additional aliquot of streptavidin-phycoerythrin stain. A confocal scanner was used to collect fluorescence signal after excitation at 570 nm. All protocols were conducted as described in the NuGEN Ovation manual and the Affymetrix GeneChip Expression Analysis Technical Manual.

RNA Expression Bioinformatics. 3 independent biological replicates for each condition were assayed on microarrays. Unsupervised hierarchical clustering by sample was performed to confirm that replicates within each condition grouped with most similarity, and to identify any outlier samples. Significance Analysis of Microarrays (SAM v3.0, www-stat.stanford.edu/~tibs/SAM/) was used to generate lists of statistically significant differentially expressed genes in pairwise comparisons of replicate averages between conditions. 13,156 genes were observed to have higher RNA abundance in cells with constitutive *LIN28B* expression versus control, and 15,771 genes were lower than in control. Candidate genes were further filtered by fold-change (threshold = 5 fold) and the resulting gene lists were tested for over-representation of Gene Ontology annotation categories using the DAVID Bioinformatics Resources (david.abcc.ncifcrf.gov).

Results

Mature *let-7a* and *let-7b* levels correlate with *LIN28B* expression in colon tumors

We measured expression of *let-7a* and *let-7b* pri-microRNA via qPCR for of the intronic portion of the *let-7a-3-b* cluster present on human chromosome 22q13.31 in 22 human colon adenocarcinomas paired with normal adjacent colonic mucosa. This revealed that levels of mature *let-7a* and *let-7b* in tumors do not correlate with *let-7a-3-b* cluster pri-microRNA expression (Table 1), alluding to a post-transcriptional mechanism of *let-7* down-regulation. Consequently, we measured via RT-qPCR mRNA expression of the *let-7* inhibitor, *LIN28B* (Figure 1). We then compared *LIN28B* expression to mature *let-7a* and *let-7b* levels in the 22 human colon tumor samples paired with normal colon (Figure 1; Table 1). We found that *LIN28B* expression negatively correlates with levels of both mature *let-7a* ($r=-0.47$, $p=0.0297$) and *let-7b* ($r=-0.41$, $p=0.0637$) in colon tumors.

Constitutive *LIN28B* expression transforms immortalized colonic epithelial cells, and promotes cellular migration and invasion in a *let-7* dependent manner

In order to further elucidate the role of *LIN28B* in colon tumorigenesis, we constitutively expressed human *LIN28B* in rat-derived immortalized colonic epithelial (IEC-6) via retroviral transduction. Expression of ectopic *LIN28B* in IEC-6 cells reduces levels of *let-7a* and *let-7b* isoforms by greater than 50% (Figure 2a-b). Surprisingly,

although *let-7* and its targets have known roles in cellular proliferation, proliferation rates were not affected by constitutive *LIN28B* expression (data not shown). We then examined the ability of *LIN28B* to promote migration and invasion via *in vitro* transwell assays. We found that constitutive *LIN28B* expression promotes migration and invasion of IEC-6 cells in culture (Figure 2c). Furthermore, IEC-6 cells, which at baseline do not form colonies in soft agar, do so with constitutive *LIN28B* expression (Figure 2d). These observations are also true for 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 3a-b). Furthermore, *LIN28B* increases migration, invasion, (Figure 3c) and soft agar colony formation in colon cancer cell lines (Figure 3d).

We next sought to determine whether *LIN28B* promotes tumorigenesis in a *let-7* dependent manner. To that end, we co-expressed a decoy *let-7a* hairpin molecule (the loop structure of this molecule is derived from mir-30, thereby eliminating the Lin28b binding site) in cells constitutively expressing *LIN28B*. We succeeded in increasing mature *let-7a* levels by more than 100% in these cells (Figure 4a). We then subjected cells co-expressing *LIN28B* and *let-7* to *in vitro* migration and invasion Boyden chamber assays. We found that concomitant *let-7* expression reduces cellular migration and invasion in the presence of *LIN28B* overexpression, as determined via Boyden chamber *in vitro* assays (Figure 4b-c). Notably, cells co-expressing *LIN28B* and *let-7* exhibit more migration and invasion than empty-vector controls (Figure 4b-c). This may indicate that *LIN28B*'s effect on cellular migration and invasion is only partially dependent upon *let-7* inhibition, portending potential *let-7* independent functions of *LIN28B*.

Microarray analysis reveals up-regulation of stem cell related genes, canonical Wnt signaling: potential *let-7* independent functions of *LIN28B*

In order to elucidate underlying mechanisms of *LIN28B*-mediated tumorigenesis, we subjected DLD-1 and LoVo colon cancer cell lines constitutively expressing *LIN28B* to microarray analyses. We identified more than 184 transcripts (164 up-regulated; 20 down-regulated) that are more than 5-fold changed with constitutive *LIN28B* expression (Table 2; Figure 5). Many genes up-regulated by *LIN28B* have reported roles in stem cell biology. For example, *KIT* (also called CD117 or c-KIT), which has been used as a stem cell marker and is crucial to maintenance of the hematopoeitic stem cell compartment (Osawa et al, 1996; Ratajczak et al, 1992), is dramatically increased by *LIN28B* expression (Figure 6b). In addition, we found the intestinal stem cell markers *LGR5* (leucine-rich repeat-containing G-protein coupled receptor 5) and *PROM1* (prominin 1) to be increased with *LIN28B* expression. Of note, *LGR5* is a transcriptional target of canonical Wnt signaling (Barker et al, 2007; Van der Flier et al, 2007). Additional Wnt targets including *DKK1* (Dickkopf-related protein 1) (Chamorro et al, 2005; Niida et al, 2004) and *CCND2* (cyclin D2) (Shtutman et al, 1999; Tetsu & McCormick, 1999) are up-regulated by *LIN28B* overexpression in colonic epithelial cells as well (Figure 6a). These results were verified by measuring transcript levels via RT-qPCR analysis and correspond to increases in β-catenin levels observed in *LIN28B*-DLD-1 and *LIN28B*-LoVo cells as well (Figure 6b).

Regulation of gene expression is mediated by *LIN28B* in a *let-7* dependent manner, but may also occur via *let-7* independent mechanisms

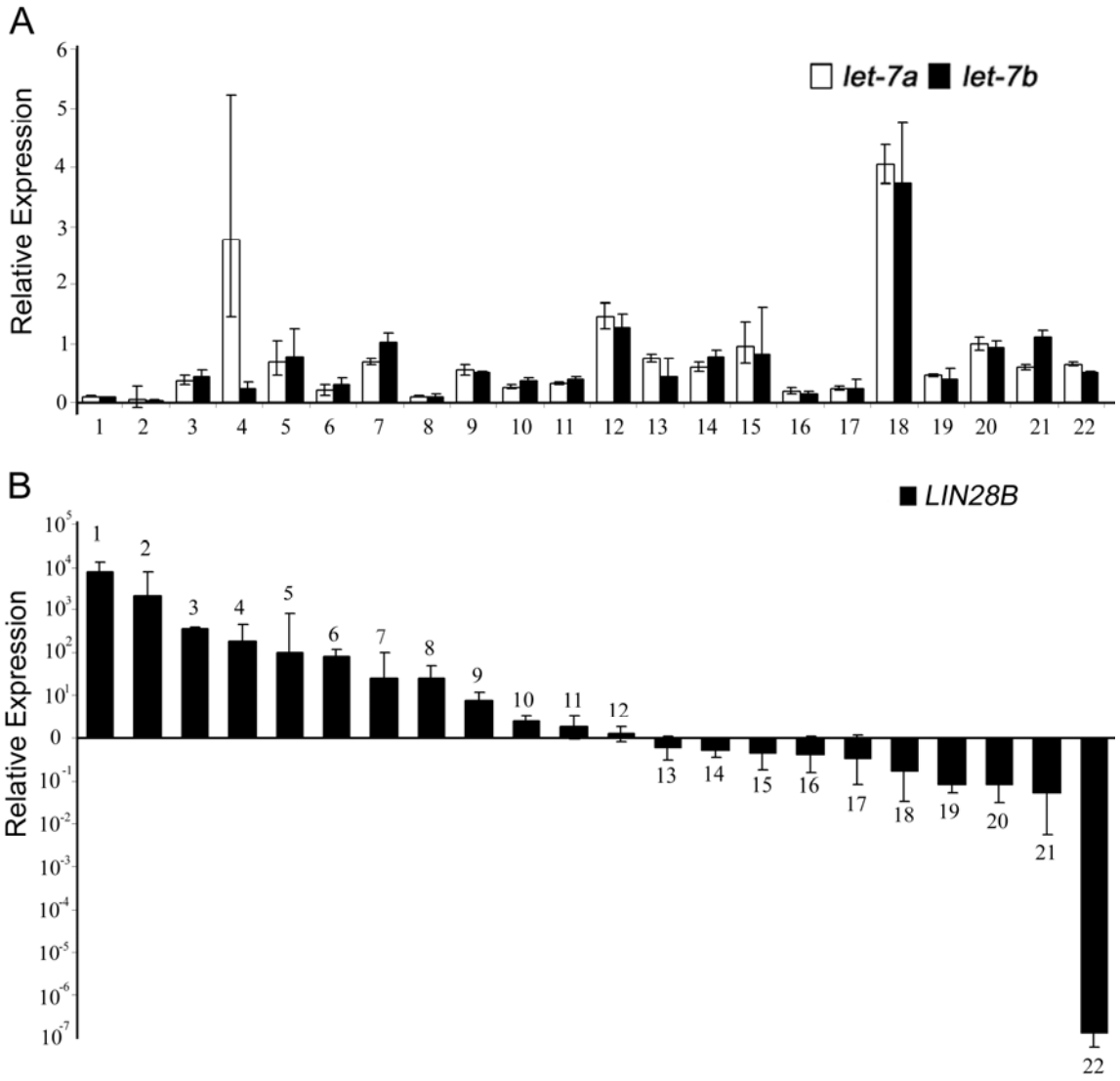
Since LIN28B represses *let-7* biogenesis, one would expect high Lin28b levels to relieve suppression of *let-7* targets, resulting in their increased expression. Constitutive expression of LIN28B in IEC-6, DLD-1 (data not shown), and LoVo colon cancer cells reduces *let-7* levels, resulting in increases in of *let-7* targets, including *IGF2BP1*, *CDC34*, and *HMGA2* (Figure 7a). Therefore, constitutive *LIN28B* expression increases *let-7* targets.

However, some genes that we have shown to be increased with constitutive *LIN28B* expression, including *LGR5*, *PROM1*, *KIT*, and *DKK1*, are not predicted by Target Scan and miRanda algorithms to be putative *let-7* targets (Griffiths-Jones et al, 2006; Griffiths-Jones et al, 2008; Grimson et al, 2007; Lewis et al, 2005; mirBase, 2010; TargetScan, 2010) (Figure 7b). This may indicate indirect regulation via let-7, or potentially let-7 independent functions of LIN28B. We were particularly intrigued by increased *LGR5* and *PROM1* transcript levels with *LIN28B* constitutive expression, as the ability of Lin28b to promote expression of intestinal stem cell markers may indicate a role for Lin28b in maintenance of the colonic stem cell compartment. *LGR5* and *PROM1* lack conventional *let-7* binding sites in their 3' UTR sequences (Griffiths-Jones et al, 2006; Griffiths-Jones et al, 2008; Grimson et al, 2007; Lewis et al, 2005; mirBase, 2010; TargetScan, 2010). In order to determine whether these genes are regulated by *LIN28B* in a *let-7* dependent fashion, we measured *LGR5* and *PROM1* gene expression in cells co-expressing *LIN28B* and a *let-7a* decoy hairpin molecule which escapes Lin28b-mediated

inhibition. We found that *let-7* targets including *HMGA2* and *IGF2BP1* are reduced by *let-7* restoration; however, *LGR5* and *PROM1* remain elevated (Figure 7). This suggests the possibility that regulation of these genes by *LIN28B* occurs via a *let-7* independent mechanism.

Chapter 2 Tables & Figures

Figure 1



Mature *let-7a* and *let-7b,* and *LIN28B* expression in colon tumors. RNA was isolated from tumors paired with adjacent normal mucosa; mature *let-7a* and *let-7b*, and *LIN28B* were detected via RT-qPCR. Relative expression of mature *let-7a* and *let-7b*, and *LIN28B* in tumors was determined by normalization to adjacent colonic epithelium. (A) Levels of mature *let-7a* and *let-7b* in 22 tumors compared to normal colonic epithelium. (B) *LIN28B* expression in the same 22 tumors compared to normal colonic epithelium.

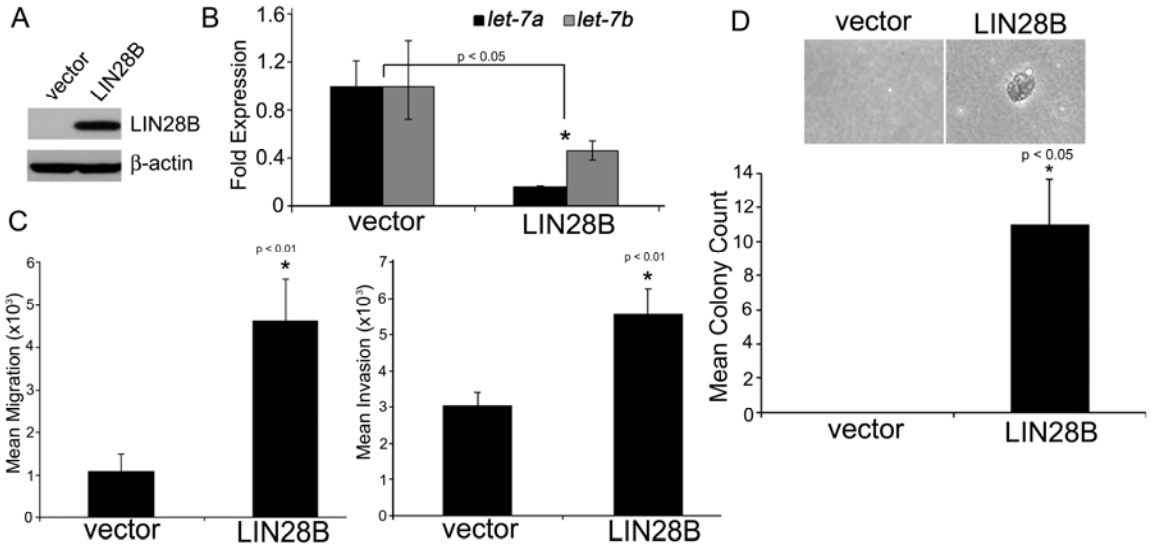
Table 1

RNA expression ratio (tumor:normal epithelium)	**mature *let-7***	***let-7a-3-b* cluster**
>0.6	10	16
<0.6	12	6
Total	**22**	**22**

RNA expression ratio (tumor:normal epithelium)	***LIN28B***
>2	10
0-2	12
Total	22

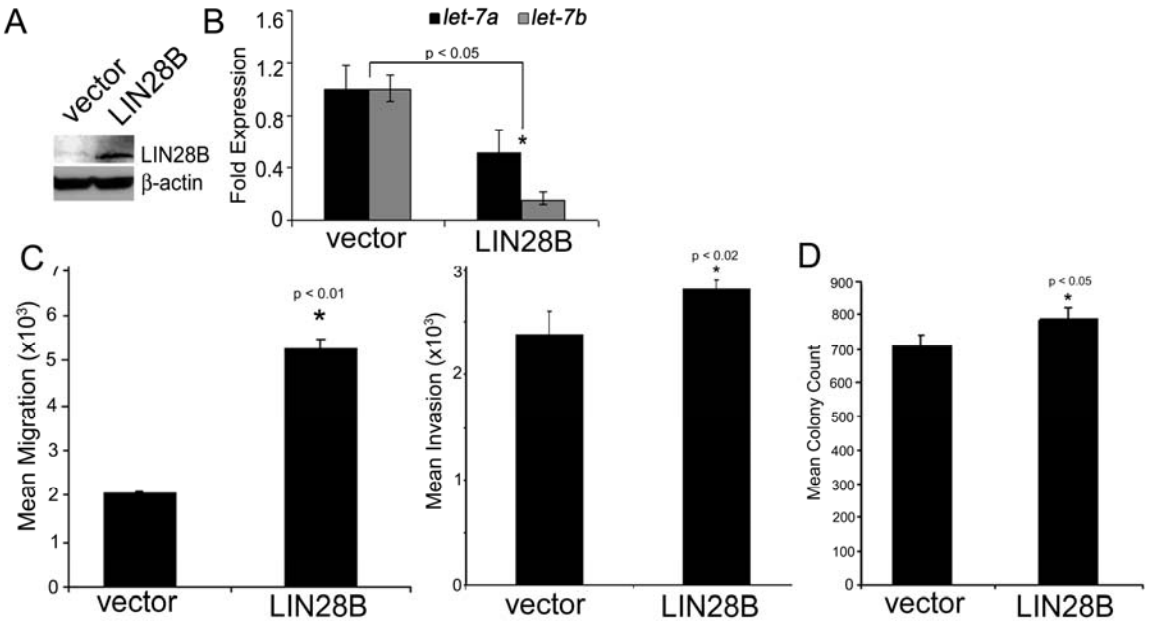
Decreased levels of mature *let-7a* and *let-7b* isoforms with concomitant *LIN28B* overexpression in a subset of colon tumors. Summary of data presented in Figure 1. RNA was isolated from tumors matched with adjacent normal colonic epithelium and utilized for RT-qPCR analysis. We compared levels of mature *let-7a* and *let-7b*, with *let-7a-3-b* cluster and *LIN28B* expression in 22 pairs of colon tumors and matched adjacent normal colonic epithelium. *let-7a-3-b* expression levels do not correlate with mature *let-7a* and *let-7b* levels. However, *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.

