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The Role of miR-21 and miR-31 in Cellular Responses Mediated by TGF-β: A Dissertation

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IN CELLULAR RESPONSES MEDIATED BY TGF-β

A Dissertation Presented

By

CHARISA LYNN COTTONHAM

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THE ROLE OF miR-21 AND miR-31
IN CELLULAR RESPONSES MEDIATED BY TGF-β

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CHARISA LYNN COTTONHAM

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Interdisciplinary Graduate Program

May 9, 2011

This work is dedicated to

My Sweet Pea, My Baby-Girl

Nailah Cherie.

You continue to be my ray of sunshine.

I am forever thankful for your patience and understanding.

Mommy loves you… always!

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ABSTRACT

The function of transforming growth factor β (TGF-β) in cancer is notoriously complex. Initially TGF-β limits tumorigenesis, but at later stages in tumor progression TGF-β promotes the malignant spread of tumor cells. Past studies to understand the pro-metastasis utility of TGF-β centered upon its ability to regulate protein-coding genes. Recently, a small class of non-coding RNAs known as microRNAs (miRNAs) emerged as novel posttranscriptional regulators of gene expression. The significance of miRNA function in cellular processes from embryonic development to the maintenance of homeostasis in adult tissues is becoming increasingly clear. Also apparent is the strong association between aberrant miRNA expression and human diseases, such as cancer. The contribution of miRNAs to TGF-β-mediated cellular responses remains an open question. Thus, I became interested if miRNAs offered an additional layer of regulation in TGF-β signaling through which this cytokine exerts its pro-metastasis function.

To address this inquiry, in the first part of this dissertation I investigated whether miRNAs influenced the ability of TGF-β to induce cellular responses directly involved with carcinoma metastasis, such as epithelial-mesenchymal transition (EMT). Here, I identified two miRNAs, miR-21 and miR-31, that are upregulated during EMT in LIM 1863 organoids, a colon carcinoma model of EMT driven by TGF-β. We performed *in*

vitro studies to characterize the function of miR-21 and miR-31 and found that these two miRNAs positively impact the induction of EMT, migration and invasion by TGF-β. Furthermore, we uncovered TIAM1 (T lymphoma and metastasis gene 1) as a novel target of both miR-21 and miR-31 and show that downregulation of TIAM1 is critical for the pro-migration and pro-invasion activities of miR-21 and miR-31. Together these findings reveal miR-21 and miR-31 as downstream effectors of TGF-β signaling by facilitating EMT, migration and invasion of colon carcinoma cells.

How TGF-β regulates miR-21 and miR-31 became important questions and thus the focus of the second part of this thesis. Interestingly, I found that TGF-β and TNF-α synergize to increase miR-21 and miR-31 levels in LIM 1863 organoids and that the synthesis of new factors induced by TGF-β/TNF-α are required for this upregulation. Moreover, I report that regulation of miR-21 by TGF-β/TNF-α occurs at multiple levels of biogenesis. More specifically data provided here show that Smad4 binds to the promoter of *miR-21* to upregulate its expression thereby specifying *miR-21* as a typical TGF-β target gene. This mechanism is different from one recently observed in smooth muscle cells in which TGF-β did not stimulate *miR-21* transcription, but interestingly, Smad4 enhanced the Drosha-mediated processing of the miR-21 precursor. These two mechanisms suggest that TGF-β regulation of miR-21 is contextual and highlight the complexity of TGF-β signaling.

As a whole, my findings establish important roles for miR-21 and miR-31 in TGF-β-mediated cellular responses that facilitate the pro-metastasis utility of TGF-β in colon cancer. Also, I describe a novel mechanism by which TGF-β/TNF-α signaling elevates the level of miR-21 and miR-31. Future studies that identify additional targets of miR-21 and miR-31 may offer further insight into the molecular mechanisms underlying cellular regulation by TGF-β. This information will be vital for the design of therapeutic interventions for colon cancer patients.

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PREFACE

Portions of this dissertation have been published elsewhere, as noted below. The contribution of co-authors are also described.

- Cottonham CL, Kaneko S, and Xu L (2010) miR-21 and miR-31 Converge on TIAM1 to Regulate Migration and Invasion of Colon Carcinoma Cells. *Journal of Biological Chemistry* **285:** 35293-35302.

  Satoshi Kaneko carried out some of the Northern blots

- Cottonham CL, Kaneko S and Xu L (submitted) Transcriptional Activation of the miR-21 gene by Smad4.

  Satoshi Kaneko carried out some of the Northern blots

CHAPTER I

INTRODUCTION

The Complexity of TGF-β in Cancer

Cancer is a devastating disease in which transforming growth factor β (TGF-β) plays important, yet complex roles (Massague, 2008). On the one hand TGF-β is recognized as a tumor suppressor in that genetic lesions affecting TGF-β pathway components are found with high incidence, particularly in pancreatic and colon cancers (Markowitz and Roberts, 1996; Wood et al., 2007; Jones et al., 2008). On the other hand, many clinical and basic studies point to elevated TGF-β signaling in late stage cancer, and suggest a pro-invasion/pro-metastasis role for TGF-β (Bierie and Moses, 2006a; Padua and Massagué, 2009). The dichotomous nature of TGF-β in cancer stems from its ability to elicit diverse cellular responses. For example, the ability of TGF-β to potently stimulate growth arrest and apoptosis in multiple cell types, especially epithelial cells, ascribes an anti-tumorigenesis function to this cytokine (Moses et al., 1990; Tang et al., 1998). Later in neoplastic progression, TGF-β alters cell biology such that sessile tumor cells gain the ability to disseminate to secondary sites within the body (Derynck et al., 2001; Padua and Massagué, 2009). The pro-metastasis function of TGF-β is attributed to its modulating actions on the plasticity and microenvironment of tumor cells (Derynck et al., 2001; Padua and Massagué, 2009).

Studies in a mouse model of skin carcinogenesis first demonstrated the contrasting activities of TGF-β in cancer. In this mouse model, long-term exposure (~15 weeks) of the skin to chemical carcinogens induces the formation of benign papillomas, of which some progress to malignant squamous carcinoma (Cui et al., 1996). An even smaller

percentage of these tumors progress to a very aggressive spindle cell carcinoma (Cui et al., 1996). Interestingly, targeted expression of the TGF-β1 gene in keratinocytes reduced the overall formation of these chemically-induced skin papillomas (Cui et al., 1996). However, in the tumors that form, heightened TGF-β expression increased the rate of malignant conversion (Cui et al., 1996). These findings indicated that TGF-β possesses drastically different roles in cancer, acting as both a tumor suppressor and a pro-invasion/metastasis factor. Furthermore, the duality of TGF-β function during tumor development and progression is also observed in a number of other cancers, including those arising from the breast and colon (Oft et al., 1998; Tang et al., 2003; Elliott and Blobe, 2005). This suggests that the complex role of TGF-β function in cancer is broad acting. Therefore, it is important to delineate the downstream effectors mediating different TGF-β responses at early versus late stages of cancer progression. Such information will be critical for designing strategies targeting specific aspects of TGF-β responses to combat cancer.

TGF-β Signaling from Membrane to Nucleus

To gain an understanding of how TGF-β can both suppress tumorigenesis and facilitate cancer progression, it must first be understood how cells recognize, interpret and ultimately convert the TGF-β signal into cellular responses. Biologically active TGF-β resides outside of the cell and, as a consequence, the TGF-β signal is relayed into and throughout the cell by intracellular effectors. Smad proteins are recognized as the primary

effectors of TGF-β signaling, although non-Smad factors (i.e., MAP kinase, Rho, and PI-3 kinase/AKT) have been demonstrated to mediate the TGF-β stimulus, (Derynck and Zhang, 2003; Feng and Derynck, 2005; Moustakas and Heldin, 2005).

In the Smad-mediated TGF-β signal pathway, signaling initiates at the cell surface when TGF-β binds to its transmembrane receptor complex comprised of the type I (TβRI) and type II (TβRII) serine/threonine receptor kinases (Shi and Massagué, 2003) (Fig. 1.1.). TGF-β initially binds to TβRII, which then recruits TβRI, thereby forming a ligand-induced receptor complex (Shi and Massagué, 2003). In this complex, TβRII rapidly phosphorylates TβRI, generating the active receptor-signaling complex (Shi and Massagué, 2003). To propagate the signal, TβRI phosphorylates the C-terminus of receptor Smads (R-Smads), i.e., Smad2 and Smad3 (Shi and Massagué, 2003). This Smad activation step is regulated by various mechanisms, including the activities of the inhibitory Smad, Smad7 (Massagué et al., 2005). Smad7 competitively interferes with the binding of Smad2/3 to TβRI, preventing the subsequent phosphorylation of these receptor Smads by TβRI (Massagué et al., 2005). In the absence of Smad7, phosphorylated R-Smads partner with Smad4, forming the activated Smad complex (Lagna et al., 1996; Zhang et al., 1996; Hata et al., 1997). Activated Smads translocate into the nucleus by interacting with nuclear import factors Imp7/8 and nucleoporins located in the nuclear pore complex (Xu et al., 2002; Xu et al., 2007; Yao et al., 2008; Chen and Xu, 2010).

Figure 1.1

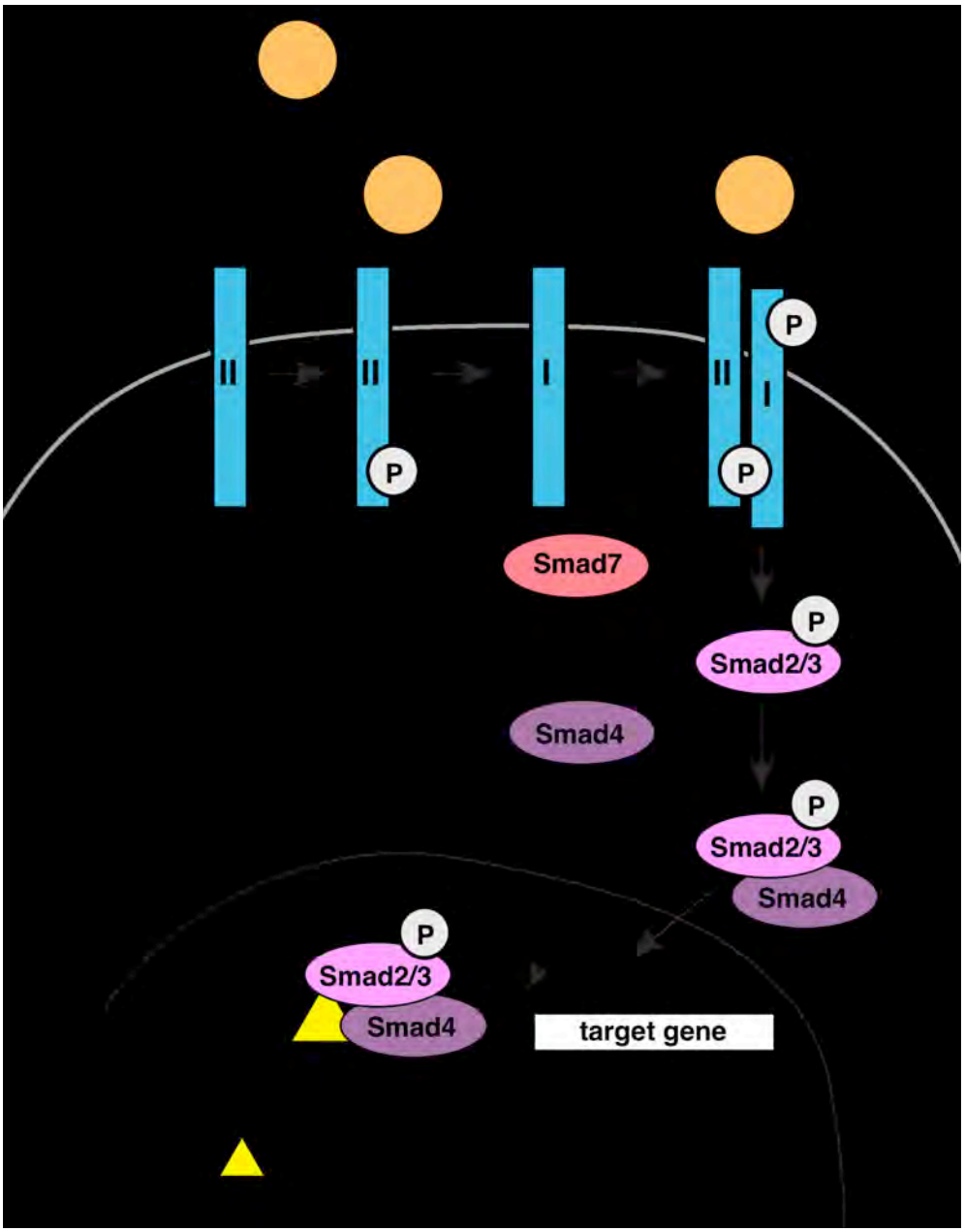


Figure 1.1 | The canonical TGF-β signaling pathway. TGF-β stimulates a cascade of events that lead to the translocation of Smad proteins into the nucleus to regulate target gene expression. See text for details.