Figure 2



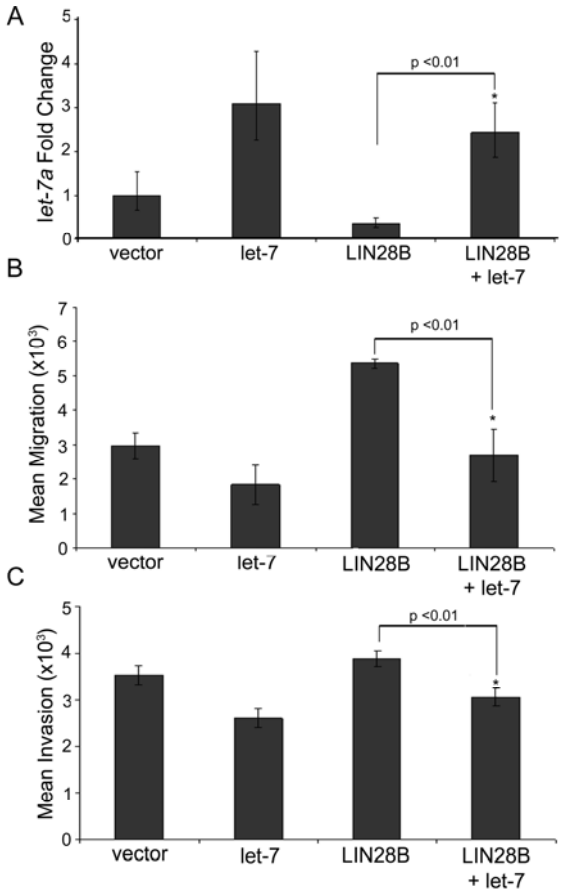
***LIN28B* expression promotes invasion, migration, and soft-agar colony formation in IEC-6 cells.** (A) *Verification of exogenous LIN28B expression.* Western blot for Lin28b in IEC-6 cells transduced with MSCV-PIG-LIN28B retrovirus. (B) *LIN28B reduces mature* let-7 *isoforms*. RT-qPCR for mature *let-7* in transduced IEC-6 cells; normalization to empty vector control. Decreased levels of mature *let-7a* and *let-7b* occur in IEC-6 cells following *LIN28B* constitutive expression. (C) *Lin28b increases migration and invasion*. Constitutive *LIN28B* expression in immortalized colonic epithelial (IEC-6) cells promotes cell migration and invasion in Boyden chamber assays. (D) LIN28B *induces transformation.* Photomicrograph (100x magnification) of soft-agar cultures from empty vector and *LIN28B*-IEC6 cells with quantitation of colonies. *LIN28B* expression confers the ability of immortalized IEC-6 cells to form colonies in soft-agar.

Figure 3



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

Figure 4



Restoration of mature *let-7* attenuates *LIN28B*-mediated migration and invasion. (A) *Decoy* let-7 *hairpins escape* LIN28B-*mediated inhibition.* RNA was isolated form empty vector and *LIN28B*-LoVo cells transduced to express *let-7a* decoy hairpin molecules and RT-qPCR performed to measure mature *let-7a*. Mature *let-7a* is increased with decoy expression in empty vector and *LIN28B*-expressing cells. (B and C) LIN28B-*mediated migration and invasion is* let-7 *dependent*. *In vitro* migration and invasion Boyden chamber assays for cells expressing empty vector, *let-7*, *LIN28B*, or co-expressing *let-7* and *LIN28B*. Increased *let-7* reduces migration and invasion of LoVo cell constitutively expressing *LIN28B*.

Table 2

Increased transcripts

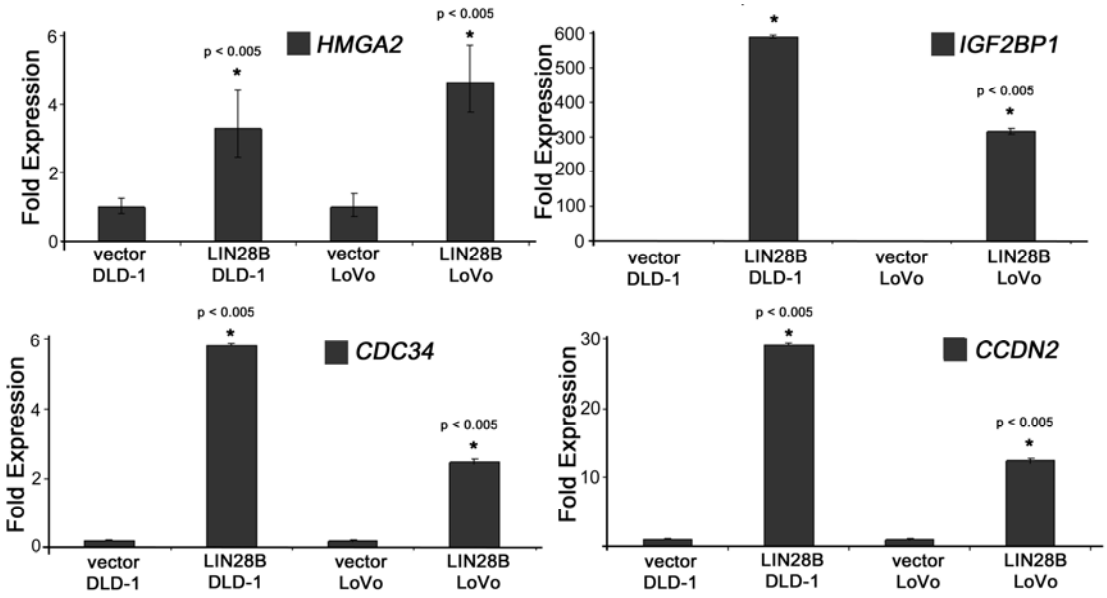
Gene Symbol	Fold	Gene Symbol	Fold	Gene Symbol	Fold
FN1	147.1	*ZNF512*	10.1	*AREG*	7.0
SPINK1	41.8	*LAMA1*	10.0	*SPP1*	6.9
DKK1	37.1	*FGG*	10.0	*FERMT2*	6.9
KIT	36.6	*RBMS1*	10.0	*ADFP*	6.8
C4orf18	34.4	*CEACAM6*	9.8	*IRF8*	6.8
ANKRD1	33.5	*TGME49_101130*	9.7	*BST2*	6.8
LCP1	26.8	*CD302*	9.7	*CTSE*	6.7
GPC3	24.9	*RPS4Y1*	9.6	*CCND2*	6.6
HAVCR1	22.6	*HEPH*	9.6	*CD109*	6.5
PROM1	22.6	*NAALAD2*	9.6	*AREG*	6.5
TTR	22.0	*RPL39L*	9.6	*BNIP3*	6.5
IGF2	21.0	*BICC1*	9.6	*PHYHIPL*	6.5
SMARCA1	20.4	*DDC*	9.6	*IL1R2*	6.4
ANO6	19.4	*DPP4*	9.6	*CRABP1*	6.3
PEG10	19.1	*KYNU*	9.4	*FAM105A*	6.1
APOA2	18.0	*PLA2G7*	9.4	*PLA2G2A*	6.1
SLC40A1	17.7	*IGF2BP1*	9.4	*AMIGO2*	5.9
LIN28B	17.6	*GCNT4*	9.3	*LIPG*	5.9
ARHGAP28	17.3	*MEF2C*	9.2	*ANTXR2*	5.9
SLC19A3	16.5	*APOH*	9.2	*COL5A2*	5.9
PDZK1	14.8	*GLDC*	9.1	*SERPINE2*	5.9
BCAT1	14.4	*RIMKLB*	9.1	*ST6GAL1*	5.9
UCHL1	14.1	*AKR1C3*	8.9	*SYCP2*	5.9
HGD	14.1	*GPX2*	8.9	*THBS1*	5.9
HGD	14.1	*C6orf150*	8.8	*PRKAA2*	5.9
IL33	13.9	*CEACAM5*	8.8	*SPINK5*	5.9
TDO2	13.4	*GCNT3*	8.8	*PPARG*	5.8
DSC3	12.9	*C3orf57*	8.8	*AS3MT*	5.8
ELOVL5	12.8	*CES1*	8.7	*FABP1*	5.8
SERPINB5	12.7	*PRSS35*	8.6	*SMC1B*	5.8
APOD	12.5	*CPNE8*	8.5	*AFP*	5.7
PVRL3	12.2	*GIPC2*	8.4	*SPON1*	5.7
PTGS2	11.9	*ZNF22*	8.3	*PTPRM*	5.7
EFNB2	11.8	*RNF128*	8.2	*CLDN1*	5.6
POF1B	11.7	*GJC1*	8.0	*C12orf27*	5.6
FREM2	11.6	*INPP4B*	7.8	*EPHA4*	5.5
PRTG	11.6	*SLC38A4*	7.8	*ENPP1*	5.5
LY75	11.6	*PLAC8*	7.8	*FAM3D*	5.5
LRP2	11.5	*FAM127A*	7.7	*MNS1*	5.4
NT5E	11.5	*S100P*	7.6	*NRCAM*	5.3
FLRT3	11.4	*SLPI*	7.5	*SCGN*	5.3
TGFBI	11.4	*CKB*	7.5	*TSTA_053980*	5.3
CALB1	11.4	*ABCC9*	7.4	*C6orf15*	5.3
ABCC2	11.4	*CPA2*	7.4	*DKFZP564O0823*	5.2
ALDH1A1	11.3	*SLC38A5*	7.4	*TSPAN8*	5.2
PI3	11.2	*SPTLC3*	7.3	*CRTAM*	5.2
SGCE	11.1	*DAB2*	7.3	*ACTL8*	5.2
AKR1C2	11.0	*STK31*	7.3	*A1CF*	5.1
REG4	11.0	*LGR5*	7.2	*HIST1H2AE*	5.1
APOB	10.9	*AGR2*	7.2	*MUC13*	5.1
SPINK6	10.8	*TSPAN2*	7.2	*AKR1B10*	5.1
PON3	10.7	*FMOD*	7.1	*GJA1*	5.0
IQGAP2	10.6	*NOX1*	7.1	*RASSF9*	5.0
AHSG	10.5	*HMGCS2*	7.0	*SLCO4C1*	5.0
CFTR	10.2	*LEPREL1*	7.0		

Decreased transcripts

Gene Symbol	Fold
NOTUM	-5.1
MKX	-5.2
NIPSNAP3B	-5.2
KLRK1	-5.3
ABCB1	-5.5
LOC253724	-5.6
LIX1	-6.1
RAB38	-6.3
ZNF738	-6.9
RPS6KA6	-7.0
DPYD	-7.8
PRKD1	-8.5
FGF20	-9.0
ODAM	-10.4
SORBIDRAFT	-10.5
APCDD1	-10.7
DPEP1	-11.2
H19	-13.2
SP3P	-16.0
PTN	-22.7

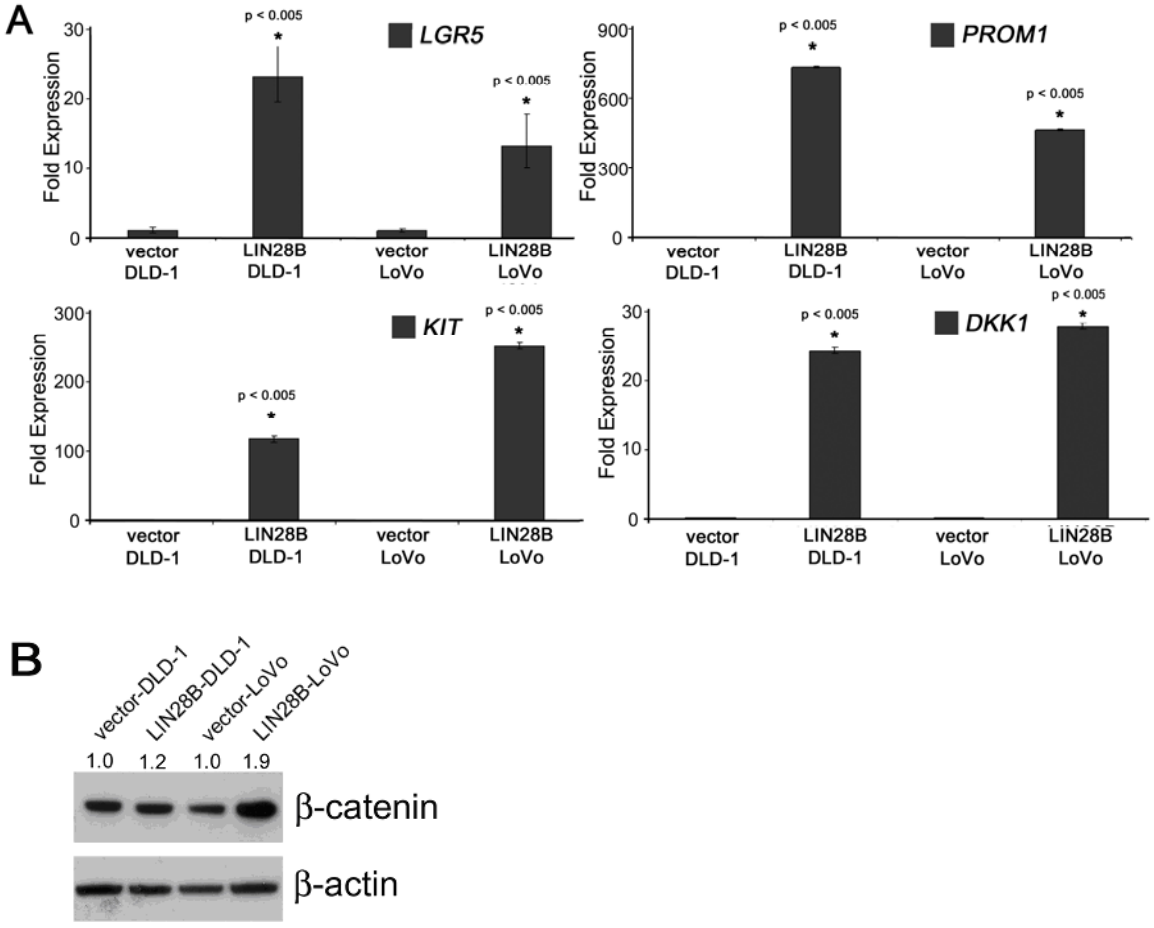
Transcripts modulated by constitutive *LIN28B* expression *in vitro*. Microarray analysis reveals 164 transcripts are up-regulated 5-fold or more, and 20 transcripts more than 5-fold down-regulated by constitutive *LIN28B* expression *in vitro*.

Figure 5



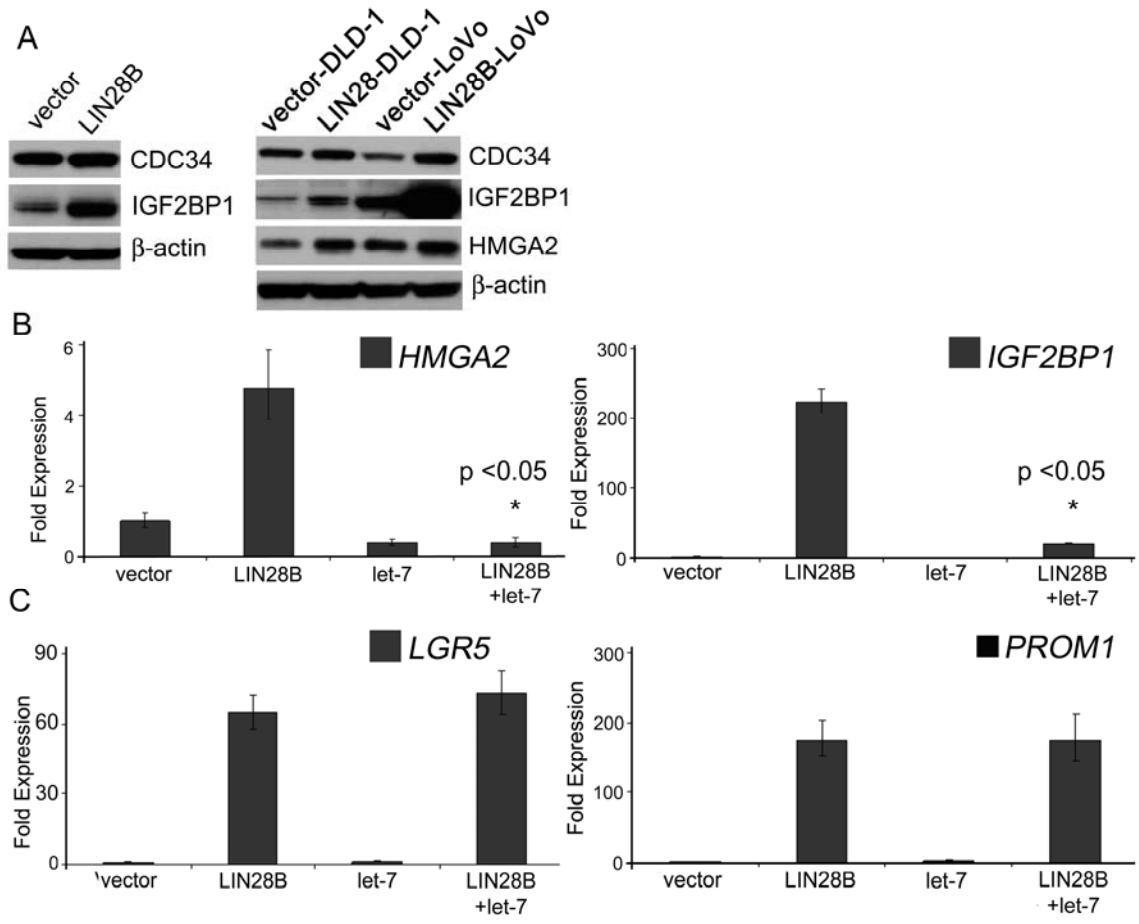
Constitutive *LIN28B* expression relieves *let-7* target suppression. RNA was isolated from empty vector and *LIN28B*-expressing DLD-1 and LoVo and RT-qPCR was done to evaluate changes in let-7 targets: *HMGA2*, *IGF2BP1*, *CCND2*, and *CDC34*; target transcript levels are normalized to empty vector cells. *LIN28B* overexpression in colon cancer cells relieves *let-7*-mediated repression, thereby increasing target transcripts.

Figure 6



Constitutive *LIN28B* expression up-regulates non-*let-7* targets. (A) *Transcripts up-regulated by* LIN28B *that do not contain* let-7 *complimentary sites.* RT-qPCR of RNA isolated from empty vector and *LIN28B*-expressing DLD-1 and LoVo cells for *LGR5*, *PROM1*, *KIT*, and *DKK1*; target transcript levels are normalized to empty vector cells. *LIN28B* increases expression of transcripts that are not predicted *let-7* targets in colon cancer cells. (B) Western blot for β-catenin on protein extracts from empty vector and *LIN28B*-expressing DLD-1 and LoVo cells. Total β-catenin is up-regulated with constitutive *LIN28B* expression.

Figure 7



***LIN28B*-mediated up-regulation of a subset of genes is *let-7* dependent.** (A) *Derepression of* let-7 *targets in cells that constitutively express* LIN28B. Western blot for *let-7* targets *CDC34*, *IGF2BP1* and *HMGA2* in IEC-6, DLD-1, and LoVo cells that constitutively express *LIN28B*. (B) let-7-*dependent gene upregulation by* LIN28B. LIN28B Increased mature *let-7* levels in LIN28B-LoVo cells restores repression of *let-7* targets (C) LIN28B-*mediated upregulation of non*-let-7 *targets is not ameliorated* by let-7 *restoration*. Upregulation of *LGR5* and *PROM1* in LIN28B-expressing cells is maintained following *let-7* restoration.

Chapter 2 Summary

We found a correlation between increased *LIN28B* expression and decreased *let-7a* and *let-7b* mature microRNAs in colon tumors, implicating a role for *LIN28B* in colon cancer pathogenesis. In order to further elucidate this role, we constitutively expressed *LIN28B* in immortalized colonic epithelial and human colon cancer cell lines. We demonstrated that *LIN28B* promotes cellular migration, invasion, and soft-agar colony formation in a manner partially dependent on *let-7* inhibition. Microarray analyses revealed several genes up-regulated with *LIN28B* constitutive expression including the intestinal stem cell markers *LGR5* and *PROM1*. These genes remain up-regulated following *let-7* restoration, suggesting a novel *let-7* independent mechanism of regulation by *LIN28B*. This work suggests *LIN28B* is a potential therapeutic and/or diagnostic target. Therefore, further analysis of *LIN28B* function *in vivo* is indicated and thus presented in Chapter 3.

Chapter 3

In vivo roles of *LIN28B*

Guide to Chapter 3 Tables and Figures

Background and Significance

Both *LIN28* and *LIN28B* are implicated in multiple developmental processes, largely as a consequence of their ability to repress *let-7* biogenesis. In *C. elegans*, *let-7* is critical to the larval fate transition, and vulval specification (Johnson et al, 2005); *Lin-28* mutants (where *let*-7 repression is dysfunctional) reiterate larval fates (Moss et al, 1997). In human embryonic stem cells, *let-7* levels increase during differentiation, partially as a consequence of reduced *LIN28* expression (Viswanathan et al, 2008). On a broader scale, *LIN28B* mutations are associated with delayed onset of puberty in humans (Tommiska et al, 2009), which may be modeled via transgenic expression of *LIN28* in mice (Zhu et al). Lin28 transgenic mice also display gut pathology (Zhu et al, 2010), further suggesting involvement in intestinal development.

Functions of *LIN28* have been described in both neurogliogenesis and skeletal myogenesis (Balzer et al, 2010; Polesskaya et al, 2007). In neuronal cells, *LIN28* is down-regulated during differentiation; constitutive *LIN28* expression in undifferentiated cells blocks gliogenesis in favor of neurogenesis (Balzer et al, 2010; Polesskaya et al, 2007). Additionally, *LIN28* is induced during myogenic differentiation and enforced expression in myoblasts drives skeletal muscle myogenesis (Balzer et al, 2010; Polesskaya et al, 2007). The functions of *LIN28* in both neurogenesis and myogenesis are dependent upon its ability to promote *IGF2* (Insulin-like growth factor 2) mRNA stabilization and transcription (Balzer et al, 2010; Polesskaya et al, 2007). *IGF2* is also upregulated during osteoblast differentiation of mesenchymal stromal cells (Hamidouche et al, 2010). More importantly, loss of *IGF2* imprinting results in a shift toward a less

differentiated intestinal epithelium (Sakatani et al, 2005). As we have demonstrated, expression of *LIN28B* relieves *let-7*-mediated repression of *IGF2BP1* (Chapter 2), which may increase translation of *IGF2* by stabilizing its mRNA (Nielsen et al, 1999). The ability of *LIN28B* to indirectly regulate *IGF2* suggests roles for *LIN28B* in modulating cellular differentiation programs.

Moreover, we previously showed that *LIN28B* expression results in up-regulation of intestinal stem cell markers including *PROM1* and *LGR5*, which may indicate specific roles of *LIN28B* in intestinal stem cells. Within the intestine, expression of the cell surface protein *PROM1* is restricted to the crypt 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, the ability of *LIN28B* to up-regulate them suggests roles for *LIN28B* in establishment and/or maintenance of intestinal stem cells. Of note, *LGR5* is an established target of the Wnt pathway (Barker et al, 2007; Van der Flier et al, 2007), and constitutive *LIN28B* expression induces additional targets of canonical Wnt signaling (Chapter 2). Since Wnt signaling is critical to maintaining the intestinal stem cell niche (Kim et al, 2005; Reya & Clevers, 2005), the ability of *LIN28B* to up-regulate Wnt targets may further indicate its involvement in intestinal stem cell biology.

In order to elucidate further the functions of *LIN28B*, we evaluated the effect of constitutive *LIN28B* expression *in vivo* by employing xenograft transplantation models. We found that *LIN28B* overexpressing tumors display increased differentiation with

evidence of enhanced glandular formation, in conjunction with increased *LGR5* and *PROM1* expression. These findings suggest *LIN28B* might be involved in both cellular re-programming and tumor progression. Of note, a subset of *LIN28B*-overexpressing tumors display metastasis to the liver and lungs, whereas control cells do not. Additionally, we find increased Lin28b expression (via immunohistochemistry) correlates with reduced survival and increased probability of recurrence in patients with colon cancer. This work supports roles for *LIN28B* in progression of colon cancer.

Experimental Procedures

Xenograft injections. *LIN28B* was expressed constitutively in DLD-1 and LoVo colon cancer cell lines via retroviral transduction as described in Chapter 2. Immunocompromised nude mice were irradiated with 5 Gray 2-3 hours prior to injection. Empty vector and *LIN28*B-expressing DLD-1 and LoVo cells were trypsinized and washed in PBS and resuspended at a concentration of 2×10^7 cells/ml. 50 μl of cell suspensions were mixed with 50 μl of BD Matrigel™ Basement Membrane Matrix (BD Biosciences, Franklin Lakes, NJ) to achieve a volume of 100 μl containing 1×10^6 cells per injection. Cells were injected subcutaneously into the rear flanks of nude, athymic, RAG-1–/– mice sedated with ketamine (100 mg/kg) and xylazine (10 mg/kg. Following injection, mice were monitored periodically and sacrificed 6 weeks post-injection. All mice were cared for in accordance with University Laboratory Animal Resources requirements.

Detection of GFP fluorescence in live mice by image analysis. We anesthetized xenografted nude mice with ketamine (100 mg/kg) and xylazine (10 mg/kg) and measured fluorescence intensity using a small animal imaging system (Kodak, Rochester, NY). Net tumor intensity was calculated over a fixed region of interest (ROI) that encompasses the dimensions of the largest tumor. Statistical significance of comparisons between empty vector and *LIN28B* tumors was determined by applying student's t-test, where $p<0.05$ is statistically significant.

Histopathological analysis of primary tumors and metastases. Moderate differentiation, and poor differentiation were scored as a percentage of viable tumor epithelium on hematoxylin and eosin stained xenograft section. Mucin production in areas of poor and moderate differentiation was determined via mucicarmine staining; scored as a percentage of viable tumor epithelium. Statistical significance of comparisons between empty vector and *LIN28B* tumors was determined by applying student's t-test, where $p<0.05$ is considered statistically significant. Metastases observed via gross and histopathologicial examinations were verified in conjunction with a board-certified pathologist, and by immunohistochemical detection of green fluorescent protein (GFP) expressed from the MSCV-PIG vector.

Gene expression analysis of xenograft tumors. Mice were euthanized in accordance with ULAR (University Laboratory Animal Resources) standards prior to excision of xenograft tumors. Extraneous tissues were dissected away and discarded, tumors were then immediately placed into RNAlater (Qiagen, Valencia, CA) and maintained on ice until storage at -80^{o} C. Tumor tissue was homogenized mechanically in RNAlater (Qiagen, Valencia, CA), and homogenized particulates were collected via centrifugation at 4^{o} C. RNA was isolated from homogenized tissues using a mirVana RNA isolation kit (Ambion, Austin, TX). cDNA was synthesized from 3 μg isolated RNA per sample using random oligomers and SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA). Synthesized cDNA was then subjected to gene expression analysis via real-time qPCR using the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Carlsbad, CA). Transcripts were amplified and detected using

PROM1 and *LGR5* specific probes, with β-actin serving as an endogenous control (Ambion, Austin, TX). 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.

Microarray analysis. We isolated RNA using a mirVana kit (Ambion, Austin, TX) from excised empty vector tumors, *LIN28B*-LoVo tumors, and *LIN28B*-LoVo mesenteric metastases. We then submitted triplicate RNA isolates to the Penn Microarray Facility. Quality control tests of the submitted total RNA samples were performed using Agilent Bioanalyzer and Nanodrop spectrophotometry. 100ng of total RNA was then converted to first-strand cDNA using reverse transcriptase primed by a poly(T) oligomer that incorporated a synthetic RNA sequence. Second-strand cDNA synthesis was followed by ribo-SPIA (Single Primer Isothermal Amplification, NuGEN Technologies Inc. San Carlo, CA) for linear amplification of each transcript. The resulting cDNA was fragmented, assessed by Bioanalyzer, and biotinylated. cDNA yields were added to Affymetrix hybridization cocktails, heated at 99ºC for 2 min and hybridized for 16 h at 45ºC to Affymetrix Human Gene 1.0 ST Array GeneChips (Affymetrix Inc., Santa Clara CA). The microarrays were then washed at low (6X SSPE) and high (100mM MES, 0.1M NaCl) stringency and stained with streptavidin-phycoerythrin. Fluorescence was amplified by adding biotinylated anti-streptavidin and an additional aliquot of streptavidin-phycoerythrin stain. A confocal scanner was used to collect fluorescence signal after excitation at 570 nm. All protocols were conducted as described in the

NuGEN Ovation manual and the Affymetrix GeneChip Expression Analysis Technical Manual.

RNA Expression Bioinformatics. 3 independent biological replicates for each condition were assayed on microarrays. Unsupervised hierarchical clustering by sample was performed to confirm that replicates within each condition grouped with most similarity, and to identify any outlier samples. Significance Analysis of Microarrays (SAM v3.0, www-stat.stanford.edu/~tibs/SAM/) was used to generate lists of statistically significant differentially expressed genes in pairwise comparisons of replicate averages between conditions. 11,242 genes were observed to have higher RNA abundance in *LIN28B* metastases versus control, and 17,579 genes were lower than in control. Candidate genes were further filtered by fold-change (threshold = 4 fold) and the resulting gene lists were tested for over-representation of Gene Ontology annotation categories using the DAVID Bioinformatics Resources (david.abcc.ncifcrf.gov).

Tumor Tissue Microarray Analysis. Samples of colorectal adenocarcinomas tumors and adjacent normal colonic mucosa were obtained from patients diagnosed and staged with colorectal adenocarcinomas under Institutional Review Board approval. Two cores of normal mucosa and two cores of tumor tissue for each represented individual were paraffin-embedded in ordered microarrays (gift from Toni Castells and Miriam Cuatrecasas, University of Barcelona). Tumor microarrays were sectioned in preparation for immunohistochemical staining.

Immunohistochemistry. Paraffin-embedded tissue microarrays and xenograft tumors were incubated at 60^{o} C prior to de-waxing and rehydration. Tissues were incubated in xylene (5 min. x 2), followed by incubations in incremental dilutions of ethanol: 100% (2 min. x 2), 95% (1 min.), 80% (1 min.), 70% (1 min.), ddH_2O (1 min.). We then performed antigen retrieval on sections by placing them in 10 μM citric acid (pH 6) and microwaving for 15 minutes. Samples were allowed to cool for 15 minutes prior to rinsing. Endogenous peroxidases were quenched in 15 ml hydrogen peroxide and 185 ml of water. Tissues were rinsed in water and washed with PBS prior to treatment with Avidin D and Biotin using Vectastain (Vector Labs, Burlingame, CA). Samples were washed again with PBS prior to treatment with Starting Block (Thermo Scientific, Rockford, IL) for 10 minutes. Tissues were incubated in Lin28b (1:200; Cell Signaling, Boston, MA) Lgr5 (1:250; Lifespan Biosciences, Seattle, WA) or prom1 (1:100 Cell Signaling, Boston, MA) primary antibodies diluted in PBT (10X PBS, 10% BSA, 10% Triton X-100 in ddH_2O) at 4^{o} C overnight. The following day, samples were washed with PBS, and treated with ABC Peroxidase Staining Kit (Pierce, Rockford IL) as per the manufacturer's protocol. For detection, DAB reagent was prepared from a DAB Peroxidase Detection Kit (Vector Labs, Burlingame, CA) during the last PBS washes by adding 1 drop of buffer, 2 drops of DAB solution, and 1 drop of peroxide solution and applied to sections. We monitored color development using a light microscope in order to determine an appropriate incubation period for each primary antibody tested. The development process was terminated removing DAB (into a chemical hazard waste container) and rinsing the sections with ddH_2O for 1 min prior to counterstaining with hematoxylin.

Tumor tissue microarray biostatistical analysis. Each core of normal and tumor mucosa stained for Lin28b via immunohistochemistry was scored according to intensity (collaboration with Toni Castells and Miriam Cuatrecasas, University of Barcelona). 1 was used to signify low Lin28b intensity, 2 for intermediate intensity, and 3 for high. Logrank tests (Mantel–Cox and Breslow) were performed to compare survival and recurrence distributions of intensity groups (1-2 vs 3). Correlation of staining intensity with survival and recurrence was determined via Chi-square analysis; 95% confidence interval calculated for confirmation of statistical significance

Results

LIN28B tumors exhibit moderate differentiation with glandular formation, in conjunction with increased intestinal stem cell marker expression

In order to determine *in vivo* roles of *LIN28B* in tumorigenesis, we xenografted our previously described *LIN28B*-DLD-1 and *LIN28B*-LoVo cells (Chapter 2) subcutaneously into the rear flanks of nude mice (1 million cells per injection). We injected a total of five cohorts and monitored tumor development in each over the course of six weeks. A total of 83 mice (41 in the empty vector; 42 *LIN28B* tumors) were evaluated in this study (Table 1).

Prior to euthanizing mice and performing necropsies, we approximated tumor size for each cohort at two week intervals. We determined fluorescent intensity of tumors as a quantitative measure of green fluorescent protein expression, which is exogenously expressed from the MSCV-PIG retroviral vector. At each interval, we found *LIN28B* tumors emit less fluorescence than empty vector controls (Figure 1a). This observation corresponds to a smaller mass of *LIN28B* xenografts as determined by weighing extracted tumors post-sacrifice (Figure 1b).

We confirmed *LIN28B* overexpression in smaller tumors by immunohistochemistry (Figure 2a-b), and established that mature *let-7* microRNA levels are decreased in xenografts where *LIN28B* is constitutively expressed (Figure 3). Interestingly, Ki-67 staining is comparable between empty vector and *LIN28B* tumors, suggesting the reduction in tumor size observed for *LIN28B* tumors did not occur as a

consequence of a cellular proliferation defect (data not shown). Similarly, immunostaining for caspase-3 did not reveal differences between empty vector and *LIN28B* tumors, suggesting the smaller tumor size is not a result of increased apoptosis (data not shown).

Yet, the histological architecture of *LIN28B* tumors is somewhat surprising in that they exhibit fewer necrotic areas, moderate differentiation, glandular formation, and increased mucin production (Figure 2d). This stands in contrast to empty vector tumors which are poorly differentiated, have large necrotic foci, and rarely exhibit mucinous gland-like structure (Figure 2c; Figure 3a-d). Immunoassaying for the colon cancer marker Cdx2 did not reveal differences between empty vector and *LIN28B*-expressing tumors (data not shown). Yet notably, the intestinal stem cell markers *LGR5* and *PROM1* are also up-regulated with *in vivo* constitutive *LIN28B* expression (Figure 4). These findings suggest roles for *LIN28B* in regulating differentiation and/or modulating cellular re-programming.

Constitutive *LIN28B* expression promotes metastasis of xenograft tumors

Although *LIN28B* xenograft tumors are smaller and appear more differentiated than their empty vector counterparts (Figure 5), they are capable of metastasizing to multiple organ sites (Figure 6). We observed liver and lung metastases in mice bearing tumors with constitutive *LIN28B* expression, at times, upon gross examination (Figure 6).We also noted metastases to perisplenic adipose tissue, lymph nodes, and mesentery. These metastases were confirmed with a histopathologist and via immunohistochemical

detection of green fluorescent protein expression, which is unique to xenografted tissues (Figure 6c-d). Of note, we did not observe metastases in any mice of the empty vector control group. Overall, we identified metastases in 10% of the *LIN28B* xenografts (Table 2). Thus, constitutive expression *LIN28B* confers metastatic ability in at least a subset of tumors (Fisher's exact one-tailed probability = 0.061).

The gene expression profile induced by constitutive *LIN28B* expression *in vitro* overlaps with the profile *in vivo*

We sought to elucidate potential mechanisms whereby *LIN28B* promotes metastasis by comparing the gene expression profile of *LIN28B* metastases to primary tumors. Accordingly, we performed microarray analysis on total RNA isolated from empty vector tumors, *LIN28B*-LoVo tumors, and *LIN28B*-LoVo metastases. We identified more than 22 genes that are more than 4-fold up-regulated, and 57 genes that are more than 4-fold down-regulated by constitutive *LIN28B* expression *in vivo* (Table 3). Many genes that are up-regulated with constitutive *LIN28B* expression *in vitro* are also increased with constitutive *LIN28B* expression *in vivo*, including the stem cell markers *LGR5*, *PROM1*, and the *let-7* targets *HMGA2* (High-mobility group AT-hook 2) and *IGF2BP1* (Insulin-like growth factor 2 mRNA-binding protein 1); these results were verified by RT-qPCR analysis (data not shown). Overall, the expression profile of empty vector tumors varies dramatically from that of *LIN28B* tumors. Notably, the expression profile of *LIN28B* metastases also differs from that of *LIN28B* primary tumors. Some genes that are specifically modulated in *LIN28B* metastases versus primary tumors, including *TNS4* (Tensin 4), *CHI3L1* (Chitinase 3-like-1), and KLK6 (Kallikrein 6), have described roles

in migration, invasion, and metastasis, and are therefore prime candidates for future studies (Bockholt & Burridge, 1993; Ghosh et al, 2004; Mizoguchi, 2006).

LIN28B overexpression correlates with reduced patient survivability

Our work suggests that *LIN28B* may serve as a prognostic factor in patients with colon cancer. To that end, we evaluated Lin28b protein levels in colon cancers and adjacent normal mucosa resected from patients receiving adjuvant 5-fluorouracil (Figure 8a). We performed immunohistochemistry for Lin28b; staining was scored according to intensity by a pathologist. We then performed logrank tests (Mantel–Cox and Breslow) to compare survival and recurrence distributions of intensity groups. We found that low intensity of Lin28b staining in stage I and II colon tumors correlates with higher patient survival (Mantel-Cox p value = 0.046; Breslow, p value = 0.013), and a lower probability of tumor recurrence (Mantel-Cox p value = 0.036; Breslow p value = 0.108) (Figure 8b-c). This indicates a worse prognosis for patients with early stage tumors that exhibit high intensity Lin28b staining.