In the nucleus, Smads act as transcription factors to mediate TGF-β-induced changes in a multitude of target genes (Kang et al., 2003). Smad3 and Smad4 are recruited to and directly bind DNA sequences within TGF-β target genes; Smad2 does not possess DNA-binding capability (Massagué et al., 2005). Sequences containing 5'-CAGAC-3' and 5'-AGAC-3' are optimal for Smad binding (Massagué et al., 2005). These DNA sequences are known as Smad-binding elements (SBEs) and are found in many genes that are transcriptionally regulated by TGF-β (Massagué et al., 2005). In addition to SBEs, Smads recognize and bind to GC-rich sequences (Massagué et al., 2005). Furthermore, Smads physically interact with different DNA-binding cofactors (i.e. AP-1, ATF3, STATs, and others) within the transcriptional complex (Feng and Derynck, 2005). In mammalians there are over 30 well-characterized DNA-binding cofactors that cooperate with Smad proteins to increase the diversity and complexity of gene responses that TGF-β elicits (Feng and Derynck, 2005). Modification of gene expression by TGF-β leads to altered cellular responses, many of which underlie the tumor suppressor function of TGF-β.

Tumor Suppressor Activities

Cytostatic Response

TGF-β induces two classes of Smad-dependent gene responses that effectively limit epithelial cell proliferation: transcriptional activation of cyclin-dependent kinase (CDK) inhibitors and downregulation of the c-Myc gene. Cell cycle progression is regulated by

CDKs, whose activity is increased by cyclins and decreased by CDK inhibitors (CKIs). In epithelial cells TGF-β induces the expression of two genes coding for CKIs, $p15^{INK4b}$ and $p21^{CIP1}$ (Hannon and Beach, 1994; Datto et al., 1995; Reynisdóttir et al., 1995). Increases in $p15^{INK4b}$ and $p21^{CIP1}$ interfere with cyclin-CDK complexes that drive progression through the G1 phase of the cell cycle. Specifically, $p15^{INK4b}$ inhibits complexes of cyclin-D/CDK4 and cyclin-D/CDK6, whereas $p21^{CIP1}$ inhibits these complexes in addition to those containing cyclin-E/CDK-2 (Siegel and Massagué, 2003). Thus, TGF-β-mediated increases in $p15^{INK4b}$ and $p21^{CIP1}$ arrest cycling cells at the G1 restriction point, preventing DNA synthesis and subsequent mitotic division.

In addition to modulating gene expression to inactivate CDKs, TGF-β also downregulates genes coding for transcription factors that promote cellular growth and proliferation, such as c-Myc and Id proteins. c-Myc drives cell cycle progression by transcriptionally upregulating genes, such as cyclin-D and E2F, that are required for entry into the S-phase of cell cycle progression, during which DNA is duplicated in preparation of mitosis (Siegel and Massagué, 2003). The level of the proto-oncogene c-Myc is rapidly reduced upon stimulation with TGF-β (Alexandrow and Moses, 1995). Interestingly, c-Myc transcriptionally represses *CDKN2B* and *CDKN1A*, genes coding for $p15^{INK4b}$ and $p21^{CIP1}$, respectively (Seoane et al., 2002; Seoane et al., 2004). Thus the rapid TGF-β-mediated downregulation of c-Myc enables *CDKN2B* and *CDKN1A* to be activated by TGF-β. In addition to negatively regulating c-Myc, TGF-β also downregulates Id proteins (Id1, Id2 and Id3), which were originally found to negatively regulate

differentiation (Norton, 2000) and later observed to promote cell cycle progression (Kang et al., 2003).

Induction of Apoptosis

TGF-β also mediates gene expression to induce apoptosis, a process in which injured or abnormal cells undergo programmed cell death. Numerous studies established the ability of TGF-β to activate pro-apoptotic genes to elicit apoptosis. For example, in a Smad-dependent manner TGF-β transcriptionally activates *TGF-β-inducible early response gene (TIEG1)*, a transcription factor that induces apoptosis in epithelial cells (Tachibana et al., 1997). The death-associated protein kinase (DAPK) was also found to be a direct transcriptional target of Smad proteins and to act upstream of mitochondrial-based apoptotic events in hepatoma cells (Jang et al., 2002). Additionally, an investigation utilizing hematopoietic cells found that Smads mediate TGF-β-induced apoptosis by upregulating Src homology 2 domain-containing inositol-5-phosphatase (SHIP), which ultimately inhibits the pro-survival kinase, Akt (Valderrama-Carvajal et al., 2002). Thus, by regulating genes involved with cell cycle progression and cell death signaling, TGF-β tightly controls cell growth and proliferation and works to restrict tumor formation.

Downfall of a Tumor Suppressor, Rise of an Oncogenic Factor

Inhibition of the TGF-β cytostatic or apoptosis programs increases the potential for unchecked proliferation and survival, which facilitates oncogenesis. For example, breast

carcinoma cells lacking both the $p15^{INK4b}$ and c-Myc responses are refractory to growth inhibition by TGF-β (Chen et al., 2001). By what means do cells become desensitized to the potent tumor suppressive effects of TGF-β? This query is addressed by two mechanisms derived from the cumulative findings of several studies. First, TGF-β pathway effectors can become largely inactivated by genetic alterations, i.e. deletions or mutations. Alternatively, the expression of key cofactors that TGF-β pathway effectors interact with to mediate the cytostasis response may be perturbed during tumor pathogenesis.

Inactivation of Core Signaling Components

The TGF-β signaling pathway is often disabled by mutations in core signaling machinery, including TGF-β receptor kinases and Smad proteins. Mutations in the type II TGF-β receptor (TβRII) are frequently found in colon cancers with microsatellite instability, which reduces TβRII expression and abolishes binding of TGF-β to this receptor (Markowitz et al., 1995). The biological consequence of losing TβRII expression was evaluated with *in vitro* and *in vivo* studies. Transient expression of TβRII in TβRII-deficient colon cancer cell lines restored the negative effects of TGF-β on growth, which could be reversed by TGF-β neutralizing antibodies (Wang et al., 1995). Furthermore, this study showed that expression of TβRII reduced the growth of TβRII-null cells. Although less frequent, inactivating mutations in the type I TGF-β receptor (TβRI) are also observed in cancers of the prostate (Kim et al., 1996), pancreas and bile duct (Goggins et al., 1998), colon (Pasche et al., 1999) and ovaries (Wang et al., 2000).

Insensitivity to TGF-β also arises from the inactivation of Smad proteins. Somatic mutations in Smad4/DPC4 (deleted in pancreatic cancer, locus 4) occurs in 50% of pancreatic cancers (Hahn et al., 1996) and approximately 30% of colon cancers (Miyaki et al., 1999). A lower percentage (<10%) of somatic Smad4 mutations are found in breast, head, neck, prostate, esophageal, gastric, and ovarian cancers (Schutte et al., 1996). Germ line mutations in Smad4 have also been found in familial juvenile polyposis, which predisposes to hamartomatous polyps and gastrointestinal cancer (Howe et al., 1998). Furthermore, a functional association of Smad4 loss and tumorigenesis was observed when Smad4 heterozygous mutant mice developed gastric polyps that, as the mice aged, progressed to more aggressive tumors, ultimately becoming invasive (Xu et al., 2000).

Besides Smad4, mutations in receptor Smads (Smad2 and Smad3) are also advantageous for tumor development. Initially known as *JV18-1*, mutations in *Smad2* were found in cancers of the colon (Vogelstein et al., 1988) and lung (Uchida et al., 1996). Furthermore, in a screen of 66 sporadic colon carcinomas, four types of missense mutations were identified in Smad2 that resulted in non-functional protein due to either a lack of Smad2 expression or an inability of the mutant protein to be phosphorylated upon TGF-β treatment (Eppert et al., 1996). An indication that Smad3 may be a tumor suppressor was offered by the finding that Smad3 expression was low or undetectable in gastric tissues that later developed into cancer. Re-expression of Smad3 restored TGF-β responsiveness as indicated by $p15^{INK4b}$ and $p21^{CIP1}$ gene induction (Han et al., 2003).

Similarly, Smad3 protein expression was found reduced or barely detectable in adolescents diagnosed with T-cell acute lymphoblastic leukemia (T-ALL), while Smad3 mRNA expression was readily detectable (Wolfraim et al., 2004). Unlike Smad2 (Waldrip et al., 1998) or Smad4 homozygous mutant mice (Sirard et al., 1998), Smad3 null mice are viable (Zhu et al., 1998). Moreover, $Smad3^{-/-}$ mice develop sporadic colon carcinomas that are hyperproliferative and metastatic (Zhu et al., 1998).

Together, these observations demonstrate that various cancers exhibit diminished expression and function in TGF-β receptors and Smad proteins. Functional studies discussed above provide convincing evidence that loss of TGF-β pathway components renders cells unresponsive to the anti-proliferative cues elicited by TGF-β. Uncoupling of the TGF-β signal from its ability to induce growth arrest or apoptosis yields a selective advantage to developing tumors. This implicates an important role for the TGF-β pathway in maintaining cellular homeostasis to prevent abnormal cell proliferation, and in so doing reduces neoplastic risk. Thus, it appears that inactivation of the TGF-β pathway is one of many molecular events that underlies carcinoma development.

Retention of Selective Gene and Cellular Responses

Some tumor cells, such as those found in breast carcinoma and glioblastoma, are able to evade the growth inhibitory effects of TGF-β without grossly disrupting the TGF-β pathway (Massagué and Gomis, 2006). In this case, oncogenic factors perturb TGF-β

induction of cell cycle arrest while enabling TGF-β to elicit other cellular responses. For example, in non-transformed mammary epithelial cells TGF-β appropriately exerts a cytostatic response. However, transformation of these cells with oncogenes Ha-*ras* and *ErbB2* potently inhibits the ability of TGF-β to induce cell cycle arrest (Chen et al., 2001). Further inquiry revealed that loss of the anti-proliferative response is due to a failure of Smads to transcriptionally repress *c-Myc* (Chen et al., 2001). The authors postulate that oncogenes such as Ras alter the expression of cofactors that Smads normally partner with at the *c-Myc* promoter to downregulate the expression of this pro-growth gene (Chen et al., 2001). Interestingly, TGF-β failed to downregulate *c-Myc* in these transformed mammary cells, yet continued to control the transcription of other genes, including those coding for extracellular matrix components, transcription factors, cytokines and signal transducers- all of which facilitate tumor development and progression (Chen et al., 2001).