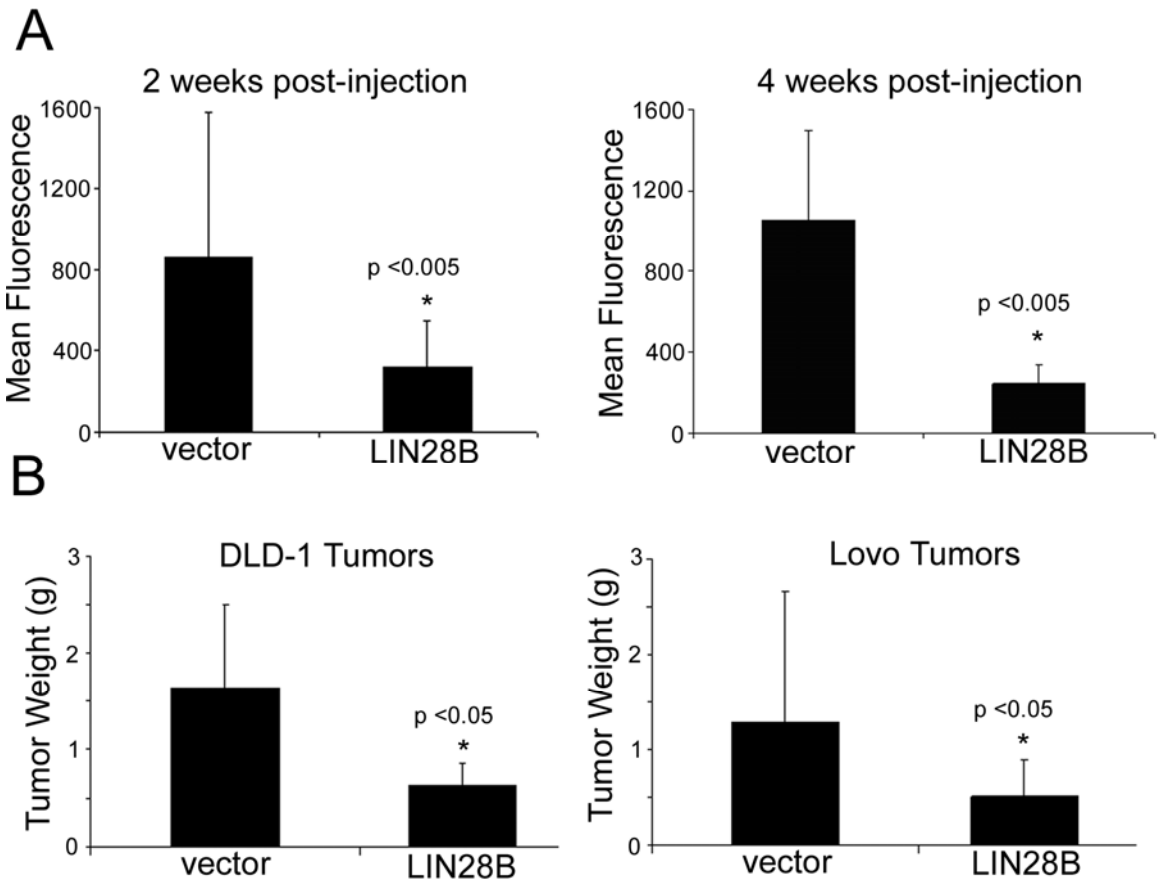
Chapter 3 Tables & Figures

Table 1

	Vector	LIN28B	Total
DLD-1	18	19	37
LoVo	23	23	46
	41	42	83

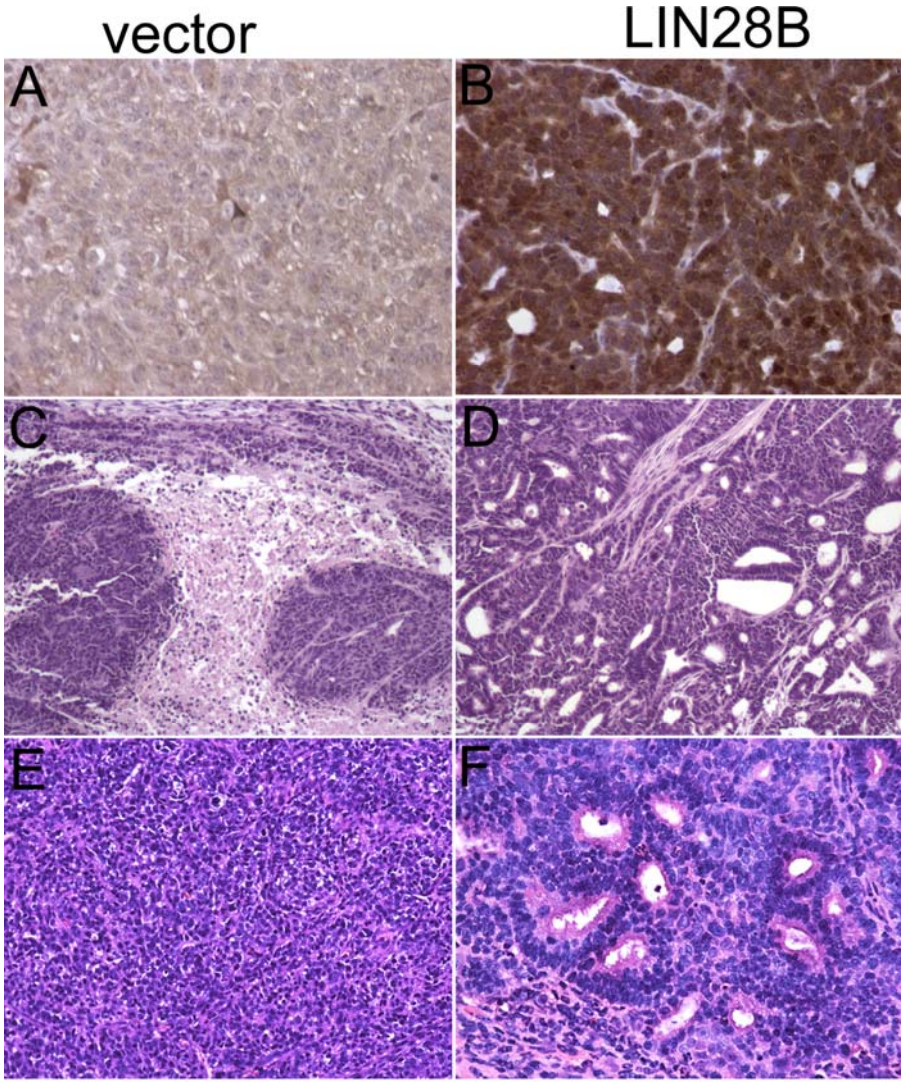
Xenograft tumors evaluated in mice. Vector-DLD-1, *LIN28B*-DLD-1, vector-LoVo, or *LIN28B*-LoVo cells were injected subcutaneously in to the rear flanks of nude mice. A total of 41 empty vector and 42 *LIN28B*-expressing tumors were evaluated.

Figure 1



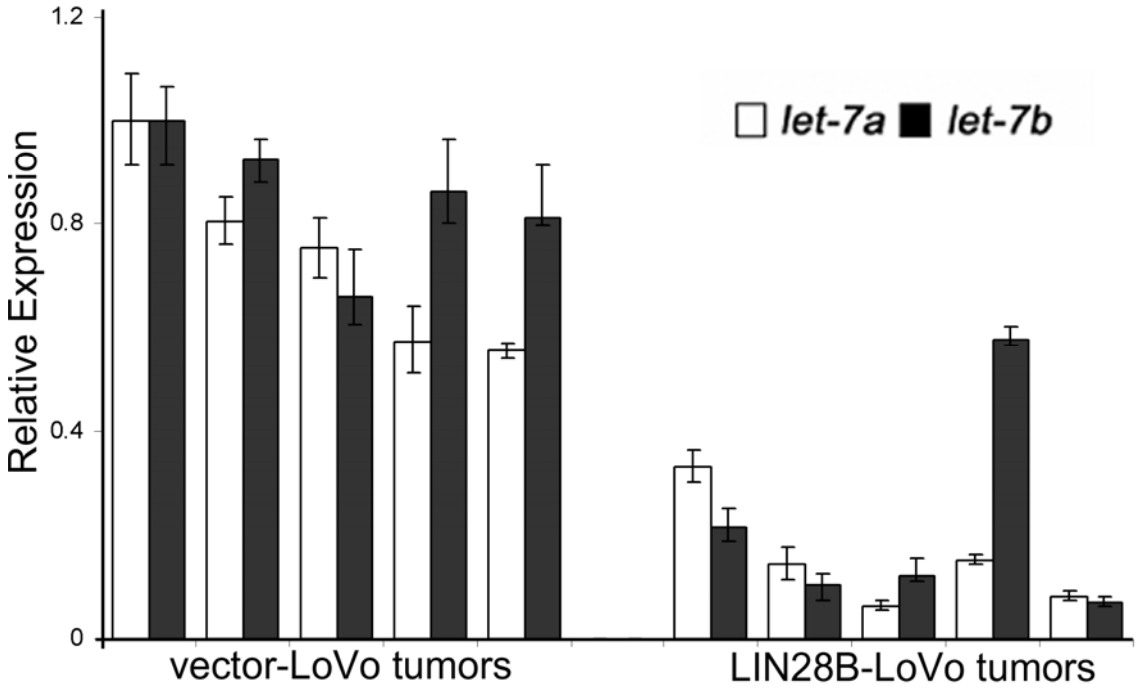
Smaller size of *LIN28B* tumors. (A) LIN28B *tumors emit less fluorescence than controls*. GFP intensity of xenograft tumors was assessed at bi-weekly intervals; at each interval, fluorescence intensity for *LIN28B* tumors was less than controls. Representative 2 and 4 week analyses of a single cohort injected with vector-LoVo or LIN28B-LoVo cells. (B) LIN28B *tumors are smaller than controls*. Tumor weight 6 weeks post-injection and extraction.

Figure 2



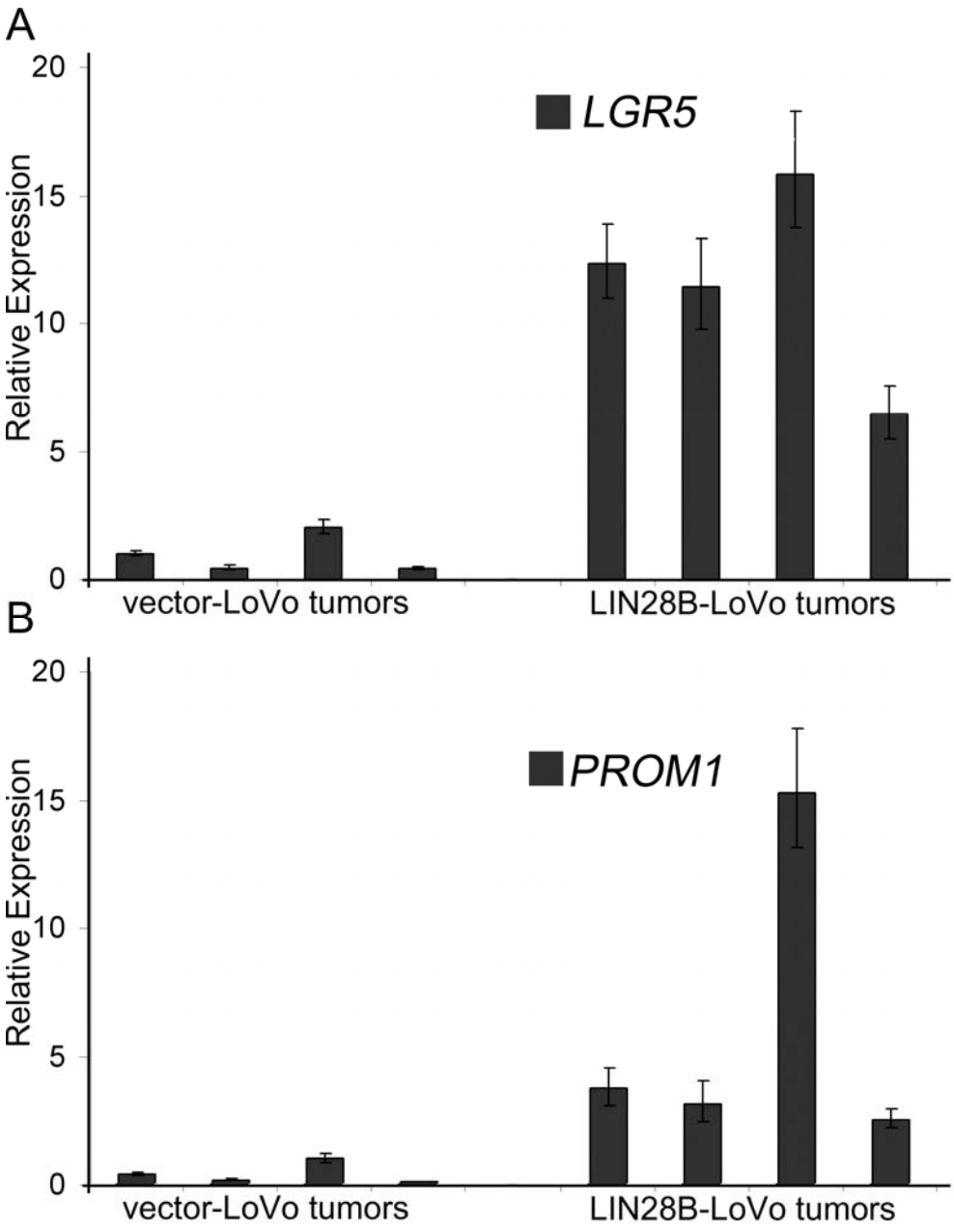
Histopathological examination of empty vector and LIN28B-expressing primary tumors. (A and B) *Confirmation of* LIN28B *overexpression in tumors*. Immumnohistochemical detection of Lin28b in vector-LoVo and LIN28B-LoVo xenografts; representative of 83 tumors tested (200x magnification). (C-F) *Hematoxylin and eosin staining of xenograft tumors*. *LIN28B* tumors exhibit fewer necrotic areas, moderate differentiation, glandular formation, and increased mucin production. (100x magnification in C and D, 200x in E and F).

Figure 3



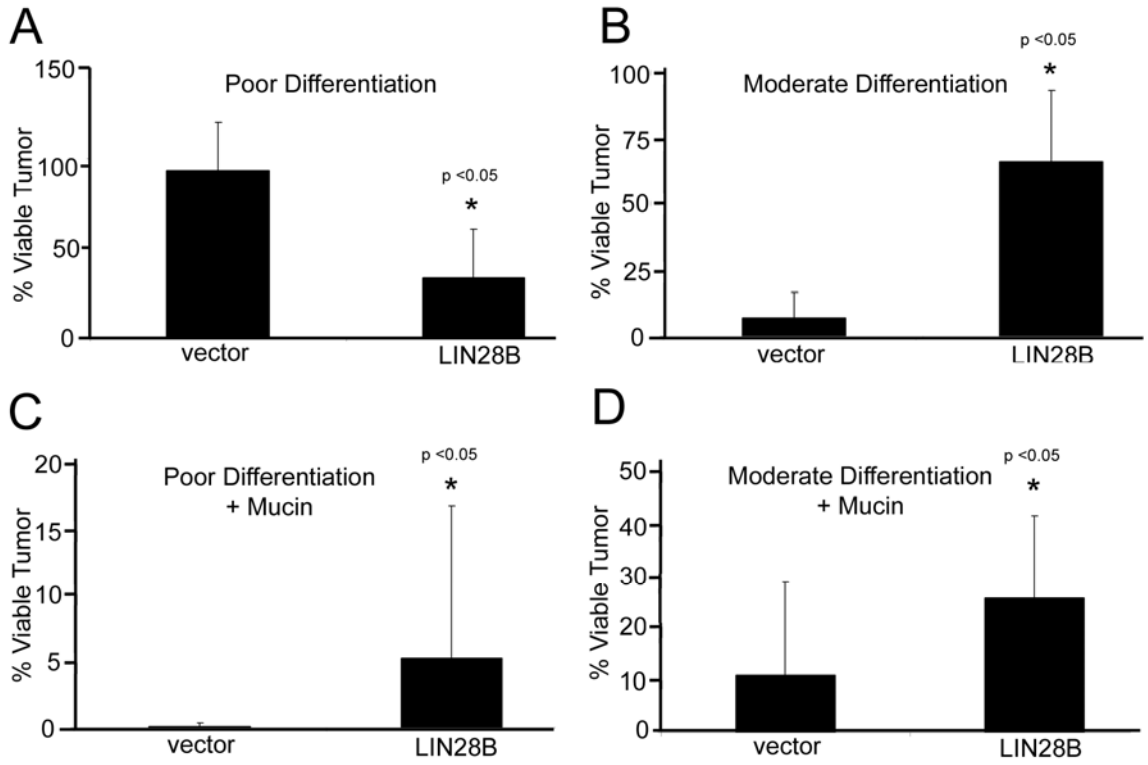
***LIN28B* represses *let-7* in xenograft tumors.** Empty vector and LIN28B-LoVo tumors were homogenized and RNA isolated for RT-qPCR to evaluate levels of mature *let-7a* and *let-7b*. Constitutive *LIN28B* expression suppresses *let-7 in vivo*.

Figure 4



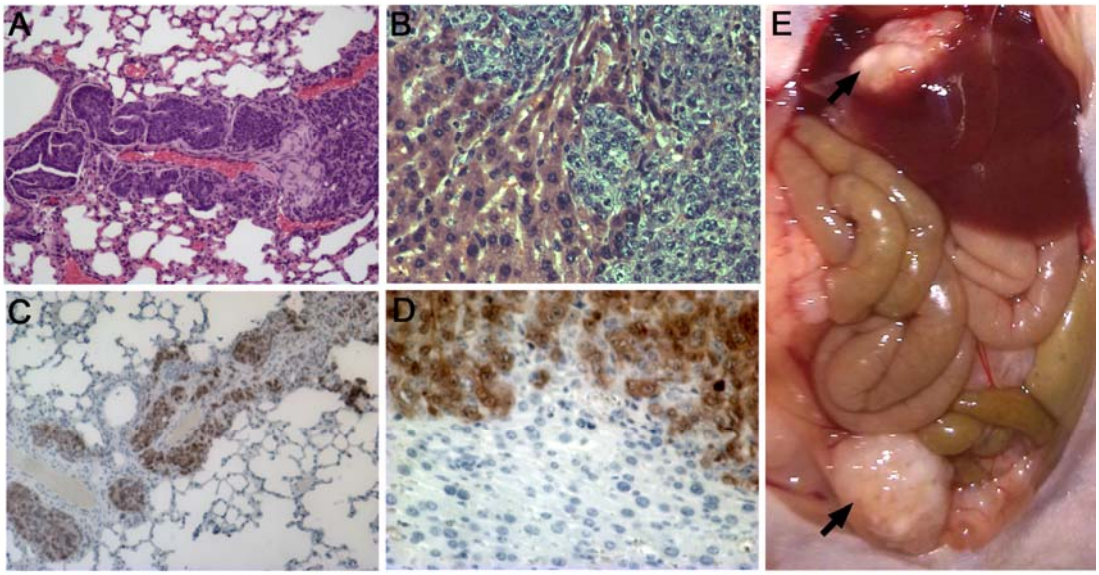
***LGR5* and *PROM1* are up-regulated by constitutive *LIN28B* expression *in vivo*.** Empty vector and LIN28B-LoVo tumors were homogenized and RNA isolated for RT-qPCR to evaluate *LGR5* and *PROM*.