Like breast carcinoma cells, glioblastoma cells are insensitive to the TGF-β cytostatic response. Loss the TGF-β growth arrest response in glioblastoma is often due to increased expression or activity of the oncogene phosphatidylinositol 3-kinase (PI3K) or decreased levels of the tumor suppressor phosphatase and tensin homolog (PTEN), which both yield a hyperactive PI3K/Akt pathway (Massagué and Gomis, 2006). Elevated PI3K/Akt activity inhibits FoxO transcription factors from serving as cofactors with Smads to mediate TGF-β activation of $p21^{CIP1}$ and subsequent induction of cell cycle arrest (Seoane et al., 2004). Once the cytostatic gene response is defunct, glioblastoma

cells respond to the TGF-β stimulus with increased production of platelet-derived growth factor (PDGF), which promotes growth, and thereby tumorigenesis (Seoane et al., 2004; Massagué and Gomis, 2006).

These findings, along with those observed in mammary epithelial cells, demonstrate how oncogenic factors (i.e. Ras, PI3K, PTEN, etc.) increase the complexity of cellular responsiveness to TGF-β. The presence of such oncogenic factors in developing neoplasms lead to the selective inactivation a subset of TGF-β gene responses, despite functional TGF-β receptors and Smad proteins. Particularly disrupted are gene responses that impact TGF-β induction of cytostasis, albeit cells remain sensitive to other TGF-β-stimulated responses. Carcinoma cells employ mechanisms like these to escape the growth inhibitory effects of TGF-β, allowing for other TGF-β-mediated cellular responses (i.e., migration and invasion) to be utilized to the benefit of the incipient tumor, and as a result, TGF-β becomes a pro-tumor agent.

Pro-Metastasis Function

Tumors that retain selective TGF-β responsiveness possess increased potential to become malignant, which suggests that TGF-β acts as a metastasis-promoting agent in cancer biology. Tumors produce excessive amounts of TGF-β (Derynck et al., 1987; Dickson et al., 1987; Akhurst et al., 1988). A high TGF-β signaling activity correlates with higher tumor grade and incidence of metastasis, both of which contribute to a poor prognosis for

breast and other cancer patients (Dalal et al., 1993; Tsushima et al., 1996; Buck et al., 2004; Bierie and Moses, 2006b). Furthermore, gain-of-function studies place emphasis on the effect of inordinate levels of TGF-β on tumor progression. For example, overexpression of *TGF-β1* in the mammary gland of mice led to increased circulating tumor cells and lung metastases (Muraoka et al., 2003).

The importance of TGF-β in metastasis was further solidified by loss-of-function experiments. In a mouse model of melanoma metastasis to the liver, sustained exposure of a TβRII antagonist inhibited TGF-β signal transduction and protected mice from liver metastases (Yang et al., 2002). In line with this data, treatment of mice with pan-acting anti-TGF-β antibodies suppressed the metastasis of breast cancer cells to the lung (Biswas et al., 2007). Additional studies from Biwas *et al.* (2007) demonstrated that lung metastases from the breast were also decreased in mice expressing a conditional knockout of TβRII in their mammary glands. Furthermore, perturbation of Smads also disrupts the influence of TGF-β on tumor malignancy as reduction of Smad4 expression inhibited the metastasis of breast carcinoma cells to the bone (Kang et al., 2005) and lungs (Padua et al., 2008) of mice.

How does TGF-β mediate carcinoma malignancy? Although the precise mechanism is poorly understood, it is clear that TGF-β acts on tumor cells and their microenvironment to facilitate metastasis (Yue and Mulder, 2001; Kang, 2006). In order to metastasize, tumor cells must survive long enough to gain the capability to invade adjacent tissues and

disseminate to other locations within the body (Hanahan and Weinberg, 2000). TGF-β contributes to tumor cell survival by subverting host surveillance mechanisms aimed at detection and elimination of abnormal, tumorigenic cells (Padua and Massagué, 2009). TGF-β exerts this function by negatively impacting lymphocyte proliferation, activation and differentiation, which together dampen host immune responses, thereby allowing carcinoma cells to escape immunosurveillance and consequently thrive (Geissmann et al., 1999; Tlsty and Coussens, 2006; Padua and Massagué, 2009). Moreover, TGF-β generates a nourishing environment in which the tumor prospers by upregulating pro-angiogenic mediators vascular endothelial growth factor (VEGF) and connective-tissue growth factor (CTGF) (Sanchez-Elsner et al., 2001; Padua and Massagué, 2009). A sustained blood supply near the tumor provides a vital supply of nutrients and oxygen to the tumor and establishes a means for tumors to spread to secondary locations within the host (Hanahan and Weinberg, 2000). Furthermore, the metastasis-promoting function of TGF-β has been directly linked to its ability to initiate epithelial-mesenchymal transition (EMT) in cell culture (Zavadil and Bottinger, 2005; Moustakas and Heldin, 2007; Xu et al., 2009).

Epithelial-Mesenchymal Transition

EMT is a highly dynamic transdifferentiation process in which epithelial cells disassemble cell-cell adhesion structures, lose apicobasal polarity, reorganize the actin cytoskeleton and acquire a motile, mesenchyme-like phenotype (Greenburg and Hay, 1982; Hay, 1995; Thiery, 2002) (Fig. 1.2). EMT occurs during the development of

vertebrates; for example, EMT facilitates the remodeling of the epithelium during gastrulation to form the three-layered embryo (Thiery, 2002). The ability of TGF-β to stimulate changes in cell morphology similar to those that transpire during EMT was initially observed *in vitro*. Exposure of mouse mammary epithelial cells to TGF-β dramatically altered cell morphology from a cuboidal, epithelial appearance to an elongated, spindle shape that was characteristic of a fibroblastoid phenotype (Miettinen et al., 1994).

EMT in Physiological Contexts

The significance of TGF-β-induced EMT has also been identified *in vivo*, as TGF-β stimulates EMT throughout development and in adult tissues. Developmentally, the proper formation of the heart and palate involve an EMT that is driven by TGF-β signaling (Nawshad et al., 2005). During embryogenesis, structures that subsequently give rise to heart valves are derived from an EMT that transforms endothelia (a specialized squamous epithelia) into cardiac mesenchyme in the atrioventricular (AV) canal and outflow tract regions (Mercado-Pimentel and Runyan, 2007). TGF-β is required for this EMT in the chick heart, as anti-TGF-β antibodies disrupted the *in vitro* formation of cardiac mesenchyme from explants of the AV canal endothelium (Potts and Runyan, 1989). Moreover, knockout of *TGF-β1* or *TGF-β2* causes serious heart malformations in mouse embryos (Letterio et al., 1994; Dickson et al., 1995).

Figure 1.2

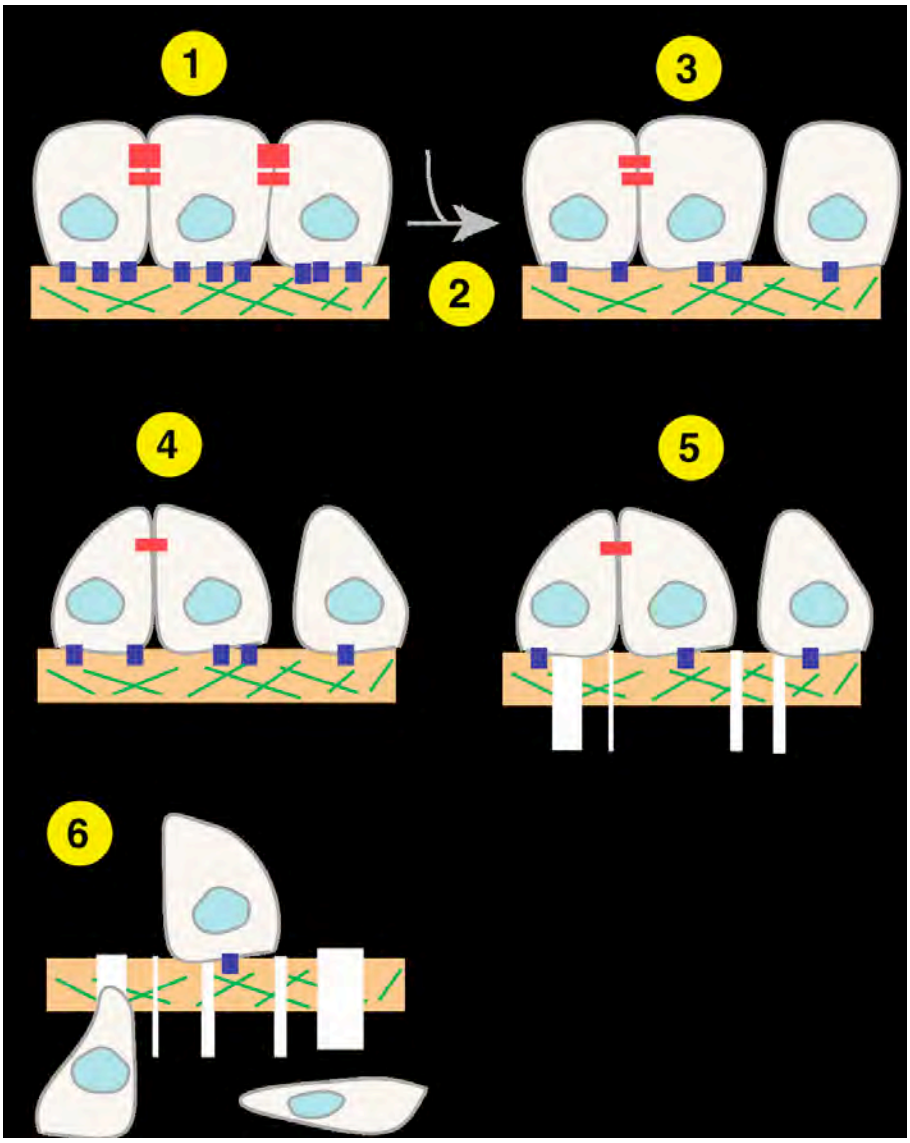


Figure 1.2 | Cell morphology during different stages of EMT. Epithelial cells undergo drastic changes in cellular morphology, architecture, and migratory capability during EMT, emerging with mesenchyme-like phenotypes and increased migratory potential. Epithelial cells are often organized into sheets **(1)** in which they laterally adhere to neighboring cells and to the basement membrane on their basal surface. **(2)** TGF-β stimulates the molecular events that drive EMT, including **(3)** downregulation of adhesion proteins (i.e., cadherins, cytokeratins, and others) **(4)** constriction at the apical surface, **(5)** disassembly and proteolytic digestion of the basement membrane **(6)**, and cell ingression and migration into the interstitial matrix.

Late in the embryogenesis of aminotes (mammals, birds, and reptiles) EMT facilitates the development of the palate. Shortly following formation of a structure known as the medial edge seam that is comprised of epithelial cells, an EMT transforms these cells into mesenchyme, allowing for palatal fusion and consequent bone formation (Fitchett and Hay, 1989). Moreover, a functional role for TGF-β-induced EMT in palatogenesis was established by the finding that TGF-β3-null mice are born with cleft palates, indicative of a failure of palatal fusion (Kaartinen et al., 1995).

TGF-β also influences EMT in the adult during tissue repair. Wound healing is a multipart process involving inflammation, *de novo* tissue formation, and tissue remodeling to facilitate reconstruction of the wound. Upon injury to the epithelium, platelets begin to produce and secrete TGF-β (Broadley et al., 1989). Increased levels of TGF-β lead to the accumulation of wound-healing effectors, i.e. inflammatory cells (neutrophils, monocytes and lymphocytes) and fibroblasts, at the site of damaged tissue (Border and Noble, 1994). Fibroblasts are vital to tissue repair, as they secrete extracellular matrix (ECM) proteins, such as fibronectin, proteoglycans and collagens, that are utilized to heal the injury (Border and Ruoslahti, 1992). Fibroblasts at the injury site arise from differing origins, including the activation of resident fibroblasts (myofibroblasts) and recruitment of fibroblasts from the bone marrow (Kalluri and Neilson, 2003). Interestingly, TGF-β stimulates EMT of the organ epithelium to also yield a population of fibroblasts at the wound to aid in the repair process (Iwano et al., 2002; Kalluri and Weinberg, 2009).

EMT as a Precursor to Carcinoma Metastasis

In addition to naturally occurring biological processes, phenotypic alterations that occur during EMT are associated with pathological conditions, namely fibrosis and carcinoma invasion and metastasis (Putz et al., 1999; Thiery, 2002; Petersen et al., 2003). Fibrosis is a disease that results from deregulated tissue repair. Abnormally sustained production of TGF-β during wound healing is a key contributor to fibrosis observed in renal, pulmonary, hepatic, cardiac, and ocular tissues (Border and Noble, 1994). Elevated levels of TGF-β cause excessive production and deposition of ECM proteins, such as collagens and fibronectin (Border and Ruoslahti, 1992). As in tissue repair, TGF-β stimulates EMT and the expression of ECM proteins that contributes to fibrogenesis (Willis and Borok, 2007). In the lens epithelium sustained TGF-β activity orchestrates EMT and subsequent fibrosis, both of which are prevented in Smad3-null mice (Saika et al., 2004). Similarly, loss of Smad3 expression in renal tubular epithelial cells impairs autoinduction of TGF-β1, decreases collagen accumulation, and consequently these cells do not undergo EMT thereby preventing fibrosis (Sato et al., 2003). These findings suggest that TGF-β induction of EMT is essential for tissue repair and deregulation of this process leads to the pathogenesis of fibrosis.

Interestingly, the effects of TGF-β on epithelial remodeling observed during wound healing and fibrosis correlate with the cellular alterations that give rise to tumor malignancy (Haddow, 1972; Dvorak, 1986; Roberts et al., 1988). These observations provocatively suggested a link between TGF-β regulation of EMT and tumor metastasis,

for which Oft *et al.* (1998) provided supporting functional data. Subcutaneous implantation of breast carcinoma cells into mice generates tumors. Isolation and re-cultivation of cells from these tumors showed phenotypic indications of EMT, i.e. loss of cuboidal shape and acquisition of mesenchyme-like characteristics (Oft et al., 1998). However, when this procedure is performed in breast carcinoma cells expressing a dominant-negative TβRII (TβRII-dn) that disrupts TGF-β signaling, the re-cultivated cells no longer underwent EMT and retained epithelial characteristics (Oft et al., 1998). Additionally, the expression of TβRII-dn reverted metastatic colon cells to an epithelial phenotype and prevented lung metastases in mice (Oft et al., 1998).

Collectively, these observations from both physiological and pathological contexts show that EMT elicited by TGF-β alters cellular plasticity, and as a result, enables motility and invasion in the affected cells. TGF-β is often elevated in tumor cells, which benefit from the pro-tumor function of TGF-β, especially the influence of TGF-β over EMT (Kang et al., 2005). TGF-β-induced conversion of epithelial cells to a more mesenchymal phenotype appears to be a direct precursor for neoplastic cells to escape the primary tumor mass and metastasize to distant sites. Thus, TGF-β arms epithelial tumors with the capacity to progress toward malignancy, and thereby acts as a pro-metastasis agent.

Transcriptional Profiling to Investigate TGF-β Control of EMT

The scientific community has made significant research efforts to uncover the molecular mechanisms that underlie the pro-metastasis function of TGF-β. As indicated previously, the role of TGF-β in tumor progression towards metastasis is directly correlated with its ability to elicit EMT. During EMT the expression of epithelial markers decrease, i.e. E-cadherin, while those associated with mesenchymal cells increase, i.e. vimentin. Thus studies to delineate how TGF-β facilitates tumor malignancy included investigations aimed at determining the means by which TGF-β orchestrated changes in gene expression during EMT.

A multitude of genes are regulated by TGF-β at the level of transcription (Zavadil et al., 2001; Kang et al., 2003). As a consequence, researchers investigated TGF-β-induced changes in the transcriptome during EMT as a means to understand the role of this cytokine in metastasis. Study of differential gene expression was primarily performed using microarray technology, which is capable of simultaneously evaluating the expression of thousands of genes in a single experiment. Microarray-based gene profiling demonstrated that TGF-β stimulates epithelial cells to undergo widespread transcriptional reprogramming during EMT involved in both development and tumor metastasis (LaGamba et al., 2005; Valcourt et al., 2005). Studies like these have also revealed that TGF-β directly activates the expression of transcription factors including SNAI1/2, Twist and ZEB1/2 (Zavadil and Bottinger, 2005; Moustakas and Heldin, 2007; Xu et al., 2009). These are master regulators of the EMT program, which suppress the

levels of epithelial markers such as E-cadherin and zonula occludens (ZO)-1, and upregulate mesenchymal markers including vimentin, fibronectin, and others (Thiery and Sleeman, 2006).

Genome-wide transcriptome analyses support observations that TGF-β crosstalks with other signal pathways to alter cellular plasticity during EMT (Zavadil et al., 2001; Thiery, 2002; Derynck and Zhang, 2003). The cooperation between Ras and TGF-β to induce EMT is clearly demonstrated at the cellular level in the mouse mammary epithelial cell model of EMT EpH4/EpRas/EpRasXT. TGF-β elicits growth arrest in EpH4 (non-transformed mammary epithelial) cells (Oft et al., 1996). However, overexpression of hyperactive (oncogenic) Ha-*ras* (EpRas cells) confers selective resistance of these cells to the growth inhibitory effects of TGF-β, and consequentially bestows tumorigenic properties (Oft et al., 1996). Importantly, in EpRas cells oncogenic Ras collaborates with TGF-β to stimulate EMT, invasion, and migration; the resulting cells are termed, EpRasXT (Oft et al., 1996). Use of small molecule inhibitors in the EpRas/EpRasXT EMT model delineated a role for a hyperactive Raf/mitogen-activated kinase (MAPK) pathway downstream of Ras during TGF-β-induced EMT (Lehmann et al., 2000; Janda et al., 2002). Importantly, microarray studies using the EpRas/EpRasXT EMT model further elucidated transcriptional programs that underlie cooperation between TGF-β and Ras in stimulation of EMT related to tumor malignancy (Jechlinger et al., 2003). This study put forth by Jechlinger *et al.* (2003) assigned distinct gene expression profiles to cellular and molecular processes that correlate with EMT, metastasis and oncogene

function. Knowledge of the cohort of genes that are regulated by TGF-β during EMT has therapeutic value. Such a point is exemplified by the finding that the potential of tumors to metastasize to the bone or lung can be depicted by which TGF-β-induced genes are expressed in patients with breast cancer (Padua et al., 2008).

Whilst microarray-based studies offer significant insight into the gene responses elicited by TGF-β, these analyses do not fully explain how TGF-β exerts its pro-metastasis function. For example, changes in the transcriptome stimulated by TGF-β do not completely correlate with alterations in protein expression that are observed during EMT and tumor metastasis. This suggests that in addition to transcriptional control of gene expression, TGF-β utilizes other regulatory mechanisms to mediate its influence on metastasis. This point is underscored by a study that aimed to identify key players involved in TGF-β–induced EMT. In comparison to previous like-minded studies that exclusively used total mRNA for microarray profiling, Jechlinger *et al.* (2003) performed microarray analyses on total, polysome-bound, and polysome-free mRNA to compare changes in gene expression elicited by TGF-β during EMT. Microarray profiling of these three mRNA samples showed that 75% of genes involved in EMT were transcriptionally regulated by TGF-β, 18% were exclusively regulated at the level of translation and 7% of genes were regulated at both levels by TGF-β during EMT in transformed mouse mammary epithelial cells (Jechlinger et al., 2003). Consistent with past investigations to understand gene regulation by TGF-β during EMT, this study showed that a large proportion (75%) of genes are controlled by TGF-β at the level of transcription. Most

notable, this study also demonstrates that a small fraction (~1/5) of the genes assayed are regulated at the level of translation during EMT induced by TGF-β. Together, these observations argue that posttranscriptional mechanisms offer additional layers of regulation in TGF-β regulation of cellular responses. Hence the question can be put forth: what are these posttranscriptional mechanisms and by what means to they contribute to TGF-β induction of EMT and thereby metastasis?

MicroRNAs: Novel Regulators of Gene Expression

MicroRNAs (miRNAs) are 20-22-nucleotide non-coding RNAs that regulate gene expression at posttranscriptional levels by binding to complementary sequences in the 3'UTR of mRNA targets, causing either translational repression or mRNA degradation (Bartel, 2004; Lim, 2005; Selbach et al., 2008; Guo et al., 2010) (Fig. 1.3).

Initial discovery of this important function of miRNAs was made with studies that investigated developmental timing in *C. elegans* (Lee et al., 1993; Wightman et al., 1993). At the time, it was clear that *lin-4* was important for development in *C. elegans*, as lin-4-null mutants often reiterated the first larval stage (L1) and, as a result, the adult stage was not reached in these mutants. Interestingly, a loss-of-function mutant, *lin-14*, had an opposite effect as the lin-4 mutant had on development, causing precocious execution of events normally found in L2, L3, L4, and adult stages.

Figure 1.3

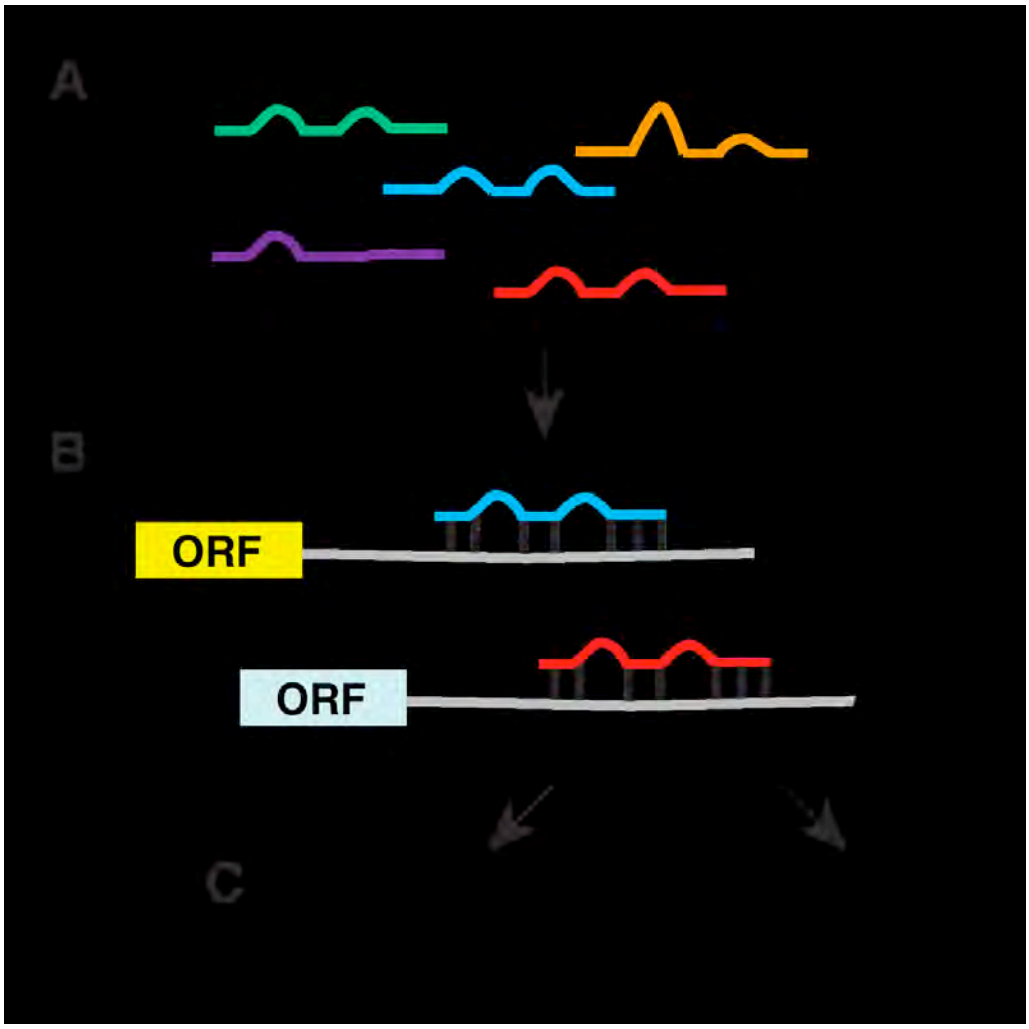


Figure 1.3 | MicroRNA control of gene expression. The small, non-coding class of RNAs, known as microRNAs (miRNAs) **(A)** bind to the 3'UTRs of target mRNAs **(B)** to either direct degradation or translational repression **(C)**, causing reduced protein expression of target genes.