Figure 5



Differentiation and mucin production in *LIN28B* tumors. (A) *Poor differentiation in xenograft tumors.* Area exhibiting poor differentiation scored as a percentage of viable tumors. *LIN28B*-expressing tumors exhibit fewer areas of poor differentiation. (B) *Moderate differentiation in xenograft tumors.* Area exhibiting moderate differentiation scored as a percentage of viable tumors. Differentiation is increased in *LIN28B*-expressing tumors. (C) *Mucin positivity in poorly differentiated tumor areas*. Mucin was detected via mucicarmine staining. Mucin positivity in poorly differentiated scored as a percentage of viable tumor. (D) *Mucin positivity in moderately differentiated tumor areas*. Mucin positivity in poorly differentiated scored as a percentage of viable tumor. Differentiation is increased in *LIN28B*-expressing tumors. Mucin production is increased in *LIN28B*-expressing tumors.

Figure 6



A subset of *LIN28B*-expressing primary tumors metastasize. (A) *Lung metastasis from a* LIN28B-*DLD1 subcutaneous xenograft.* Hematoxylin and eosin staining. (B) *Liver metastasis from a* LIN28B-*LoVo subcutaneous xenograft.* Hematoxylin and eosin staining; note invasion of metastasis into normal liver (C and D) *Metastases are GFP-positive*. Immunohistochemistry for GFP expressed from MSCV-PIG retroviral vector confirms that these tissue originated from xenograft injections. (E) *Metastases visible upon gross examination*. Arrows indicate liver and mesenteric metastases from LIN28B-LoVo tumors (magnification = 100x in panels A and B; 200x in panels C and D).

Table 2

	Vector	LIN28B
-met	41	38
+met	0	4*
Total	41	42

*p = 0.061

Metastasis in xenografted mice. Of the 83 mice we evaluated, we observed metastases in 4 animals - 10% of xenografted mice (Table 2). Thus, constitutive expression *LIN28B* confers metastatic ability in at least a subset of tumors (Fisher's exact one-tailed probability = 0.061).

Table 3

Increased transcripts

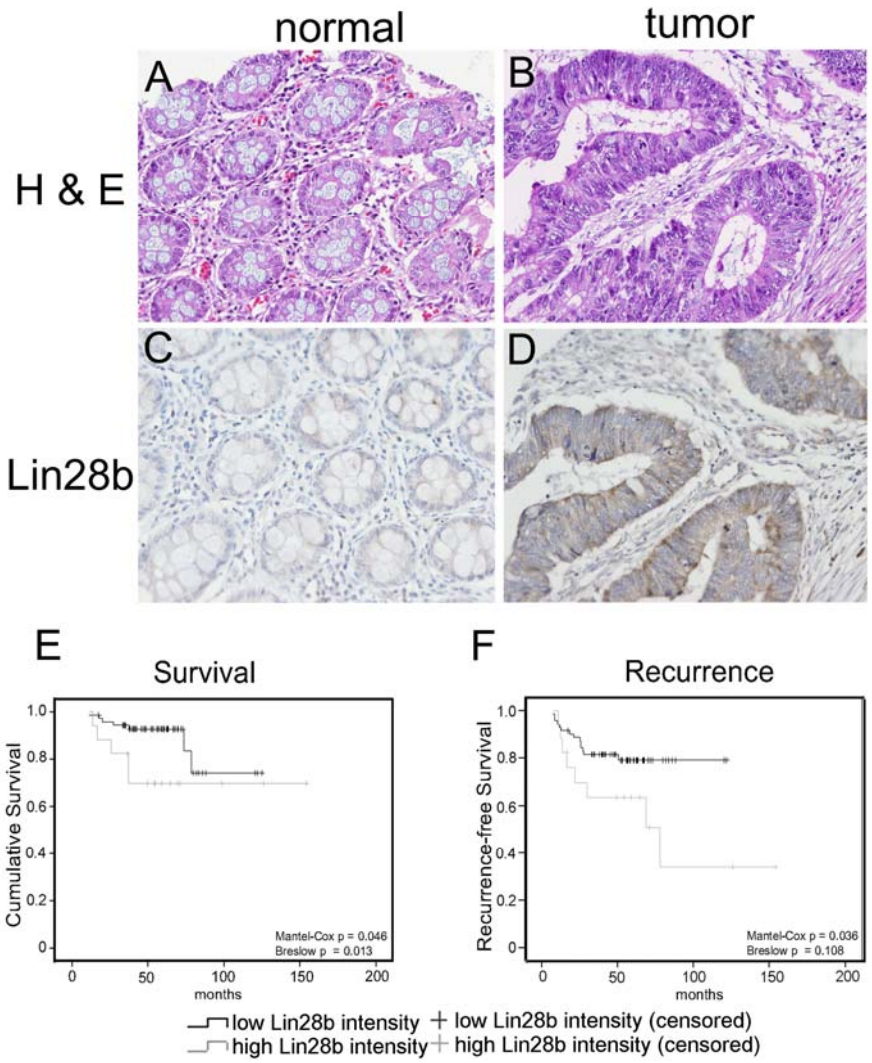
Gene Symbol	Fold
CFTR	9.61
LGR5	9.13
TBMG_03081	7.69
PROM1	7.36
SPINK1	5.30
C4orf18	5.17
TGME49_101340	5.03
SLC6A14	4.61
ppc	4.52
S100P	4.47
C9orf150	4.38
fic	4.33
SULT1C4	4.32
POF1B	4.23
TDO2	4.23
SNORD115-11	4.22
VIPR2	4.21
SLC14A1	4.20
KIT	4.08

Decreased transcripts

Gene Symbol	Fold	Gene Symbol	Fold
KRT5	-4.00	FGF20	-5.70
QPRT	-4.19	CDH12	-5.80
ZNF876P	-4.26	PRKD1	-5.94
ANGPTL4	-4.31	LOC253724	-6.13
GNE	-4.38	SNORD116-23	-6.15
CORO1A	-4.44	CAB39L	-6.29
APCDD1	-4.46	MPP1	-6.36
GPX8	-4.46	LIX1	-6.91
SP3P	-4.48	ZNF738	-6.98
PRF1	-4.50	NQO1	-7.84
IGFBP4	-4.52	PRRX1	-8.96
CRABP2	-4.56	RPS6KA6	-9.30
ZNF91	-4.60	DPEP1	-12.02
FKBP10	-4.64	OLR1	-12.88
ZC4H2	-4.67	SLC2A3	-13.40
ZFP57	-4.90	SNORD116-21	-14.51
RNU5F	-4.97	SNORD116-17	-15.56
KLK7	-4.98	SNORD116-17	-15.56
SNORD116-4	-4.99	SNORD116-15	-18.07
SNORD116-6	-5.01	SNORD116-1	-19.28
ZNF724P	-5.01	SNORD116-20	-19.33
ICAM1	-5.18	SNORD116-5	-20.00
ZNF675	-5.24	SNORD116-5	-20.00
NOTUM	-5.24	SNORD116-8	-23.54
HUNK	-5.25	SNORD116-3	-25.40
GGT6	-5.31	SNORD116-3	-25.40
MAGED1	-5.33	SNORD116-14	-26.67
C12orf59	-5.37	SNORD116-24	-27.40
ODAM	-5.47		

Transcripts modulated by constitutive *LIN28B* expression *in vivo*. Microarray analysis reveals 19 transcripts up-regulated 4-fold or more, and 56 transcripts more than 5-fold down-regulated by constitutive *LIN28B* expression *in vitro*.

Figure 7



***LIN28B* overexpression in tumors correlates with reduced survival and increased probability of tumor recurrence.** (A -D) *Representative H & E and IHC for Lin28b in normal colon and colon tumor mucosa from tumor microarrays*. Lin28b staining is intense in a subset of colon tumors. (E) *Lin28b expression and survival.* High Lin28b staining intensity in stage I and II tumors correlates with reduced patient survival. (F) *Lin28b and recurrence.* High Lin28b in stage I and II tumors correlates with increased probability of tumor recurrence.

Chapter 3 Summary

We analyzed *in vivo* roles of *LIN28B* in tumorigenesis by xenografting retrovirally transduced colon cancer cells into nude mice. Tumors that constitutively express *LIN28B* display increased differentiation with glandular formation, in conjunction with increased *LGR5* and *PROM1* expression. Moreover, *LIN28B* tumors exhibit metastasis, whereas empty vector tumors do not. This finding supports a role for *LIN28B* in progression to metastatic colon cancer. Furthermore, increased Lin28b staining intensity in early stage colon cancers correlates with reduced patient survival. Together, our findings in nude mice and human colon tumors highlight the potential diagnostic and/or therapeutic utility of targeting *LIN28B* in colon cancer.

Discussion and Future Directions

Summary of findings

We hypothesized that ***LIN28B promotes colon tumorigenesis via suppression of let-7 in the colon.*** In pursuing this hypothesis, we demonstrated initially a reduction in mature *let-7a* and *let-7b* levels in colon tumors when compared to matched adjacent normal colonic epithelium. While *let-7* pri-microRNA expression does not vary greatly, reduced *let-7* levels in tumors does correlate with increased *LIN28B* expression. Furthermore, *LIN28B* overexpression correlates with reduced survival and increased tumor recurrence in colon cancer patients.

We demonstrated potential tumor suppressive functions of *let-7a* and *let-7b* in colon cancer cell lines via constitutive expression of a *let-7a-3-b* pri-microRNA. Increased *let-7a* and *let-7b* represses *let-7* targets, while inhibiting cell migration and invasion. Importantly, *let-7* is regulated post-transcriptionally by Lin28b, an RNA-binding protein. Constitutive *LIN28B* expression in immortalized colonic epithelial and colon cancer cells promotes cellular migration, invasion, transformation, and results in de-repression of *let-7* targets. Restoration of *let-7* levels does not ameliorate all *LIN28B* effects, suggesting the possibility of *let-7* independent functions of *LIN28B*.

Constitutive *LIN28B* expression modulates many gene transcripts as revealed by microarray analyses. These genes include the intestinal stem cell markers *LGR5* and *PROM1*, which are not predicted *let-7* targets, and remain up-regulated following *let-7* restoration. The ability of *LIN28B* to up-regulate *LGR5* and *PROM1* suggests possible functions of *LIN28B* in cellular re-programming, further supported by the observation that *LIN28B* promotes differentiation *in vivo*. Additionally, we observed metastases in a

subset of *LIN28B*-expressing primary tumors, suggesting a role for *LIN28B* in progression to metastatic colon cancer.

Implications of tumor-suppressive functions of *let-7* in colon cancer

let-7 microRNAs exhibit tumor suppressive functions in a variety of cancers (Akao et al, 2006; Esquela-Kerscher & Slack, 2006; Kumar et al, 2008; Shell et al, 2007; Takamizawa et al, 2004; Viswanathan et al, 2008; Yu et al, 2007a), which may reflect the high degree of sequence homology between the 12 *let-7* isoforms (Vella & Slack, 2005). Although we have demonstrated reduced levels of mature *let-7a* and *let-7b* in colon tumors, we did not evaluate the remaining *let-7* isoforms, which may compensate for the reduction of *let-7a* and *let-7b*. However, we did show previously that *let-7a* and *let-7b* are the major *let-7* isoforms expressed in the human colon. Furthermore, we demonstrated a statistically significant inverse correlation between *LIN28B* overexpression and reduced *let-7a* and *let-7b* levels in colon tumors. Constitutive *LIN28B* expression in colonic epithelial cells results in down-regulation of all *let-7* isoforms, and, thus it is unlikely that Lin28b-mediated reductions in *let-7a* and *let-7b* in colon tumors are compensated by other *let-7* microRNAs.

Additional roles of *let-7a* and *let-7b* in colon tumorigenesis remain to be determined. Although *let-7* down-regulation occurs in two-thirds of colon tumors, *LIN28B* overexpression does not occur frequently enough to account for *let-7* repression in all these tumors. While variations in *let-7* pri-microRNA expression may account for some changes in mature *let-7* levels, it is possible that other post-transcriptional mechanisms of *let-7* repression account for these differences. For example, *LIN28*

overexpression may occur in a subset of tumors where *let-7* repression occurs, novel repressors of *let-7* biogenesis may exist, or perhaps other proteins that effect *let-7* microRNA stability are reduced in these cells.

The functional roles of *let-7 in vivo*

Genetically engineered cell lines and/or mice in which *let-7* expression may be temporally and/or spatially controlled would be excellent tools for assessing roles of *let-7* microRNAs in colon tumorigenesis. Since *let-7a* and *let-7b* are the major *let-7* isoforms expressed in the human colon, the *let-7a-3-b* bicistronic cluster on chromosome 22q13.31 is a prime candidate for genetic manipulation. In that context, one might consider a conditional knock-out of the *let-7a-3-b* locus (*let-7c-2-b* in mice), which may be developed by inserting flanking loxP sites around the microRNA cluster (this is being done in the lab currently). Similarly, a transgene driving expression of anti-sense RNAs that bind to and inhibit *let-7* isoforms may be employed. While disrupting *let-7* function alone may not initiate transformation of colonic epithelial cells, *let-7a-3-b* conditional knockouts could be combined with existing mouse models (e.g. *APC* mutation), allowing one to evaluate the roles of *let-7* microRNAs in accelerating colon tumorigenesis.

Alternatively, one might consider transgenic overexpression of *let-7* in mice, where the ability of *let-7* to inhibit colon tumorigenesis *in vivo* may be evaluated. One might predict that *let-7* overexpression would delay tumor formation in the colon or potentially reverse tumor development as observed previously with introduction of *let-7* into lung adenocarcinomas (Esquela-Kerscher et al, 2008; Trang et al, 2010). The ability

of *let-7* to inhibit the development of colon tumors could be evaluated by breeding *let-7* transgenic mice with established models of colon cancer.

Potential roles of *let-7* in colon differentiation

Given that *let-7* coordinates differentiation programs (Johnson et al, 2007; Shell et al, 2007; Tsonis et al, 2007; Yu et al, 2007a), resolving the functions of *let-7* microRNAs in colonic development may aid in elucidating the roles *LIN28B* in cellular re-programming and differentiation in the colon. One might predict that *let-7* overexpression would promote intestinal differentiation, while inhibition of *let-7* microRNAs might delay or inhibit differentiation. Genetic manipulations that permit temporal and/or spatial control of *let-7* expression in mice or epithelial cell lines would be helpful. Colonic organogenesis and epithelial differentiation may be evaluated in *let-7* conditional knockout and/or transgenic mice. Alternatively, the functions of *let-7* in colonic differentiation may be evaluated *in vitro* by utilizing cell lines where *let-7* expression is inducible. Here, models of intestinal differentiation, such as in CaCo-2 or fetal colonic epithelial cells grown in standard two-dimensional or organotypic culture, may be employed. Both cell types spontaneously differentiate in culture to what closely recapitulates the mature colonic epithelium (Kalabis et al, 2008; Kalabis et al, 2003; Sambuy et al, 2005). Thus, overexpression or inhibition of *let-7* in these cells may serve as useful tools for evaluating functions of *let-7* in intestinal differentiation. One might predict that *let-7* overexpression would shorten the time required for either cell type to spontaneously differentiate, which may be assessed by evaluating markers of enterocyte differentiation, including villin, carbonic anhydrase, and intestinal alkaline phosphatase

(Bekku et al, 1998; Dahlqvist & Lindberg, 1965; Maunoury et al, 1992), as well as microvillus brush border development via electron microscopy.

Elucidating *let-7* independent functions of *LIN28B*

Manipulating *let-7* expression via overexpression or inhibition may be implemented as a means of determining *let-7* independent functions of *LIN28B* as well. For example, we have identified a number of genes that are modulated with constitutive *LIN28B* expression. Still, it remains to be determined whether *LIN28B* affects these genes in a *let-7* dependent manner. Each gene of interest may be evaluated in the context of constitutive *LIN28B* expression coupled with *let-7* restoration, as we have done for *HMGA2*, *IGF2BP1*, *LGR5* and *PROM1*. This would aid in determining whether these genes are regulated by Lin28b in a *let-7* dependent or independent fashion, whether directly or indirectly. Alternatively, a comparative bioinformatics approach which compares our microarray results with predicted *let-7* targets may be undertaken. Such a bioinformatics study may offer insights to additional *let-7* independent functions of *LIN28B*, and elucidate whether *LIN28B* functions primarily as an inhibitor of *let-7*, or if *let-7* repression occurs as one of several *LIN28B* functions (as our microarray results suggest).

Mutations that specifically ameliorate the ability of *LIN28B* to inhibit *let-7* would be useful also in further elucidating the mechanisms of *LIN28B*'s functions. This may be achieved by introducing point mutations into the cold-shock domain of *LIN28B*, which has been utilized 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, thereby precluding assessment of specific *let-7* independent functions. A more specific approach involves first determining the specific domains and/or amino acid residues of *LIN28B* essential for repression of *let-7* biogenesis, which may be accomplished by introducing mutations and/or deletions into *LIN28B* systematically, then targeting these domains or amino acids exclusively. Cells expressing the resulting *LIN28B* mutant may be evaluated in migration, invasion, xenograft assays, and microarray studies. Targeting the specific domains of *LIN28B* required for *let-7* inhibition in genetically engineered mice would further aid in determining whether the ability to repress *let-7* is essential to *LIN28B*'s roles in organ development and tumorigenesis.

LIN28B overexpression in colon cancer

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 virtually all colon tumors. We may conclusively determine whether increased c-myc correlates with high Lin28b in colon tumors by performing immunohistochemistry for c-myc in our tissue microarrays.