These observations indicated that lin-4 negatively regulated lin-14. Examination of the functional relationship between lin-4 and lin-14 by Ambros and Ruvkun revealed that 1) the gene encoding lin-4 did not generate protein, but yielded transcripts of 22nt and 61nt in length and 2) regions within lin-4 were complementary to regions within the 3'UTR of lin-14 (Lee et al., 1993; Wightman et al., 1993).

This ground-breaking mechanism of posttranscriptional gene regulation by miRNAs is present in animals, plants and viruses (Bartel, 2004; Pfeffer et al., 2004). In humans there are currently over 1000 known miRNAs (Griffiths-Jones et al., 2008). The list of human miRNAs will most likely continue to grow as the technological advance of high-throughput sequencing continues to identify presently unknown miRNAs. Moreover, approximately 30% of the human genome is estimated to be regulated by miRNAs (Lewis et al., 2005), making the function of miRNAs in cellular physiology and disease current areas of intense research.

MicroRNAs in Cancer

It is now beyond question that miRNAs have critical roles in diverse physiological processes, including development, differentiation, apoptosis and proliferation. Abnormal miRNA function underlies a number of pathological conditions, such as diabetes (Poy, 2004) and mental retardation (Jin et al., 2004). Rapidly emerging evidence strongly suggest critical roles of miRNAs in the pathogenesis of cancer (Garzon et al., 2009).

Alterations in miRNA expression profiles can distinguish between normal and cancerous tissues (Lu et al., 2005; Volinia et al., 2006). Furthermore, miRNA expression patterns are highly correlative with the prognosis of cancer patients (Garzon et al., 2009).

Tumor Suppressors

Depending on the tissue of origin, miRNAs can function as tumor suppressors or oncogenes. For example, deletion of the chromosomal region harboring genes encoding for both miR-15a and miR-16-1 causes low expression of these miRNAs in chronic lymphoblastic leukemia (CLL) (Calin et al., 2002). Decreased levels of miR-15 and miR-16 allows for heightened expression of their common target BCL2 and subsequent survival of malignant B cells (Cimmino et al., 2005). Experimental data also demonstrates a tumor suppressor role for the let-7 family of miRNAs. These miRNAs are poorly expressed in lung, breast, and cervical cancers and are characterized as an anti-growth factor. Further investigation revealed that the let-7 family member miR-84 targets Ras, a gene known to transform cells and ascribe enhanced growth properties (Johnson et al., 2005). Subsequent studies identified that additional oncogenes were targets of the let-7 family of miRNAs, including HMGA2 (Lee and Dutta, 2007) and c-Myc (Sampson et al., 2007), which further supports a tumor suppressor role for let-7 in cancer. Other miRNAs associated with tumor suppressor function are those belonging to the miR-29 and miR-34 clusters. The miR-29 cluster comprised of three miRNAs, miR-29a, miR-29b, and miR-29c, is downregulated in CLL, AML and carcinomas of the bile duct, breast, and lung (Garzon et al., 2009). Both *in vitro* and *in vivo* analyses

demonstrated that loss of activity of the miRNAs in the miR-29 cluster results in elevated levels of oncogenes TCL1, MCL1, and three DNA methyl transferases (Pekarsky et al., 2006; Fabbri et al., 2007; Mott et al., 2007). The miR-34 cluster miRNAs (miR-34a, miR-34b, miR-34c) are downregulated in pancreatic, colon, and breast cancers and target several cell cycle regulators including CDK4, CDK6, cyclin E2, and cell cycle-associated transcription factor E2F3, effectively limiting cellular proliferation (Chang et al., 2007; He et al., 2007a; Raver-Shapira et al., 2007). Interestingly, the miR-34 cluster is a component of the p53 tumor suppressor pathway as these miRNAs are transcriptionally activated by p53, a transcription factor that regulates genes involved with inducing cell cycle arrest and apoptosis in stressed and damaged cells (Chang et al., 2007; He et al., 2007a; Raver-Shapira et al., 2007). Thus it appears that miRNAs play important roles in maintaining homeostasis, as loss of their expression and activity facilitates tumorigenesis.

Oncogenic miRNAs

The first miRNA attributed with oncogenic function was miR-155 (Metzler et al., 2004; Kluiver et al., 2005). MiR-155 is highly expressed in hematologic cancers CLL, DLBCL, AML, BL and also found in solid tumors arising in the breast and lung (Garzon et al., 2009). In cooperation with c-Myc, miR-155 spurs tumor development (Tam, 2001). Additional support for miR-155 as a causative agent in tumorigenesis is provided by the observation that mice transgenic for miR-155 expression in B-cells developed acute lymphoblastic leukemia (ALL) due to increased proliferation of B-cell precursors (Costinean et al., 2006; Costinean et al., 2009). This effect of miR-155 on B-cell

development is attributed to its ability to target and downregulate SHIP and CCAAT enhancer-binding protein β (C/EBPβ), two negative regulators of interleukin-6 signaling, a pathway that promotes B-cell differentiation (Costinean et al., 2009; O'Connell et al., 2009; Pedersen et al., 2009).

Alongside miR-155, several other miRNAs are linked to facilitating tumor development and progression, including miR-21. MiR-21 is upregulated in carcinomas of the breast, colon, pancreas, lung, prostate, liver and stomach (Volinia et al., 2006). In addition, cancers of hematological origin, including AML and CML exhibit increased miR-21 expression. (Garzon et al., 2005; Garzon et al., 2008). Recently, Medina *et al.* (2010) generated a miR-21 transgenic mouse to investigate the role of miR-21 in cancer *in vivo*. In this mouse model, overexpression of miR-21 caused a pre-B malignant lymphoid-like phenotype, which could be reversed by decreasing miR-21 expression (Medina et al., 2010). These studies by Medina and colleagues also demonstrate that a single miRNA, miR-21 in this case, can impact several aspects of cancer biology, including initiation, maintenance, survival and metastasis of tumors (Medina et al., 2010).

Other notable miRNAs that are associated with oncogenic activity include the miR-17-92 cluster and members of the miR-371-373 cluster. The miR-17-92 cluster, composed of miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1, and miR-92a-1, is the protypical polycistronic miRNA gene and for its role in cancer, is considered a classic oncomiR cluster (He, 2005). The miR-17-92 cluster is highly upregulated in lymphomas, breast,

lung, colon, stomach, and pancreatic cancers (Garzon et al., 2009). One manner in which this cluster contributes to tumorigenesis is through its translational repression of factors that regulate cellular proliferation (i.e. $p21^{CIP1}$; induces cell cycle arrest) and apoptosis (i.e. E2F1; induces apoptosis), subsequently leading to increased survival and proliferation (Mendell, 2008). Interestingly, the miR-17-92 cluster is transcriptionally activated by c-Myc (O'Donnell et al., 2005). This finding suggests a link between the miR-17-92 cluster and loss of the tumor suppressor function of TGF-β, as c-Myc is downregulated by TGF-β to mediate cytostasis in normal cells (Alexandrow and Moses, 1995). However some tumors, such as those arising from the breast, TGF-β fails to downregulate c-Myc (Chen et al., 2001). Such failure maintains c-Myc expression and activities, including transactivation of the *miR-17-92* cluster, thereby facilitating evasion of TGF-β-induced cell cycle arrest (Petrocca et al., 2008a; Petrocca et al., 2008b). Like the miR-17-92 cluster, members of the miR-371-373 cluster facilitate tumorigenesis. In testicular germ cell tumors that were transformed by hyperactive Ras, miR-372 and miR-373 were able to surmount p53-mediated senescence by targeting the tumor suppressor LATS2 (Voorhoeve et al., 2006). All together these studies describe both tumor suppressor and oncogene functions for miRNAs. Thus precise control of miRNA expression has important pathophysiological implications.

Biosynthesis of MicroRNAs

Genes encoding miRNAs are primarily transcribed by RNA polymerase II (pol II) into long primary transcripts known as pri-miRNAs (Cai et al., 2004; Lee et al., 2004) (Fig. 1.4). Pri-miRNAs are processed into ~70 nt hairpin-shaped precursor miRNAs (pre-miRNAs) by the nuclear Microprocessor complex consisting of Drosha, DGCR8, and other regulatory factors such as p68 and p72 (Lee, 2003; Denli et al., 2004; Gregory et al., 2004; Han et al., 2004; Landthaler et al., 2004). Exportin 5 exports pre-miRNAs into the cytoplasm where they are further processed by Dicer into ~22 bp miRNA/miRNA* duplexes (Hutvagner et al., 2001; Yi et al., 2003; Lund et al., 2004). One strand of this duplex is preferentially loaded onto Argonaute proteins generating the RNA-induced silencing complex (RISC) containing the mature miRNA. As a part of the RISC, the mature miRNA directs base-pairing with target mRNAs to inhibit their translation or induce degradation (Gregory et al., 2005; Bartel, 2009). Multiple steps in this sequence of miRNA biogenesis (i.e., transcription, maturation, and stability) can be regulated by a variety of mechanisms, including signal pathways (Kim et al., 2009; Krol et al., 2010).

Figure 1.4

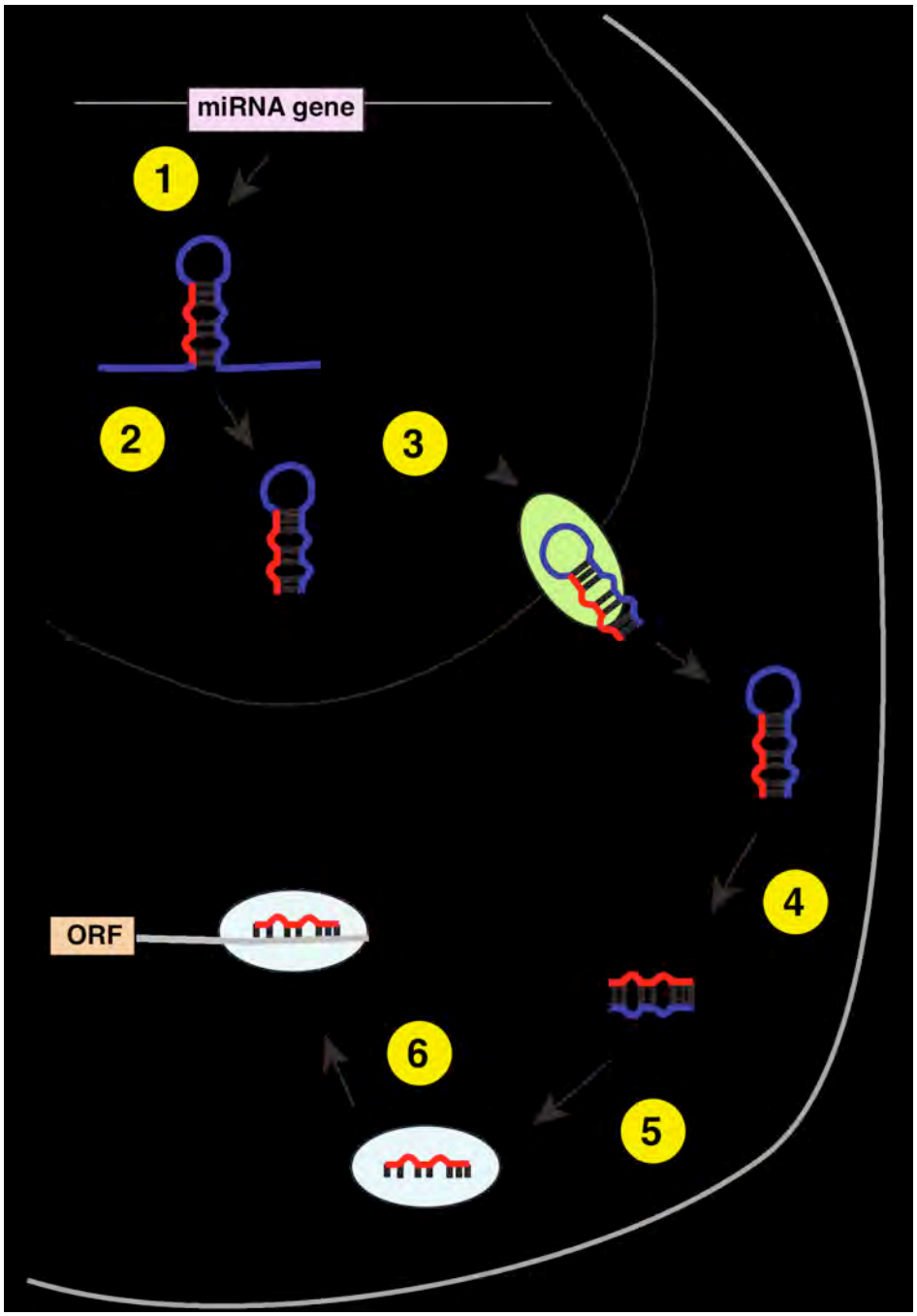


Figure 1.4 | MicroRNA biogenesis. (1) Genes encoding miRNAs are transcribed by RNA polymerase II into primary transcripts that are **(2)** processed by the Drosha-DGCR8 microprocessor into hairpin-shaped precursor miRNAs. **(3)** The pre-miRNA is exported into the cytoplasm where **(4)** Dicer and Argonaute proteins (AGOs) further process it into a mature miRNA, which is then **(5)** incorporated into the RNA-induced silencing complex (RISC). In the RISC, **(6)** AGOs facilitate miRNA-directed regulation of mRNA expression.

MicroRNAs in TGF-β Signaling and Tumor Metastasis

Previous reports illustrate that miRNAs are involved in TGF-β signaling and cellular responses (Table 1.1). Furthermore, earlier profiling studies identified miRNAs whose levels undergo significant changes during TGF-β-induced EMT, suggesting the possible involvement of miRNAs in this process (Zavadil et al., 2007). In particular, several independent studies identified the miR-200 family as important suppressors of EMT in a number of different models (Burk et al., 2008b; Gregory et al., 2008a; Korpal et al., 2008b; Park et al., 2008a). MiR-200 inhibits EMT by directly recognizing complementary sites in the 3'-UTR of ZEB1/2 and repressing the translation of these positive regulators of EMT (Burk et al., 2008b; Gregory et al., 2008a; Korpal et al., 2008b; Park et al., 2008a). MiR-200 itself is repressed by TGF-β, through an unknown mechanism (Korpal et al., 2008b). MiRNAs such as miR-9 and miR-335 promote metastasis by directly suppressing the levels of E-cadherin (miR-9) or SOX4 (miR-335) (Tavazoie et al., 2008; Ma et al., 2010). MiR-10b has also been suggested to facilitate breast cancer metastasis, but this was contradicted by a more recent report in which high miR-10b appeared to suppress motility and invasiveness of breast cancer cells (Ma et al., 2007; Moriarty et al., 2010). MiR-31 acts to repress breast cancer cell migration and invasion, and a low miR-31 level correlates with high metastatic potential (Valastyan et al., 2009; Valastyan et al., 2010). The anti-metastasis function of miR-31 is attributable to downregulation of three targets: integrin α5, radixin and rhoA (Valastyan et al., 2009; Valastyan et al., 2010). Although these observations show that miRNAs impose another

layer of regulation on breast cancer metastasis, more studies are needed to fully resolve the function of miRNAs in TGF-β signaling.

Table 1.1 | MicroRNAs in TGF-β Signaling

MicroRNA	Context	Targets	Function	References
miR-106b-25 cluster	Cancer Cell cycle Apoptosis	Bim, E2F1, p21	Disrupts cell cycle arrest and apoptosis by TGF-β	(Petrocca et al., 2008b)
miR-15, miR-16	Development	AcvR2a	Hinders Nodal-induced mesoderm development	(Martello et al., 2007)
miR-17-92 cluster	Cancer Cell cycle	Bim, E2F1, p21, PTEN	Disrupts TGF-β induction of cell cycle arrest and apoptosis	(Ventura, 2008)
miR-133, miR-590	Atrial fibrillation	TGF-β1, TβRII	Limits collagen induction by TGF-β	(Shan et al., 2009)
miR-155	Cancer EMT	RhoA	Facilitates TGF-β disassembly of adherens junctions	(Kong et al., 2008)
miR-192	Fibrosis	ZEB-1, ZEB-2	Perturbs TGF-β downregulation of E-cadherin	(Krupa et al., 2010)
miR-200 family	Cancer EMT	SIP1, ZEB	Facilitates TGF-β downregulation of E-cadherin	(Burk et al., 2008b; Gregory et al., 2008a)
miR-21	Cancer	PDCD4, PTEN, TIMP1, TPM1, and more	Aids with survival and metastasis of cancer cells	(Garzon et al., 2009)
miR-24	Erythropoeisis	Alk4	Disrupts Smad2 activation by Activin	(Wang et al., 2008)
miR-LAT	Apoptosis HSV infection	Smad3, TGF-β1	Confers resistance to TGF-β-mediated apoptosis in neurons	(Gupta et al., 2006)

Thesis Objective

The mechanisms by which TGF-β promotes the malignant spread of carcinoma cells in the later stages of tumor progression are not fully understood. So far, protein-coding target genes of Smads are the focus of most studies that investigate TGF-β regulation of cellular responses. However, an increasing number of reports demonstrate the significance of miRNA function in a variety of physiological processes, including those regulated by TGF-β. These observations led to the **hypothesis of my thesis: miRNAs play an important role in TGF-β-mediated cellular responses by acting as effectors or modifiers of TGF-β signaling.**

To investigate this idea, I studied the function of miRNAs in TGF-β-induced epithelial-to-mesenchymal transition (EMT), a process germane to carcinoma metastasis (Hanahan and Weinberg, 2000). Specifically, I pursued the following:

Aim 1: **To identify and characterize the function of miRNAs that impact EMT induced by TGF-β**

Aim 2: **To determine how TGF-β signaling regulates the expression of candidate miRNAs**

These two aims explored the significance of miRNAs in TGF-β signaling- a novel angle to study cellular regulation by TGF-β. The overall goal of this thesis was to increase our comprehension of the role of miRNAs in TGF-β-regulated cellular responses, especially those pertaining to tumor metastasis.

CHAPTER II

CHARACTERIZATION OF miR-21 AND miR-31 IN THE PRO-METASTASIS FUNCTION OF TGF-β

Introduction

TGF-β promotes cell migration and invasion, an attribute that is linked to the pro-metastasis function of this cytokine in late stage cancers. However, the molecular determinants underlying the influence of TGF-β on metastasis are poorly understood. MiRNAs regulate cellular processes that advance tumors to malignancy, such as EMT, migration and invasion (Garzon et al., 2009). Thus it is feasible that miRNAs are an important component of the pro-metastasis action of TGF-β. In this study we utilized the LIM 1863 colon carcinoma organoid that undergoes epithelial-mesenchymal transition (EMT) in response to TGF-β to investigate role of miRNAs in EMT. EMT in LIM 1863 organoids is markedly accelerated by TNF-α, and we found that the levels of miR-21 and miR-31 were prominently elevated under the synergistic actions of TGF-β/TNF-α. Consistent with this, overexpression of either miR-21 or miR-31 significantly enhanced the effect of TGF-β alone on LIM 1863 morphological changes. More importantly, transwell assays demonstrated the positive effects of both miR-21 and miR-31 in TGF-β regulation of LIM 1863 motility and invasiveness. Elevated levels of miR-21 and miR-31 also enhanced motility and invasiveness of other colon carcinoma cell lines. We present compelling evidence that TIAM1, a guanidine exchange factor of the Rac GTPase, is a direct target of both miR-21 and miR-31. Indeed in LIM 1863 cells, suppression of TIAM1 is required for miR-21/miR-31 to enhance cell migration and invasion. Therefore we have uncovered miR-21 and miR-31 as downstream effectors of TGF-β in facilitating invasion and metastasis of colon carcinoma cells.

Results

The colon carcinoma cell line LIM 1863 as a model of TGF-β-induced EMT

The Ludwig Institute in Melbourne (LIM) established the LIM 1863 colorectal carcinoma cell line, which originated from a portion of the ileocecal valve isolated from a 74-year-old Caucasian female with a poorly differentiated ulcerated carcinoma that extended through the thickness of the muscle wall (Whitehead et al., 1987). *In vitro,* LIM 1863 cells are arranged around a central lumen, forming three-dimensional spheroids, which are commonly referred to as "organoids," that propagate in suspension by cleavage (Whitehead et al., 1987; Hayward and Whitehead, 1992). Importantly, these colonic organoids retain both morphological and functional differentiation traits analogous to those observed in the intestinal crypt *in vivo*. For example, LIM 1863 cells possess progenitor cells that undergo consistent differentiation to yield mature columnar cells with a polarized, structurally complete brush border and goblet cells that secrete mucus (Whitehead et al., 1987). Additionally, cells appear to proliferate near the outer edges of the organoid and migrate toward the central lumen before shedding as dead cells into the culture medium (Hayward and Whitehead, 1992), indicating the LIM 1863 organiod possesses both zones of proliferation and differentiation similar to the normal colon. Together, these findings show that LIM 1863 organoids are unique amongst available colon carcinoma cell lines in their retention of normal colonic features.

Additional studies investigated the effects of various growth factors, including TGF-β, on LIM 1863 organoids. Like many epithelial cells, LIM 1863 organoids are sensitive to the anti-proliferative cues elicited by TGF-β, as indicated by reduced ^{3}H-Thymidine incorporation in cells treated with TGF-β (Hayward et al., 1995). In addition, TGF-β induced profound changes in the morphology of LIM 1863, causing adhesion and spreading of organoids (Hayward et al., 1995). In 5-7 days after TGF-β stimulation, LIM 1863 cells assume a monolayer morphology (Fig. 2.1). Co-treatment with TNF-α can accelerate the morphological changes to complete in 48 hours, but by itself TNF-α has no effect (Bates and Mercurio, 2003). This morphological change is reversible upon removal of TGF-β and TNF-α. These cellular characteristics and other accompanying molecular changes (i.e., downregulation of epithelial marker, E-cadherin and upregulation of mesenchymal marker, N-cadherin) led to the conclusion that this is a typical TGF-β-induced epithelial-mesenchymal transition (EMT) (Bates and Mercurio, 2003) (Bates and Mercurio, 2003). Furthermore, TGF-β-induced EMT confers migratory capability to LIM 1863 cells (Bates and Mercurio, 2003), establishing the LIM 1863 cell line as a unique three-dimensional culture system to study TGF-β regulation of tumor cell migratory and invasive properties (Vincan et al., 2007a; Vincan et al., 2008).