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, as *LIN28B* is also a predicted *let-7* target (Griffiths-Jones et al, 2006; Griffiths-Jones et al, 2008; Grimson et al, 2007; Lewis et al, 2005; mirBase, 2010; TargetScan, 2010). 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 function. Of note, SNPs that disrupt *let-7* binding occur within the 3' UTR of the established *let-7* target *KRAS (Nelson et al, 2010)*. The presence of these SNPs increases risk for non-small cell lung cancer by providing an alternative mechanism for up-regulating oncogenic *KRAS* in tumors (Chin et al, 2008). Accordingly, SNPs may exist in the 3' UTR of *LIN28B*, which may account for increases in *LIN28B* transcripts observed in tumors. Sequencing the 3' UTR of both *LIN28B* loci present in patients with colon cancers may offer insight into this possibility.

The ability of c-myc to transactivate *LIN28* has not been described, and may account for differential implications of *LIN28* and *LIN28B* in colon cancer. However, it is important to note that distinct functions have not been described for either homolog, and it is possible that the two are completely redundant. Perhaps there are critical differences between the expression patterns of *LIN28* and *LIN28B* in tissues, which may be determined by conducting *in situ* hybridization and comparing the mRNA expression profiles of each homolog to one another. It is especially important to identify distinct functions of these homologs if *LIN28B* is to be targeted therapeutically in colon cancer, as inadvertent inhibition of *LIN28* may result in intolerable toxicities. Also of note, if *LIN28* and *LIN28B* function redundantly in colon tumorigenesis, *LIN28* may be up-regulated in tumors where *LIN28B* is targeted as a means of compensation. Accordingly, both the distinct and overlapping functions of *LIN28* and *LIN28B* are important considerations when designing small-molecule inhibitors of either.

LIN28B in transformation, cell migration, invasion, and metastasis

We found that constitutive *LIN28B* expression promotes transformation, cell migration, and invasion *in vitro*, and causes a subset of xenograft tumors to metastasize. This coupled with the inverse correlation between *LIN28B* expression and patient survivability suggests roles for *LIN28B* in progression of early stage polyps to invasive and/or metastatic colon carcinoma. The vast majority of colon cancer deaths occur as a result of invasive or metastatic disease (ACS, 2010). Thus, unraveling the mechanisms underlying *LIN28B*'s ability to foster transformation, migration, invasion, and metastasis is of interest, as this may reveal novel therapeutic targets for colon cancer.

Whether LIN28B is required for any of these cellular processes could be determined via deletion or inhibition of *LIN28B* expression. We attempted to knock-down *LIN28B* in colon cancer cell lines using RNA interference without success. This is perhaps attributable to low *LIN28B* transcript levels that accompany the presence of detectable Lin28b protein in DLD-1 and LoVo cells (data not shown). However, the CaCo-2 cell line exhibits high levels of *LIN28B* mRNA (data not shown). Thus, one might consider expanding this work by inhibiting *LIN28B* in CaCo-2 cells through the use of siRNAs. An unfortunate drawback to this method is that CaCo-2 cells are incapable of migration and invasion in Boyden chamber assays (data not shown), and thus may not undergo metastasis when xenografted. Still, a requirement for *LIN28B* in cellular transformation may be elucidated by performing soft-agar colony formation assays with CaCo-2 cells. Furthermore, an siRNA approach in CaCo-2 cells may aid in determining additional mRNA transcripts that *LIN28B* modulates.

Clues to the how *LIN28B* overexpression might foster transformation, migration, invasion, and metastasis may be obtained from mining our microarray data. The most provocative genes include those which are specifically modulated in *LIN28B* metastases as compared to *LIN28B*-expressing primary tumors; as a transcript that is induced in metastatic tumors may constitute a potential mechanism by which metastasis occurs. We explored our microarray data comparing empty vector, *LIN28B*-expressing primary tumors and *LIN28B* metastases for genes involved in cell migration, invasion, and metastasis; recognizing that cell migration and invasion are distinct processes from metastasis, yet regularly associated with it.

Three genes that are more than 4-fold changed in metastases as compared to primary tumors are particularly interesting: *TNS4* (Tensin 4), *CHI3L1* (Chitinase 3-like-1), and *KLK6* (Kallikrein 6). *TNS4* is a focal adhesion protein involved in cell adherence to extra-cellular matrix substrates (Bockholt & Burridge, 1993). *CHI3L1* is expressed during intestinal inflammation, and enhances bacterial cell invasion into intestinal mucosa (Mizoguchi, 2006). *KLK6* degrades extracellular matrix and may enhance the metastatic potential of tumor cells (Ghosh et al, 2004). All three of these genes are involved in attachment to and degradation of basement membrane, thus may encourage migration, invasion, or seeding of tumor cells at distant sites. It may be determined whether these genes play a role in the ability of *LIN28B* to promote migration, invasion, and metastasis by inhibiting each (via an si-RNA approach, for example) in colon cancer cells constitutively expressing *LIN28B*, and conducting migration, invasion, and xenograft assays. If the ability of *LIN28B* to promote migration, invasion, and/or metastasis is dependent on any one of these genes, one would expect knockdown to

ameliorate the *LIN28B*-mediated effects. Of note, *KLK6* is a predicted *let-7* target, containing several conserved (although poorly) *let-7* binding sites (Griffiths-Jones et al, 2006; Griffiths-Jones et al, 2008; Grimson et al, 2007; Lewis et al, 2005; mirBase, 2010; TargetScan, 2010); thus, the ability of *LIN28B* to increase *KLK6* may is likely mediated via *let-7* repression. However, *TNS4* and *CHI3L1* are not predicted *let-7* targets, thus their regulation by *LIN28B* may represent additional *let-7* independent functions (Griffiths-Jones et al, 2006; Griffiths-Jones et al, 2008; Grimson et al, 2007; Lewis et al, 2005; mirBase, 2010; TargetScan, 2010).

Tumors expressing *LIN28B* are not only capable of metastasis, they exhibit increased expression of the intestinal stem cell markers *LGR5* and *PROM1*. Both *LGR5* and *PROM1* have been implicated previously in colon tumorigenesis: adenomas may arise in the colon from $PROM1^{+}$ crypt cells (Zhu et al, 2009), and overexpression of *LGR5* in colon tumors correlates with advanced cancer, invasion, and metastasis (Uchida et al, 2010). *LGR5* and *PROM1* up-regulation with *LIN28B* overexpression may be consistent with the emerging principle that stem cells are involved in maintenance of tumors. Intriguingly, targeting stem cells within colon tumors is potentially an effective therapeutic strategy, and *LIN28B* may serve as a potential marker of tumors to be targeted using this approach.

LIN28B in cellular re-programming

It is atypical for genes that promote tumorigenesis to simultaneously enhance differentiation. Thus, it is difficult to reconcile our findings that primary tumors constitutively expressing *LIN28B* are smaller and more differentiated than their empty

vector counterparts, yet metastasize more frequently. Considering the role of *LIN28* in induction of pluripotency, we hypothesize that *LIN28B* tumors appear more differentiated as a result of cellular re-programming. Given that constitutive *LIN28* expression in undifferentiated cells blocks gliogenesis in favor of neurogenesis (Balzer et al, 2010), it is plausible that *LIN28B* expression restricts cells to a particular cell fate. Alternatively, the presence of high *LIN28B* may induce pluripotency, causing cells to dedifferentiate into stem cells, with subsequent differentiation into default lineages.

A similar phenotype has been described in the intestinal epithelium where loss of the *Math1* (also called Atoh1) transcription factor leads to depletion of goblet, enteroendocrine, and Paneth cells, while permitting enterocyte differentiation (Yang et al, 2001). One could address whether *LIN28B* is accelerating differentiation or inducing pluripotency in epithelial cells via co-expression of *Math1* in cells constitutively expressing *LIN28B*, and monitoring the frequency at which cells commit to secretory lineages. An *in vitro* approach might involve fetal colonic epithelial cells, which are capable of differentiating to colonocytes, goblet, and enteroendocrine cells when grown in standard two-dimensional or organotypic culture (Kalabis et al, 2008; Kalabis et al, 2003).

The ability of *LIN28B* to induce *LGR5* and *PROM1* expression also suggests potential functions of *LIN28B* in intestinal and colonic epithelial stem cells. Within the intestine, expression of the cell surface protein *PROM1* is restricted to the crypt 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, upregulation of these factors by *LIN28B* suggests a possible role for *LIN28B* in establishment and/or maintenance of intestinal stem cells, or in modulating cell fate decisions.

LIN28B may modulate cell fate decisions via regulation of *IGF2* expression. *LIN28* plays a role in both neurogenesis and myogenesis through its ability to promote *IGF2* transcription (Balzer et al, 2010; Polesskaya et al, 2007). *IGF2* is involved in differentiation of variety of cell types, including osteoblasts and mesenchymal cells (Hamidouche et al, 2010). Loss of *IGF2* imprinting, which results in increased *IGF2* expression, shifts the intestinal epithelium toward a less differentiated state and increases expression of the intestinal stem cell marker Musashi 1 (Sakatani et al, 2005). It is possible that *LIN28B* induces dedifferentiation and expression of the intestinal stem cell markers *LGR5* and *PROM1* by relieving *let-7*-mediated repression of *IGF2BP1*, thereby stabilizing *IGF2* mRNA and increasing its translation. Accordingly, it would be interesting to determine whether *IGF2* is required for *LIN28B*'s function by inhibiting *IGF2* in cells that constitutively express *LIN28B* and subsequently evaluating *LGR5* and *PROM1* expression. If *IGF2* is required for the ability of *LIN28B* to induce dedifferentiation, or increase *LGR5* and *PROM1* transcription, one would expect the absence of *IGF2* to eliminate these effects.

LIN28B in epithelial-mesenchymal transition

Epithelial-mesenchymal transition (EMT) is a program of cellular development characterized by loss of cell adhesion and increased cell mobility, and exemplified by decreased E-cadherin with concomitant increased vimentin and N-cadherin (Roussos et

al, 2010; Singh & Settleman, 2010). EMT occurs during normal embryogenesis, but may be re-initiated in cancer cells resulting in invasion and metastasis (Roussos et al, 2010; Singh & Settleman, 2010). We find that constitutive *LIN28B* expression fosters migration, invasion, and metastasis, all of which suggest roles for *LIN28B* in epithelial-mesenchymal transition (EMT).

TGF-β (Transforming growth factor-beta) promotes EMT in normal cells, and supports EMT-mediated metastasis in tumors (Singh & Settleman, 2010; Wendt et al, 2009). Constitutive *LIN28B* expression up-regulates the TGF-β pathway target *TGFBI* (Transforming growth factor β-induced, also called *BIGH3*) as determined by our microarray analysis. This suggests that increased TGF-β signaling activity may occur with constitutive *LIN28B* expression. Moreover, TGF-β induces the *let-7* target *HMGA2* during EMT (Thuault et al, 2006), further linking TGF-β signaling and EMT with *LIN28B*. Conceivably, TGF-β signaling is a mediator of *LIN28B*-associated EMT and metastasis.

In order to elucidate the roles of *LIN28B* in EMT, one might first determine whether changes in E-cadherin, vimentin, and N-cadherin occur with constitutive *LIN28B* expression. Additionally, it could be determined whether TGF-β pathway activation occurs with constitutive *LIN28B* expression by assessing phosphorylation of SMAD transcription factors in *LIN28B*-expressing cells; SMAD proteins are the transducers of extracellular TGF-β signaling, thus, an increase in their phosphorylation signifies TGF-β pathway activation (Heldin et al, 1997; Nakao et al, 1997). Provided a link exists between *LIN28B* and TGF-β signaling, disrupting it may constitute a novel therapeutic strategy for colon cancer treatment.

Murine models as tools for further elucidating roles of *LIN28B* in intestinal development and tumorigenesis

Genetically engineered mice would offer excellent resources for fully elucidating the roles of *LIN28B* in colon development and tumorigenesis. To that end, flanking loxP sites may be engineered into the mouse *LIN28B* locus; alternatively, one might consider introducing mutations that disrupt specific functions of *LIN28B*, such as its ability to bind RNA or inhibit *let-7* biogenesis. Targeted deletion of *LIN28B* in the colon would allow one to determine the requirement, if any, of *LIN28B* in intestinal development. We hypothesize a central role for *LIN28B* in intestinal cell fate decisions and thus, we hypothesize deletion or expression of functionally null *LIN28B* mutants in the colon would result in a vastly perturbed or absent intestinal epithelium. Correspondingly, one might expect deletion of *LIN28B* in the colon to inhibit or delay tumor formation, which may be determined by interbreeding *LIN28B* knockout mice with existing mouse models of colon cancer (Taketo & Edelmann, 2009).

Roles of *LIN28B* in colon organogenesis and tumorigenesis may also be evaluated via constitutive expression in the colon. This may be achieved by creating a conditionally activated transgene to drive *LIN28B* transcription. Spatially controlled conditional activation of gene expression has been achieved in the past by employing a lox-STOP-lox cassette, which effectively prohibits expression of a gene of interest until the cassette is excised (Olive et al, 2004; Tuveson et al, 2004). Genetically engineered mice in which constitutive *LIN28B* transcription is subjected to the control of a lox-STOP-lox cassette would be useful tools in determining the contributions of *LIN28B* to intestinal differentiation. Using this model, one could target *LIN28B* overexpression to the

intestinal epithelium by via cre-mediated excision of the lox-STOP-lox cassette, which may achieved by cross-breeding with Fabp1-cre or Villin-cre or mice (el Marjou et al, 2004; Wong et al, 2000). Major benefits of this approach over traditional transgenic models include the ability to activate *LIN28B* overexpression in adult mice (using Cre-ERT), and to expand studies of *LIN28B* to other tissues (by taking advantage of the vast array of Cre transgenes that exist). Conversely, one could place *LIN28B* under the direct control of the Villin promoter (currently being done in the lab). Based on the results of our xenograft tumors, one might expect increased *LIN28B* expression in the colonic epithelium to alter the secretory lineage. In addition, targeting *LIN28B* overexpression in the colonic epithelium may accelerate progression of adenocarcinomas in existing mouse models of colon cancer, thereby creating a valuable murine model of metastatic colon cancer.

Conclusions

We have demonstrated reduction in mature *let-7a* and *let-7b* levels in colon tumors as compared to matched adjacent normal colonic epithelium. Subsequently, we demonstrated potential tumor suppressive properties of *let-7a* and *let-7b* via constitutive expression of a *let-7a-3-b* pri-microRNA in colon cancer cell lines. Increased expression of *let-7a* and *let-7b* represses *let-7* targets, while decreasing cell migration and invasion *in vitro*.

Reduced levels of *let-7a* and *let-7b* in colon tumors correlate with increased expression of the *let-7* inhibitor, *LIN28B*. We have demonstrated that overexpression of *LIN28B* occurs in approximately 50% of colon tumors, and that high Lin28b in early stage colon cancer correlates with increased probability of recurrence and reduced patient survival.

Constitutive *LIN28B* expression in immortalized colonic epithelial and colon cancer cell lines promotes cell migration, invasion, and transformation *in vitro*. In addition, constitutive *LIN28B* expression modulates levels of a number of mRNA transcripts, including *let-7* targets, and the intestinal stem cell markers *LGR5* and *PROM1*, which are not predicted *let-7* targets. Restoration of *let-7* levels in cells expressing *LIN28B* constitutively ameliorates some, but not all of these described effects, thereby suggesting *let-7* independent functions of *LIN28B*. Additionally, roles for *LIN28B* in cellular reprogramming and metastasis are suggested by constitutive *LIN28B* expression *in vivo*, which results in more differentiated xenograft tumors that display a metastatic phenotype.

The overarching hypothesis of this work, **LIN28B *promotes tumorigenesis via suppression of* let-7 *in the colon***, appears to be correct and highlights the importance of

both *let-7* and *LIN28B* in the molecular pathogenesis of colon cancer. This novel work suggests roles for *let-7* dependent and independent functions of *LIN28B* in cell migration and invasion, modulation of gene expression, cellular reprogramming, and tumor metastasis. Future studies will elucidate further *LIN28B*'s functional roles in colon cancer through generation of genetically engineered mice.

References

Abe N, Watanabe T, Suzuki Y, Matsumoto N, Masaki T, Mori T, Sugiyama M, Chiappetta G, Fusco A, Atomi Y (2003) An increased high-mobility group A2 expression level is associated with malignant phenotype in pancreatic exocrine tissue. *Br J Cancer* **89**(11)**:** 2104-2109

ACS ACS. (2010) Cancer Facts & Figures 2010. American Cancer Society, Atlanta, GA.

Akao Y, Nakagawa Y, Naoe T (2006) let-7 microRNA functions as a potential growth suppressor in human colon cancer cells. *Biol Pharm Bull* **29**(5)**:** 903-906

Allione F, Eisinger F, Parc P, Noguchi T, Sobol H, Birnbaum D (1998) Loss of heterozygosity at loci from chromosome arm 22Q in human sporadic breast carcinomas. *Int J Cancer* **75**(2)**:** 181-186

Ambros V, Horvitz HR (1984) Heterochronic mutants of the nematode Caenorhabditis elegans. *Science* **226**(4673)**:** 409-416

Ayabe T, Satchell DP, Pesendorfer P, Tanabe H, Wilson CL, Hagen SJ, Ouellette AJ (2002a) Activation of Paneth cell alpha-defensins in mouse small intestine. *J Biol Chem* **277**(7)**:** 5219-5228

Ayabe T, Satchell DP, Wilson CL, Parks WC, Selsted ME, Ouellette AJ (2000) Secretion of microbicidal alpha-defensins by intestinal Paneth cells in response to bacteria. *Nat Immunol* **1**(2)**:** 113-118

Ayabe T, Wulff H, Darmoul D, Cahalan MD, Chandy KG, Ouellette AJ (2002b) Modulation of mouse Paneth cell alpha-defensin secretion by mIKCa1, a Ca2+-activated, intermediate conductance potassium channel. *J Biol Chem* **277**(5)**:** 3793-3800

Balzer E, Heine C, Jiang Q, Lee VM, Moss EG (2010) LIN28 alters cell fate succession and acts independently of the let-7 microRNA during neurogliogenesis in vitro. *Development* **137**(6)**:** 891-900

Balzer E, Moss EG (2007) Localization of the developmental timing regulator Lin28 to mRNP complexes, P-bodies and stress granules. *RNA Biol* **4**(1)**:** 16-25

Barker N, van Es JH, Kuipers J, Kujala P, van den Born M, Cozijnsen M, Haegebarth A, Korving J, Begthel H, Peters PJ, Clevers H (2007) Identification of stem cells in small intestine and colon by marker gene Lgr5. *Nature* **449**(7165)**:** 1003-1007

Bartel DP (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* **116**(2)**:** 281-297

Beddington RS, Robertson EJ (1989) An assessment of the developmental potential of embryonic stem cells in the midgestation mouse embryo. *Development* **105**(4)**:** 733-737

Behrens J, von Kries JP, Kuhl M, Bruhn L, Wedlich D, Grosschedl R, Birchmeier W (1996) Functional interaction of beta-catenin with the transcription factor LEF-1. *Nature* **382**(6592)**:** 638-642

Bekku S, Mochizuki H, Takayama E, Shinomiya N, Fukamachi H, Ichinose M, Tadakuma T, Yamamoto T (1998) Carbonic anhydrase I and II as a differentiation marker of human and rat colonic enterocytes. *Res Exp Med (Berl)* **198**(4)**:** 175-185

Berlingieri MT, Manfioletti G, Santoro M, Bandiera A, Visconti R, Giancotti V, Fusco A (1995) Inhibition of HMGI-C protein synthesis suppresses retrovirally induced neoplastic transformation of rat thyroid cells. *Mol Cell Biol* **15**(3)**:** 1545-1553

Bockholt SM, Burridge K (1993) Cell spreading on extracellular matrix proteins induces tyrosine phosphorylation of tensin. *J Biol Chem* **268**(20)**:** 14565-14567

Boyerinas B, Park SM, Shomron N, Hedegaard MM, Vinther J, Andersen JS, Feig C, Xu J, Burge CB, Peter ME (2008) Identification of let-7-regulated oncofetal genes. *Cancer Res* **68**(8)**:** 2587-2591

Brabletz S, Schmalhofer O, Brabletz T (2009) Gastrointestinal stem cells in development and cancer. *J Pathol* **217**(2)**:** 307-317

Brittan M, Wright NA (2002) Gastrointestinal stem cells. *J Pathol* **197**(4)**:** 492-509

Brittan M, Wright NA (2004a) The gastrointestinal stem cell. *Cell Prolif* **37**(1)**:** 35-53

Brittan M, Wright NA (2004b) Stem cell in gastrointestinal structure and neoplastic development. *Gut* **53**(6)**:** 899-910

Bryan EJ, Watson RH, Davis M, Hitchcock A, Foulkes WD, Campbell IG (1996) Localization of an ovarian cancer tumor suppressor gene to a 0.5-cM region between D22S284 and CYP2D, on chromosome 22q. *Cancer Res* **56**(4)**:** 719-721

Castells A, Gusella JF, Ramesh V, Rustgi AK (2000) A region of deletion on chromosome 22q13 is common to human breast and colorectal cancers. *Cancer Res* **60**(11)**:** 2836-2839

Castells A, Ino Y, Louis DN, Ramesh V, Gusella JF, Rustgi AK (1999) Mapping of a target region of allelic loss to a 0.5-cM interval on chromosome 22q13 in human colorectal cancer. *Gastroenterology* **117**(4)**:** 831-837

Chamorro MN, Schwartz DR, Vonica A, Brivanlou AH, Cho KR, Varmus HE (2005) FGF-20 and DKK1 are transcriptional targets of beta-catenin and FGF-20 is implicated in cancer and development. *EMBO J* **24**(1)**:** 73-84

Chan S, Gabra H, Hill F, Evan G, Sikora K (1987) A novel tumour marker related to the c-myc oncogene product. *Mol Cell Probes* **1**(1)**:** 73-82

Chang TC, Zeitels LR, Hwang HW, Chivukula RR, Wentzel EA, Dews M, Jung J, Gao P, Dang CV, Beer MA, Thomas-Tikhonenko A, Mendell JT (2009) Lin-28B transactivation is necessary for Myc-mediated let-7 repression and proliferation. *Proc Natl Acad Sci U S A* **106**(9)**:** 3384-3389

Cheng H, Leblond CP (1974a) Origin, differentiation and renewal of the four main epithelial cell types in the mouse small intestine. I. Columnar cell. *Am J Anat* **141**(4)**:** 461-479

Cheng H, Leblond CP (1974b) Origin, differentiation and renewal of the four main epithelial cell types in the mouse small intestine. III. Entero-endocrine cells. *Am J Anat* **141**(4)**:** 503-519

Cheng H, Leblond CP (1974c) Origin, differentiation and renewal of the four main epithelial cell types in the mouse small intestine. V. Unitarian Theory of the origin of the four epithelial cell types. *Am J Anat* **141**(4)**:** 537-561

Chin LJ, Ratner E, Leng S, Zhai R, Nallur S, Babar I, Muller RU, Straka E, Su L, Burki EA, Crowell RE, Patel R, Kulkarni T, Homer R, Zelterman D, Kidd KK, Zhu Y, Christiani DC, Belinsky SA, Slack FJ, Weidhaas JB (2008) A SNP in a let-7 microRNA complementary site in the KRAS 3' untranslated region increases non-small cell lung cancer risk. *Cancer Res* **68**(20)**:** 8535-8540

Chung DC, Brown SB, Graeme-Cook F, Tillotson LG, Warshaw AL, Jensen RT, Arnold A (1998) Localization of putative tumor suppressor loci by genome-wide allelotyping in human pancreatic endocrine tumors. *Cancer Res* **58**(16)**:** 3706-3711

Clevers H (2006) Wnt/beta-catenin signaling in development and disease. *Cell* **127**(3)**:** 469-480

Cole MF, Johnstone SE, Newman JJ, Kagey MH, Young RA (2008) Tcf3 is an integral component of the core regulatory circuitry of embryonic stem cells. *Genes Dev* **22**(6)**:** 746-755

Dahlqvist A, Lindberg T (1965) Fetal development of the small-intestinal disaccharidase and alkaline phosphatase activities in the human. *Biol Neonat* **9**(1)**:** 24-32

De Miguel MP, Fuentes-Julian S, Alcaina Y (2010) Pluripotent Stem Cells: Origin, Maintenance and Induction. *Stem Cell Rev*

Drucker DJ (2007) The role of gut hormones in glucose homeostasis. *J Clin Invest* **117**(1)**:** 24-32

el Marjou F, Janssen KP, Chang BH, Li M, Hindie V, Chan L, Louvard D, Chambon P, Metzger D, Robine S (2004) Tissue-specific and inducible Cre-mediated recombination in the gut epithelium. *Genesis* **39**(3)**:** 186-193

Erisman MD, Rothberg PG, Diehl RE, Morse CC, Spandorfer JM, Astrin SM (1985) Deregulation of c-myc gene expression in human colon carcinoma is not accompanied by amplification or rearrangement of the gene. *Mol Cell Biol* **5**(8)**:** 1969-1976

Esquela-Kerscher A, Slack FJ (2006) Oncomirs - microRNAs with a role in cancer. *Nat Rev Cancer* **6**(4)**:** 259-269

Esquela-Kerscher A, Trang P, Wiggins JF, Patrawala L, Cheng A, Ford L, Weidhaas JB, Brown D, Bader AG, Slack FJ (2008) The let-7 microRNA reduces tumor growth in mouse models of lung cancer. *Cell Cycle* **7**(6)**:** 759-764

Euling S, Ambros V (1996a) Heterochronic genes control cell cycle progress and developmental competence of C. elegans vulva precursor cells. *Cell* **84**(5)**:** 667-676

Euling S, Ambros V (1996b) Reversal of cell fate determination in Caenorhabditis elegans vulval development. *Development* **122**(8)**:** 2507-2515

Fabjani G, Tong D, Wolf A, Roka S, Leodolter S, Hoecker P, Fischer MB, Jakesz R, Zeillinger R (2005) HMGA2 is associated with invasiveness but not a suitable marker for the detection of circulating tumor cells in breast cancer. *Oncol Rep* **14**(3)**:** 737-741

Fearon ER, Vogelstein B (1990) A genetic model for colorectal tumorigenesis. *Cell* **61**(5)**:** 759-767

Fedele M, Battista S, Kenyon L, Baldassarre G, Fidanza V, Klein-Szanto AJ, Parlow AF, Visone R, Pierantoni GM, Outwater E, Santoro M, Croce CM, Fusco A (2002) Overexpression of the HMGA2 gene in transgenic mice leads to the onset of pituitary adenomas. *Oncogene* **21**(20)**:** 3190-3198

Fedele M, Berlingieri MT, Scala S, Chiariotti L, Viglietto G, Rippel V, Bullerdiek J, Santoro M, Fusco A (1998) Truncated and chimeric HMGI-C genes induce neoplastic transformation of NIH3T3 murine fibroblasts. *Oncogene* **17**(4)**:** 413-418

Finelli P, Pierantoni GM, Giardino D, Losa M, Rodeschini O, Fedele M, Valtorta E, Mortini P, Croce CM, Larizza L, Fusco A (2002) The High Mobility Group A2 gene is amplified and overexpressed in human prolactinomas. *Cancer Res* **62**(8)**:** 2398-2405

Ghosh MC, Grass L, Soosaipillai A, Sotiropoulou G, Diamandis EP (2004) Human kallikrein 6 degrades extracellular matrix proteins and may enhance the metastatic potential of tumour cells. *Tumour Biol* **25**(4)**:** 193-199

Griffiths-Jones S (2004) The microRNA Registry. *Nucleic Acids Res* **32**(Database issue)**:** D109-111

Griffiths-Jones S, Grocock RJ, van Dongen S, Bateman A, Enright AJ (2006) miRBase: microRNA sequences, targets and gene nomenclature. *Nucleic Acids Res* **34**(Database issue)**:** D140-144

Griffiths-Jones S, Saini HK, van Dongen S, Enright AJ (2008) miRBase: tools for microRNA genomics. *Nucleic Acids Res* **36**(Database issue)**:** D154-158

Grimson A, Farh KK, Johnston WK, Garrett-Engele P, Lim LP, Bartel DP (2007) MicroRNA targeting specificity in mammals: determinants beyond seed pairing. *Mol Cell* **27**(1)**:** 91-105

Guo Y, Chen Y, Ito H, Watanabe A, Ge X, Kodama T, Aburatani H (2006) Identification and characterization of lin-28 homolog B (LIN28B) in human hepatocellular carcinoma. *Gene* **384:** 51-61

Hagan JP, Piskounova E, Gregory RI (2009) Lin28 recruits the TUTase Zcchc11 to inhibit let-7 maturation in mouse embryonic stem cells. *Nat Struct Mol Biol* **16**(10)**:** 1021-1025

Hamidouche Z, Fromigue O, Ringe J, Haupl T, Marie PJ (2010) Crosstalks between integrin alpha 5 and IGF2/IGFBP2 signalling trigger human bone marrow-derived mesenchymal stromal osteogenic differentiation. *BMC Cell Biol* **11:** 44

Hart MJ, de los Santos R, Albert IN, Rubinfeld B, Polakis P (1998) Downregulation of beta-catenin by human Axin and its association with the APC tumor suppressor, beta-catenin and GSK3 beta. *Curr Biol* **8**(10)**:** 573-581

Hawkins NJ, Bariol C, Ward RL (2002) The serrated neoplasia pathway. *Pathology* **34**(6)**:** 548-555

He TC, Sparks AB, Rago C, Hermeking H, Zawel L, da Costa LT, Morin PJ, Vogelstein B, Kinzler KW (1998) Identification of c-MYC as a target of the APC pathway. *Science* **281**(5382)**:** 1509-1512

Heldin CH, Miyazono K, ten Dijke P (1997) TGF-beta signalling from cell membrane to nucleus through SMAD proteins. *Nature* **390**(6659)**:** 465-471

Heo I, Joo C, Cho J, Ha M, Han J, Kim VN (2008) Lin28 mediates the terminal uridylation of let-7 precursor MicroRNA. *Mol Cell* **32**(2)**:** 276-284

Heo I, Joo C, Kim YK, Ha M, Yoon MJ, Cho J, Yeom KH, Han J, Kim VN (2009) TUT4 in concert with Lin28 suppresses microRNA biogenesis through pre-microRNA uridylation. *Cell* **138**(4)**:** 696-708

Huang CS, O'Brien M J, Yang S, Farraye FA (2004) Hyperplastic polyps, serrated adenomas, and the serrated polyp neoplasia pathway. *Am J Gastroenterol* **99**(11)**:** 2242-2255

Hutvagner G, McLachlan J, Pasquinelli AE, Balint E, Tuschl T, Zamore PD (2001) A cellular function for the RNA-interference enzyme Dicer in the maturation of the let-7 small temporal RNA. *Science* **293**(5531)**:** 834-838

Ichii S, Nagase H, Mori T, Baba S, Nakamura Y (1993) A novel mutation of the APC (adenomatous polyposis coli) gene in a familial adenomatous polyposis (FAP) patient and presymptomatic diagnosis using PCR. *Hum Mol Genet* **2**(5)**:** 597

Jeong SH, Wu HG, Park WY (2009) LIN28B confers radio-resistance through the posttranscriptional control of KRAS. *Exp Mol Med* **41**(12)**:** 912-918

Johnson CD, Esquela-Kerscher A, Stefani G, Byrom M, Kelnar K, Ovcharenko D, Wilson M, Wang X, Shelton J, Shingara J, Chin L, Brown D, Slack FJ (2007) The let-7 microRNA represses cell proliferation pathways in human cells. *Cancer Res* **67**(16)**:** 7713-7722

Johnson SM, Grosshans H, Shingara J, Byrom M, Jarvis R, Cheng A, Labourier E, Reinert KL, Brown D, Slack FJ (2005) RAS is regulated by the let-7 microRNA family. *Cell* **120**(5)**:** 635-647

Kalabis J, Oyama K, Okawa T, Nakagawa H, Michaylira CZ, Stairs DB, Figueiredo JL, Mahmood U, Diehl JA, Herlyn M, Rustgi AK (2008) A subpopulation of mouse esophageal basal cells has properties of stem cells with the capacity for self-renewal and lineage specification. *J Clin Invest* **118**(12)**:** 3860-3869

Kalabis J, Patterson MJ, Enders GH, Marian B, Iozzo RV, Rogler G, Gimotty PA, Herlyn M (2003) Stimulation of human colonic epithelial cells by leukemia inhibitory factor is dependent on collagen-embedded fibroblasts in organotypic culture. *FASEB J* **17**(9)**:** 1115-1117

Kim KA, Kakitani M, Zhao J, Oshima T, Tang T, Binnerts M, Liu Y, Boyle B, Park E, Emtage P, Funk WD, Tomizuka K (2005) Mitogenic influence of human R-spondin1 on the intestinal epithelium. *Science* **309**(5738)**:** 1256-1259

Kinzler KW, Vogelstein B (1996) Lessons from hereditary colorectal cancer. *Cell* **87**(2)**:** 159-170

Korinek V, Barker N, Morin PJ, van Wichen D, de Weger R, Kinzler KW, Vogelstein B, Clevers H (1997) Constitutive transcriptional activation by a beta-catenin-Tcf complex in APC-/- colon carcinoma. *Science* **275**(5307)**:** 1784-1787

Kressner U, Bjorheim J, Westring S, Wahlberg SS, Pahlman L, Glimelius B, Lindmark G, Lindblom A, Borresen-Dale AL (1998) Ki-ras mutations and prognosis in colorectal cancer. *Eur J Cancer* **34**(4)**:** 518-521

Kumar MS, Erkeland SJ, Pester RE, Chen CY, Ebert MS, Sharp PA, Jacks T (2008) Suppression of non-small cell lung tumor development by the let-7 microRNA family. *Proc Natl Acad Sci U S A* **105**(10)**:** 3903-3908

Lagos-Quintana M, Rauhut R, Lendeckel W, Tuschl T (2001) Identification of novel genes coding for small expressed RNAs. *Science* **294**(5543)**:** 853-858

Lamlum H, Papadopoulou A, Ilyas M, Rowan A, Gillet C, Hanby A, Talbot I, Bodmer W, Tomlinson I (2000) APC mutations are sufficient for the growth of early colorectal adenomas. *Proc Natl Acad Sci U S A* **97**(5)**:** 2225-2228

Langelotz C, Schmid P, Jakob C, Heider U, Wernecke KD, Possinger K, Sezer O (2003) Expression of high-mobility-group-protein HMGI-C mRNA in the peripheral blood is an independent poor prognostic indicator for survival in metastatic breast cancer. *Br J Cancer* **88**(9)**:** 1406-1410

Lee Y, Ahn C, Han J, Choi H, Kim J, Yim J, Lee J, Provost P, Radmark O, Kim S, Kim VN (2003) The nuclear RNase III Drosha initiates microRNA processing. *Nature* **425**(6956)**:** 415-419

Lee Y, Jeon K, Lee JT, Kim S, Kim VN (2002) MicroRNA maturation: stepwise processing and subcellular localization. *EMBO J* **21**(17)**:** 4663-4670

Lee YS, Dutta A (2007) The tumor suppressor microRNA let-7 represses the HMGA2 oncogene. *Genes Dev* **21**(9)**:** 1025-1030

Legesse-Miller A, Elemento O, Pfau SJ, Forman JJ, Tavazoie S, Coller HA (2009) let-7 Overexpression leads to an increased fraction of cells in G2/M, direct down-regulation of Cdc34, and stabilization of Wee1 kinase in primary fibroblasts. *J Biol Chem* **284**(11)**:** 6605-6609

Lengauer C, Kinzler KW, Vogelstein B (1997) Genetic instability in colorectal cancers. *Nature* **386**(6625)**:** 623-627

Levy DB, Smith KJ, Beazer-Barclay Y, Hamilton SR, Vogelstein B, Kinzler KW (1994) Inactivation of both APC alleles in human and mouse tumors. *Cancer Res* **54**(22)**:** 5953-5958

Lewis BP, Burge CB, Bartel DP (2005) Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell* **120**(1)**:** 15-20

Luongo C, Moser AR, Gledhill S, Dove WF (1994) Loss of Apc+ in intestinal adenomas from Min mice. *Cancer Res* **54**(22)**:** 5947-5952

Maunoury R, Robine S, Pringault E, Leonard N, Gaillard JA, Louvard D (1992) Developmental regulation of villin gene expression in the epithelial cell lineages of mouse digestive and urogenital tracts. *Development* **115**(3)**:** 717-728

Mayr C, Hemann MT, Bartel DP (2007) Disrupting the pairing between let-7 and Hmga2 enhances oncogenic transformation. *Science* **315**(5818)**:** 1576-1579

mirBase. (2010) Sequences Database. University of Manchester, Manchester, Vol. 2010, pp. microrna.sanger.ac.uk/cgi-bin/sequences/mirna_entry.pl?acc=MI0000062.