Figure 2.1

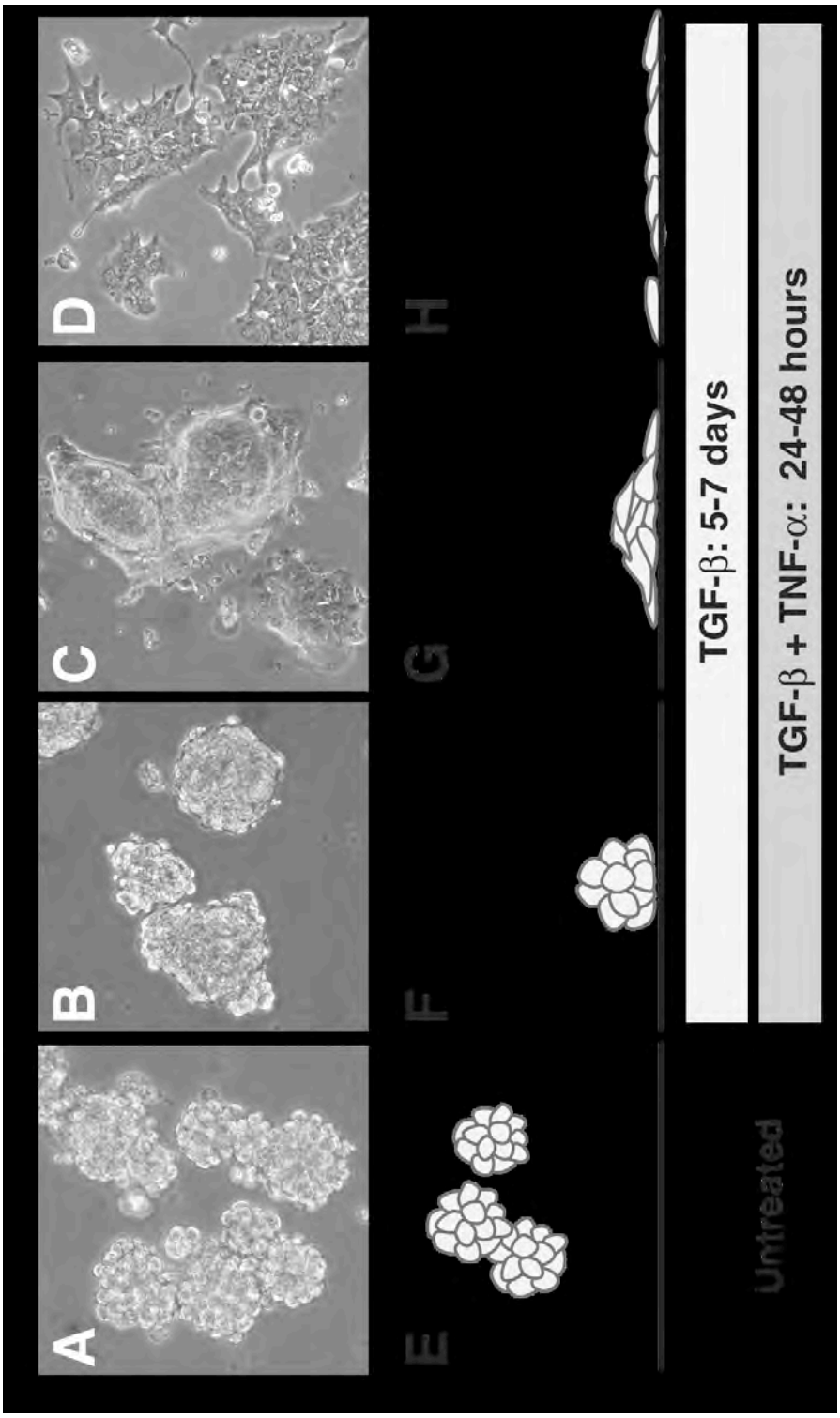
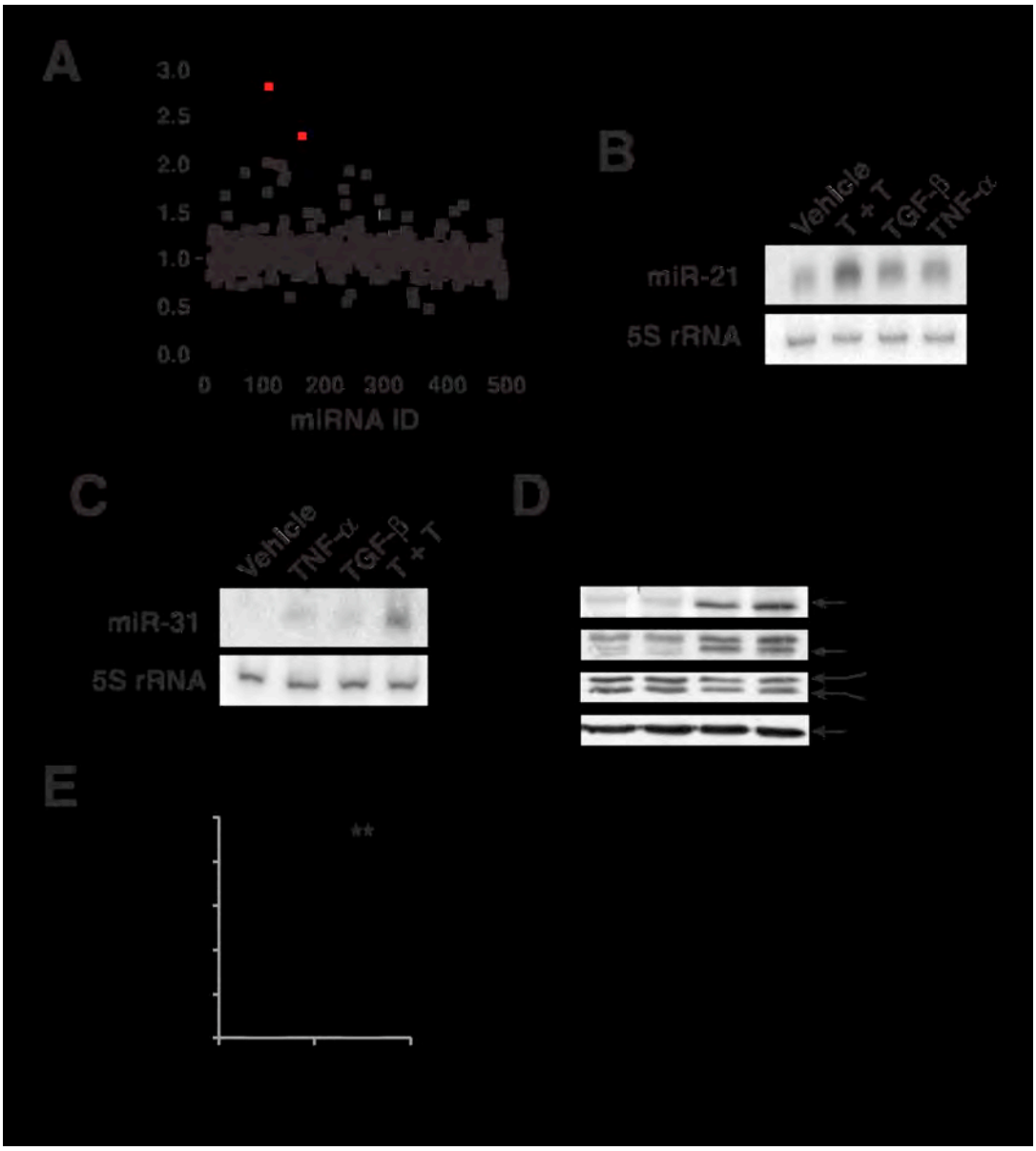


Figure 2.1 | TGF-β induces morphological changes in LIM 1863 organoids. Phase contrast microscopy **(A-D)** and schematic drawings **(E-H)** show the alterations in phenotype that LIM 1863 organoids undergo following TGF-β treatment. **(A and E)** In the absence of TGF-β, LIM 1863 cells propagate as non-adherent organoids. **(B and F)** The addition of 2ng/ml TGF-β1 to the culture media stimulates LIM 1863 organoids to adhere to tissue culture plate **(C and G)**, spread (**D and H)**, and adopt a flattened and migratory mesenchyme-like phenotype. These changes in morphology occur within 5-7 days after TGF-β stimulation and can be accelerated to occur within 24-48 hours when LIM 1863 organoids are treated with a combination of 2 ng/ml TGF-β1 and 10 ng/ml TNF-α; TNF-α alone does not alter morphology. All microscopy images were acquired at 10X magnification.

miR-21 and miR-31 are Induced Synergistically by TGF-β and TNF-α in LIM 1863 organoids

We became interested in whether miRNAs may play a role in regulating EMT of LIM 1863. Microarray profiling revealed miR-21 and miR-31 as the most elevated miRNAs after treatment of TGF-β and TNF-α (Fig. 2.2A); other differentially expressed miRNAs are listed in Table 2.1 and a comprehensive dataset of differentially expressed miRNAs is provided in the Appendix. Northern blotting further validated the induction of the mature forms of these two miRNAs by TGF-β/TNF-α (Fig. 2.2B and 2.2C). For both miR-21 and miR-31, co-treatment with TGF-β and TNF-α was more robust than each cytokine individually in elevating the levels of these two miRNAs (Fig. 2.2B and 2.2C). Given the synergistic effect of TGF-β/TNF-α in both the upregulation of miR-21/miR-31 and EMT, we reasoned that the increase in miR-21/miR-31 may have functional relevance to EMT of LIM 1863 organoid, and focused on these two miRNAs for further studies. The signal transduction of TGF-β in LIM 1863 cells appeared to be normal, as TGF-β induced a rapid C-terminal phosphorylation in both Smad2 and Smad3, and activated the transcription of a typical target gene, Smad7 (Fig. 2.2D and 2.2E).

Figure 2.2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3.0
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B
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T + T
TGF-β
TNF-α
miR-21
5S rRNA
C
Vehicle
TNF-α
TGF-β
T + T
miR-31
5S rRNA
D
E
**

Figure 2.2 | miR-21 and miR-31 are upregulated during TGF-β/TNF-α-induced EMT in LIM 1863 organoids. (A) LNA-based microarray was used to measure the relative ratio of each miRNA in LIM 1863 organoids with or without TGF-β/TNF-α (T + T) treatment. A total of 455 miRNAs were profiled. miR-21 and miR-31 were most significantly upregulated by TGF-β/TNF-α. **(B and C)** Northern blot analyses detecting miR-21 **(B)** or miR-31 **(C)** in LIM 1863 organoids with indicated cytokine treatment for 24 h. The ~21nt mature miRNAs (miR-21 or miR-31) are shown. 5S rRNA expression was used as an internal control. **(D)** Extracts from LIM 1863 cells after indicated cytokine treatments were analyzed by Western blotting using indicated antibodies. **(E)** Real-time quantitative RT-PCR analysis of Smad7 mRNA levels in LIM 1863 cells after vehicle or TGF-β/TNF-α (T+T) stimulation (mean $\pm$ S.D., **, $p < 0.01$). U6 snRNA was used as the internal standard.

Table 2.1 | Differentially expressed miRNAs during EMT in LIM 1863 cells.

	MicroRNA	Cytokine / Vehicle		
		TNF-α	TGF-β	T + T
Up	miR-21	1.58	1.75	2.80
	miR-31	1.12	1.08	2.26
	miR-202-3p	1.97	1.92	2.02
	miR-22	1.17	1.15	2.00
	miR-23a	1.19	1.37	1.98
	miR-492	1.14	1.33	1.93
	miR-517-3p	1.92	1.81	1.89
	miR-27a	1.09	1.17	1.87
	miR-24	1.14	1.29	1.82
Down	miR-487b	0.58	0.57	0.53
	miR-586	0.55	0.56	0.52

miR-21 and miR-31 are Important Regulators in TGF-β-induced EMT of LIM 1863 cells

TGF-β-induced EMT in LIM 1863 is a slow process. By the first 24 hours, most organoids attached to the tissue culture plate, but only a small percentage of them began to spread out into a monolayer (Fig. 2.3A). By counting the organoids exhibiting either a "spreading" or "not spreading" morphology, we quantified TGF-β-induced EMT and evaluated the impact of miR-21 and miR-31 overexpression. Indeed, when examined 24 hours after TGF-β addition, LIM 1863 organoids transfected with the precursors of either miR-21 or miR-31 had a significantly higher percentage adopting a "spreading" morphology, compared to organoids transfected with a non-targeting control miRNA precursor (Fig. 2.3B). The same was observed 48 hours after TGF-β stimulation (Fig. 2.3B). Interestingly if the TGF-β-containing media was removed after 8 hours of treatment and replaced with fresh media containing TGF-β, the percentage of spreading organoids was greatly reduced in cells that overexpressed miR-31, but not miR-21 or control cells (Figs. 2.4A and 2.4B), suggesting that in the presence of TGF-β, increased levels of miR-31 leads to the secretion of factors that are important for the induction of spreading by TGF-β. In line with this result, conditioned media from cells that overexpressed miR-31 caused alterations in cell morphology in naïve LIM 1863 organoids (Fig. 2.4C). Together, these data suggest that miR-21 and miR-31 are able to accelerate the EMT process initiated by TGF-β.