Miyakawa A, Wang XL, Nakanishi H, Imai FL, Shiiba M, Miya T, Imai Y, Tanzawa H (1998) Allelic loss on chromosome 22 in oral cancer: possibility of the existence of a tumor suppressor gene on 22q13. *Int J Oncol* **13**(4)**:** 705-709

Mizoguchi E (2006) Chitinase 3-like-1 exacerbates intestinal inflammation by enhancing bacterial adhesion and invasion in colonic epithelial cells. *Gastroenterology* **130**(2)**:** 398-411

Morin PJ, Sparks AB, Korinek V, Barker N, Clevers H, Vogelstein B, Kinzler KW (1997) Activation of beta-catenin-Tcf signaling in colon cancer by mutations in beta-catenin or APC. *Science* **275**(5307)**:** 1787-1790

Moss EG, Lee RC, Ambros V (1997) The cold shock domain protein LIN-28 controls developmental timing in C. elegans and is regulated by the lin-4 RNA. *Cell* **88**(5)**:** 637-646

Moss EG, Tang L (2003) Conservation of the heterochronic regulator Lin-28, its developmental expression and microRNA complementary sites. *Dev Biol* **258**(2)**:** 432-442

Motoyama K, Inoue H, Nakamura Y, Uetake H, Sugihara K, Mori M (2008) Clinical significance of high mobility group A2 in human gastric cancer and its relationship to let-7 microRNA family. *Clin Cancer Res* **14**(8)**:** 2334-2340

Murphy KG, Bloom SR (2006) Gut hormones and the regulation of energy homeostasis. *Nature* **444**(7121)**:** 854-859

Nakamura T, Tsuchiya K, Watanabe M (2007) Crosstalk between Wnt and Notch signaling in intestinal epithelial cell fate decision. *J Gastroenterol* **42**(9)**:** 705-710

Nakao A, Imamura T, Souchelnytskyi S, Kawabata M, Ishisaki A, Oeda E, Tamaki K, Hanai J, Heldin CH, Miyazono K, ten Dijke P (1997) TGF-beta receptor-mediated signalling through Smad2, Smad3 and Smad4. *EMBO J* **16**(17)**:** 5353-5362

Nelson HH, Christensen BC, Plaza SL, Wiencke JK, Marsit CJ, Kelsey KT (2010) KRAS mutation, KRAS-LCS6 polymorphism, and non-small cell lung cancer. *Lung Cancer* **69**(1)**:** 51-53

Neutra MR, O'Malley LJ, Specian RD (1982) Regulation of intestinal goblet cell secretion. II. A survey of potential secretagogues. *Am J Physiol* **242**(4)**:** G380-387

Nielsen J, Christiansen J, Lykke-Andersen J, Johnsen AH, Wewer UM, Nielsen FC (1999) A family of insulin-like growth factor II mRNA-binding proteins represses translation in late development. *Mol Cell Biol* **19**(2)**:** 1262-1270

Niida A, Hiroko T, Kasai M, Furukawa Y, Nakamura Y, Suzuki Y, Sugano S, Akiyama T (2004) DKK1, a negative regulator of Wnt signaling, is a target of the beta-catenin/TCF pathway. *Oncogene* **23**(52)**:** 8520-8526

Niwa H, Miyazaki J, Smith AG (2000) Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. *Nat Genet* **24**(4)**:** 372-376

Olive KP, Tuveson DA, Ruhe ZC, Yin B, Willis NA, Bronson RT, Crowley D, Jacks T (2004) Mutant p53 gain of function in two mouse models of Li-Fraumeni syndrome. *Cell* **119**(6)**:** 847-860

Osawa M, Hanada K, Hamada H, Nakauchi H (1996) Long-term lymphohematopoietic reconstitution by a single CD34-low/negative hematopoietic stem cell. *Science* **273**(5272)**:** 242-245

Papailiou J, Bramis KJ, Gazouli M, Theodoropoulos G (2010) Stem cells in colon cancer. A new era in cancer theory begins. *Int J Colorectal Dis*

Park SM, Shell S, Radjabi AR, Schickel R, Feig C, Boyerinas B, Dinulescu DM, Lengyel E, Peter ME (2007) Let-7 prevents early cancer progression by suppressing expression of the embryonic gene HMGA2. *Cell Cycle* **6**(21)**:** 2585-2590

Pasquinelli AE, Reinhart BJ, Slack F, Martindale MQ, Kuroda MI, Maller B, Hayward DC, Ball EE, Degnan B, Muller P, Spring J, Srinivasan A, Fishman M, Finnerty J, Corbo J, Levine M, Leahy P, Davidson E, Ruvkun G (2000) Conservation of the sequence and temporal expression of let-7 heterochronic regulatory RNA. *Nature* **408**(6808)**:** 86-89

Paulus W, Baur I, Boyce FM, Breakefield XO, Reeves SA (1996) Self-contained, tetracycline-regulated retroviral vector system for gene delivery to mammalian cells. *J Virol* **70**(1)**:** 62-67

Peng Y, Laser J, Shi G, Mittal K, Melamed J, Lee P, Wei JJ (2008) Antiproliferative effects by Let-7 repression of high-mobility group A2 in uterine leiomyoma. *Mol Cancer Res* **6**(4)**:** 663-673

Pereira L, Yi F, Merrill BJ (2006) Repression of Nanog gene transcription by Tcf3 limits embryonic stem cell self-renewal. *Mol Cell Biol* **26**(20)**:** 7479-7491

Phillips TE, Phillips TH, Neutra MR (1984) Regulation of intestinal goblet cell secretion. III. Isolated intestinal epithelium. *Am J Physiol* **247**(6 Pt 1)**:** G674-681

Pierantoni GM, Finelli P, Valtorta E, Giardino D, Rodeschini O, Esposito F, Losa M, Fusco A, Larizza L (2005) High-mobility group A2 gene expression is frequently induced in non-functioning pituitary adenomas (NFPAs), even in the absence of chromosome 12 polysomy. *Endocr Relat Cancer* **12**(4)**:** 867-874

Polesskaya A, Cuvellier S, Naguibneva I, Duquet A, Moss EG, Harel-Bellan A (2007) Lin-28 binds IGF-2 mRNA and participates in skeletal myogenesis by increasing translation efficiency. *Genes Dev* **21**(9)**:** 1125-1138

Potten CS, Booth C, Pritchard DM (1997) The intestinal epithelial stem cell: the mucosal governor. *Int J Exp Pathol* **78**(4)**:** 219-243

Potten CS, Gandara R, Mahida YR, Loeffler M, Wright NA (2009) The stem cells of small intestinal crypts: where are they? *Cell Prolif* **42**(6)**:** 731-750

Potten CS, Loeffler M (1990) Stem cells: attributes, cycles, spirals, pitfalls and uncertainties. Lessons for and from the crypt. *Development* **110**(4)**:** 1001-1020

Powell SM, Zilz N, Beazer-Barclay Y, Bryan TM, Hamilton SR, Thibodeau SN, Vogelstein B, Kinzler KW (1992) APC mutations occur early during colorectal tumorigenesis. *Nature* **359**(6392)**:** 235-237

Qiu C, Ma Y, Wang J, Peng S, Huang Y (2010) Lin28-mediated post-transcriptional regulation of Oct4 expression in human embryonic stem cells. *Nucleic Acids Res* **38**(4)**:** 1240-1248

Ratajczak MZ, Luger SM, Gewirtz AM (1992) The c-kit proto-oncogene in normal and malignant human hematopoiesis. *Int J Cell Cloning* **10**(4)**:** 205-214

Reinhart BJ, Slack FJ, Basson M, Pasquinelli AE, Bettinger JC, Rougvie AE, Horvitz HR, Ruvkun G (2000) The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans. *Nature* **403**(6772)**:** 901-906

Rey JA, Bello MJ, de Campos JM, Vaquero J, Kusak ME, Sarasa JL, Pestana A (1993) Abnormalities of chromosome 22 in human brain tumors determined by combined cytogenetic and molecular genetic approaches. *Cancer Genet Cytogenet* **66**(1)**:** 1-10

Reya T, Clevers H (2005) Wnt signalling in stem cells and cancer. *Nature* **434**(7035)**:** 843-850

Rindi G, Leiter AB, Kopin AS, Bordi C, Solcia E (2004) The "normal" endocrine cell of the gut: changing concepts and new evidences. *Ann N Y Acad Sci* **1014:** 1-12

Roussos ET, Keckesova Z, Haley JD, Epstein DM, Weinberg RA, Condeelis JS (2010) AACR special conference on epithelial-mesenchymal transition and cancer progression and treatment. *Cancer Res* **70**(19)**:** 7360-7364

Rowan AJ, Lamlum H, Ilyas M, Wheeler J, Straub J, Papadopoulou A, Bicknell D, Bodmer WF, Tomlinson IP (2000) APC mutations in sporadic colorectal tumors: A

mutational "hotspot" and interdependence of the "two hits". *Proc Natl Acad Sci U S A* **97**(7)**:** 3352-3357

Rubinfeld B, Albert I, Porfiri E, Fiol C, Munemitsu S, Polakis P (1996) Binding of GSK3beta to the APC-beta-catenin complex and regulation of complex assembly. *Science* **272**(5264)**:** 1023-1026

Rubinfeld B, Souza B, Albert I, Muller O, Chamberlain SH, Masiarz FR, Munemitsu S, Polakis P (1993) Association of the APC gene product with beta-catenin. *Science* **262**(5140)**:** 1731-1734

Rustgi AK (2007) The genetics of hereditary colon cancer. *Genes Dev* **21**(20)**:** 2525-2538

Sakatani T, Kaneda A, Iacobuzio-Donahue CA, Carter MG, de Boom Witzel S, Okano H, Ko MS, Ohlsson R, Longo DL, Feinberg AP (2005) Loss of imprinting of Igf2 alters intestinal maturation and tumorigenesis in mice. *Science* **307**(5717)**:** 1976-1978

Sambuy Y, De Angelis I, Ranaldi G, Scarino ML, Stammati A, Zucco F (2005) The Caco-2 cell line as a model of the intestinal barrier: influence of cell and culture-related factors on Caco-2 cell functional characteristics. *Cell Biol Toxicol* **21**(1)**:** 1-26

Shell S, Park SM, Radjabi AR, Schickel R, Kistner EO, Jewell DA, Feig C, Lengyel E, Peter ME (2007) Let-7 expression defines two differentiation stages of cancer. *Proc Natl Acad Sci U S A* **104**(27)**:** 11400-11405

Shtutman M, Zhurinsky J, Simcha I, Albanese C, D'Amico M, Pestell R, Ben-Ze'ev A (1999) The cyclin D1 gene is a target of the beta-catenin/LEF-1 pathway. *Proc Natl Acad Sci U S A* **96**(10)**:** 5522-5527

Sikora K, Chan S, Evan G, Gabra H, Markham N, Stewart J, Watson J (1987) c-myc oncogene expression in colorectal cancer. *Cancer* **59**(7)**:** 1289-1295

Singh A, Settleman J (2010) EMT, cancer stem cells and drug resistance: an emerging axis of evil in the war on cancer. *Oncogene* **29**(34)**:** 4741-4751

Snippert HJ, van Es JH, van den Born M, Begthel H, Stange DE, Barker N, Clevers H (2009) Prominin-1/CD133 marks stem cells and early progenitors in mouse small intestine. *Gastroenterology* **136**(7)**:** 2187-2194 e2181

Stewart J, Evan G, Watson J, Sikora K (1986) Detection of the c-myc oncogene product in colonic polyps and carcinomas. *Br J Cancer* **53**(1)**:** 1-6

Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S (2007) Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* **131**(5)**:** 861-872

Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* **126**(4)**:** 663-676

Takamizawa J, Konishi H, Yanagisawa K, Tomida S, Osada H, Endoh H, Harano T, Yatabe Y, Nagino M, Nimura Y, Mitsudomi T, Takahashi T (2004) Reduced expression of the let-7 microRNAs in human lung cancers in association with shortened postoperative survival. *Cancer Res* **64**(11)**:** 3753-3756

Taketo MM, Edelmann W (2009) Mouse models of colon cancer. *Gastroenterology* **136**(3)**:** 780-798

TargetScan. (2010) TargetScan Human Release 5.1. Whitehead Institute for Biomedical Research Cambridge, MA, Vol. 2010, p. http://www.targetscan.org/.

Tetsu O, McCormick F (1999) Beta-catenin regulates expression of cyclin D1 in colon carcinoma cells. *Nature* **398**(6726)**:** 422-426

Thuault S, Valcourt U, Petersen M, Manfioletti G, Heldin CH, Moustakas A (2006) Transforming growth factor-beta employs HMGA2 to elicit epithelial-mesenchymal transition. *J Cell Biol* **174**(2)**:** 175-183

Tommiska J, Wehkalampi K, Vaaralahti K, Laitinen EM, Raivio T, Dunkel L (2009) LIN28B in constitutional delay of growth and puberty. *J Clin Endocrinol Metab* **95**(6)**:** 3063-3066

Trang P, Medina PP, Wiggins JF, Ruffino L, Kelnar K, Omotola M, Homer R, Brown D, Bader AG, Weidhaas JB, Slack FJ (2010) Regression of murine lung tumors by the let-7 microRNA. *Oncogene* **29**(11)**:** 1580-1587

Tsonis PA, Call MK, Grogg MW, Sartor MA, Taylor RR, Forge A, Fyffe R, Goldenberg R, Cowper-Sal-lari R, Tomlinson CR (2007) MicroRNAs and regeneration: Let-7 members as potential regulators of dedifferentiation in lens and inner ear hair cell regeneration of the adult newt. *Biochem Biophys Res Commun* **362**(4)**:** 940-945

Tuveson DA, Shaw AT, Willis NA, Silver DP, Jackson EL, Chang S, Mercer KL, Grochow R, Hock H, Crowley D, Hingorani SR, Zaks T, King C, Jacobetz MA, Wang L, Bronson RT, Orkin SH, DePinho RA, Jacks T (2004) Endogenous oncogenic K-ras(G12D) stimulates proliferation and widespread neoplastic and developmental defects. *Cancer Cell* **5**(4)**:** 375-387

Uchida H, Yamazaki K, Fukuma M, Yamada T, Hayashida T, Hasegawa H, Kitajima M, Kitagawa Y, Sakamoto M. (2010) Overexpression of leucine-rich repeat-containing G protein-coupled receptor 5 in colorectal cancer. *Cancer Sci*, Vol. 101, pp. 1731-1737.

van de Wetering M, Sancho E, Verweij C, de Lau W, Oving I, Hurlstone A, van der Horn K, Batlle E, Coudreuse D, Haramis AP, Tjon-Pon-Fong M, Moerer P, van den Born M, Soete G, Pals S, Eilers M, Medema R, Clevers H (2002) The beta-catenin/TCF-4 complex imposes a crypt progenitor phenotype on colorectal cancer cells. *Cell* **111**(2)**:** 241-250

Van der Flier LG, Sabates-Bellver J, Oving I, Haegebarth A, De Palo M, Anti M, Van Gijn ME, Suijkerbuijk S, Van de Wetering M, Marra G, Clevers H (2007) The Intestinal Wnt/TCF Signature. *Gastroenterology* **132**(2)**:** 628-632

Vella MC, Slack FJ (2005) C. elegans microRNAs. *WormBook***:** 1-9

Viswanathan SR, Daley GQ, Gregory RI (2008) Selective blockade of microRNA processing by Lin28. *Science* **320**(5872)**:** 97-100

Viswanathan SR, Powers JT, Einhorn W, Hoshida Y, Ng TL, Toffanin S, O'Sullivan M, Lu J, Phillips LA, Lockhart VL, Shah SP, Tanwar PS, Mermel CH, Beroukhim R, Azam M, Teixeira J, Meyerson M, Hughes TP, Llovet JM, Radich J, Mullighan CG, Golub TR,

Sorensen PH, Daley GQ (2009) Lin28 promotes transformation and is associated with advanced human malignancies. *Nat Genet* **41**(7)**:** 843-848

Wang YC, Chen YL, Yuan RH, Pan HW, Yang WC, Hsu HC, Jeng YM (2010) Lin-28B expression promotes transformation and invasion in human hepatocellular carcinoma. *Carcinogenesis* **31**(9)**:** 1516-1522

Wendt MK, Allington TM, Schiemann WP (2009) Mechanisms of the epithelial-mesenchymal transition by TGF-beta. *Future Oncol* **5**(8)**:** 1145-1168

WHO. (2010) Cancer: Facts Sheet. World Health Organization.

Wong MH, Saam JR, Stappenbeck TS, Rexer CH, Gordon JI (2000) Genetic mosaic analysis based on Cre recombinase and navigated laser capture microdissection. *Proc Natl Acad Sci U S A* **97**(23)**:** 12601-12606

Yamanaka S, Blau HM (2010) Nuclear reprogramming to a pluripotent state by three approaches. *Nature* **465**(7299)**:** 704-712

Yang Q, Bermingham NA, Finegold MJ, Zoghbi HY (2001) Requirement of Math1 for secretory cell lineage commitment in the mouse intestine. *Science* **294**(5549)**:** 2155-2158

Yi R, Qin Y, Macara IG, Cullen BR (2003) Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. *Genes Dev* **17**(24)**:** 3011-3016

Yu F, Yao H, Zhu P, Zhang X, Pan Q, Gong C, Huang Y, Hu X, Su F, Lieberman J, Song E (2007a) let-7 regulates self renewal and tumorigenicity of breast cancer cells. *Cell* **131**(6)**:** 1109-1123

Yu J, Vodyanik MA, Smuga-Otto K, Antosiewicz-Bourget J, Frane JL, Tian S, Nie J, Jonsdottir GA, Ruotti V, Stewart R, Slukvin, II, Thomson JA (2007b) Induced pluripotent stem cell lines derived from human somatic cells. *Science* **318**(5858)**:** 1917-1920

Zhu H, Shah S, Shyh-Chang N, Shinoda G, Einhorn WS, Viswanathan SR, Takeuchi A, Grasemann C, Rinn JL, Lopez MF, Hirschhorn JN, Palmert MR, Daley GQ Lin28a transgenic mice manifest size and puberty phenotypes identified in human genetic association studies. *Nat Genet* **42**(7)**:** 626-630

Zhu H, Shah S, Shyh-Chang N, Shinoda G, Einhorn WS, Viswanathan SR, Takeuchi A, Grasemann C, Rinn JL, Lopez MF, Hirschhorn JN, Palmert MR, Daley GQ (2010) Lin28a transgenic mice manifest size and puberty phenotypes identified in human genetic association studies. *Nat Genet* **42**(7)**:** 626-630

Zhu L, Gibson P, Currle DS, Tong Y, Richardson RJ, Bayazitov IT, Poppleton H, Zakharenko S, Ellison DW, Gilbertson RJ (2009) Prominin 1 marks intestinal stem cells that are susceptible to neoplastic transformation. *Nature* **457**(7229)**:** 603-607