Figure 2.3

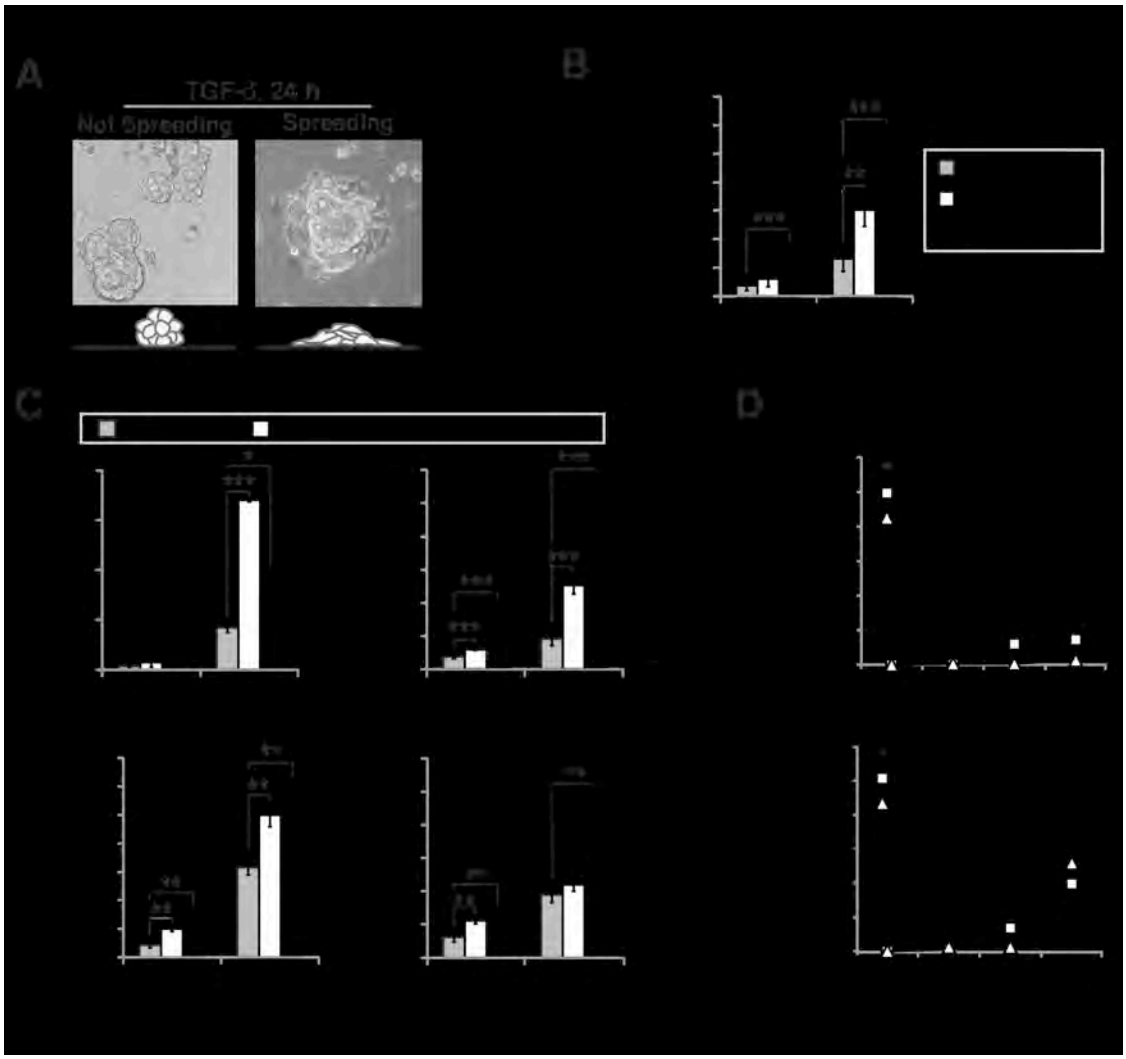

Figure 2.3 | TGF-β-induced LIM 1863 morphological changes is potentiated by miR-21 and miR-31. (A) Representative LIM 1863 organoids exhibiting "spreading" or "not spreading" morphologies after 24 h TGF-β treatment (upper). A schematic drawing of "spreading" and "not spreading" morphologies is shown (lower). **(B)** LIM 1863 organoids transfected with indicated miRNA precursors were stimulated with TGF-β, and the morphology was scored as "spreading" or "not spreading" at 24 h or 48 h post TGF-β addition. The results are plotted (mean $\pm$ S.D., > 200 organoids were counted in each experiment, data represent >3 experiments). **(C)** Same experiment as in *B* (only the 24 h time point), and plotted are the mRNA levels of indicated markers as measured by quantitative real-time PCR (mean $\pm$ S.D, n>3), using U6 snRNA as an internal reference. **(D)** Inhibition of miR-21 and miR-31 activities suppresses TGF-β/TNF-α-induced EMT marker expression. LIM 1863 cells were transfected with 250 nM 2'O-Methyl RNA inhibitors of miR-21, miR-31 or control. Eighteen hours later, cells were treated with TGF-β/TNF-α as indicated after which LAMC2 and MMP7 expression was evaluated by quantitative real-time PCR, using U6 snRNA as an internal control (mean $\pm$ S.D, n>3). FN1: fibronectin 1; IL8: interleukin 8; LAMC2: laminin γ2; MMP7: matrix metalloproteinase 7. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Figure 2.4

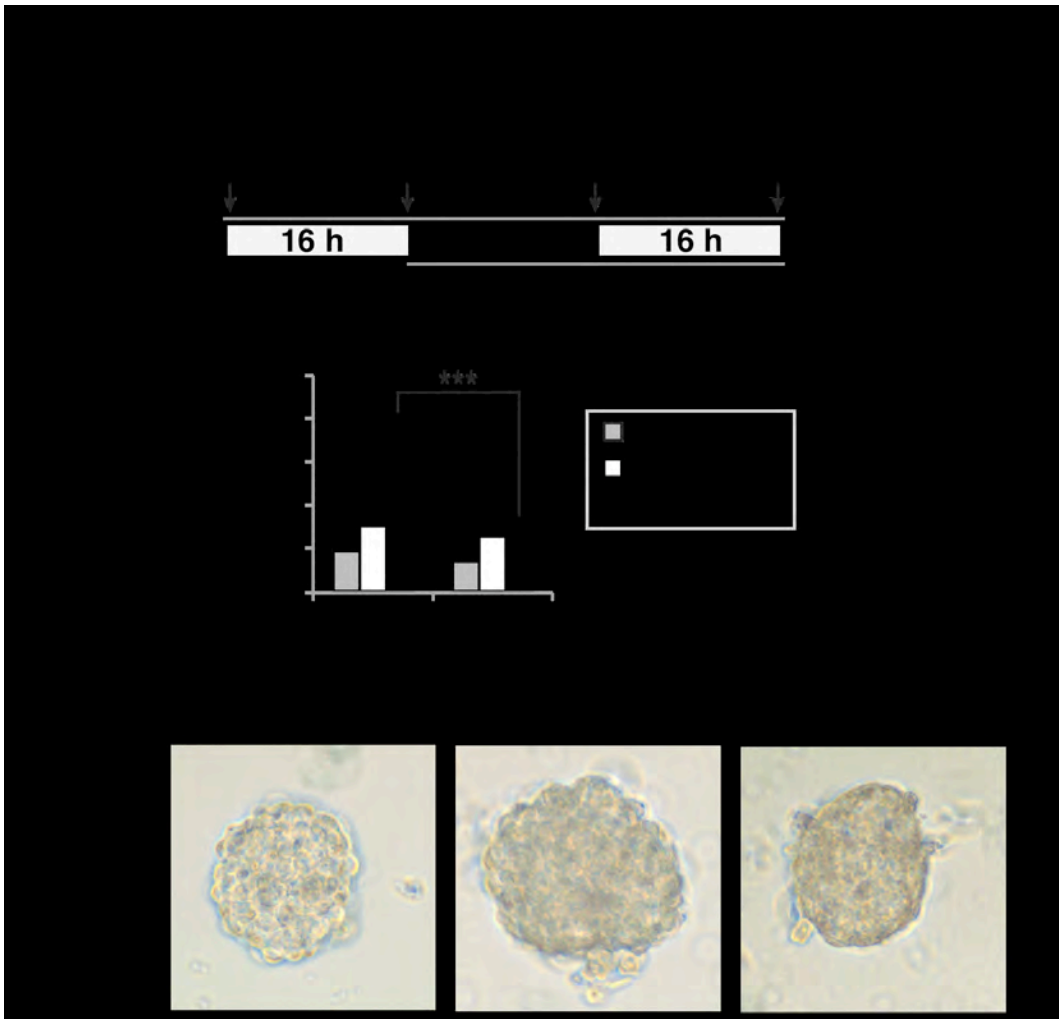

Figure 2.4 | miR-31 stimulates secretion of factors that facilitate TGF-β induction of EMT. (A) Schematic of the experimental timeline. **(B)** LIM 1863 organoids transfected with indicated miRNA precursors were stimulated with TGF-β. Eight hours post stimulation, the media was replaced with fresh media containing TGF-β. This is the "wash" sample. In the "mock" samples, the organoids remained in the same TGF-β-containing media throughout the experiment. After 24 hours from initial addition of TGF-β, the morphology was scored as "spreading" or "not spreading." The results are plotted (mean $\pm$ S.D., > 200 organoids were counted in each experiment, data represent >3 experiments). **(C)** LIM 1863 organoids were cultured for 72 hours in conditioned media (CM) from LIM 1863 organoids overexpressing miR-21, miR-31 or control and images were captured at 20X magnification.

To better quantify the EMT process, we measured the mRNA levels of a number of EMT markers. In our LIM 1863 culture, TGF-β/TNF-α induced re-distribution of E-cadherin from the cell surface to the cytoplasm without much change in its protein level, indicating that in our hands these cells do not undergo a classical EMT (Fig. 2.5). We measured the expression of other known markers including fibronectin-1 (FN1), interleukin-8 (IL-8), laminin-γ2 (LAMC2) and matrix metalloproteinase-7 (MMP7) (Bates et al., 2004; Vincan et al., 2007b). The levels of these markers were all increased 24 hours after TGF-β treatment (Fig. 2.3C). Transfection of either miR-21 or miR-31 precursors further potentiated TGF-β in increasing the mRNA levels of FN1, IL8 and LAMC2 (Fig. 2.3C). In the case of MMP7, only miR-31 had a significant effect (Fig. 2.3C). Even in cells without any exposure to TGF-β, either miR-21 or miR-31 elevated the expression of these EMT markers (Fig. 2.3C) and interleukin-6 (IL-6) (Fig. 2.6), a cytokine whose increased expression correlates with colon carcinoma metastases (Knüpfer and Preiss, 2010). These more quantitative analyses further substantiated our conclusion that a high level of miR-21 or miR-31 facilitates TGF-β-induced EMT of LIM 1863. Importantly, TGF-β-induced upregulation of Smad7 was not enhanced by either miR-21 or miR-31 overexpression, suggesting that these two miRNAs only facilitate a subset of the TGF-β responses, and do not enhance TGF-β signaling in general (Fig. 2.7).

Figure 2.5

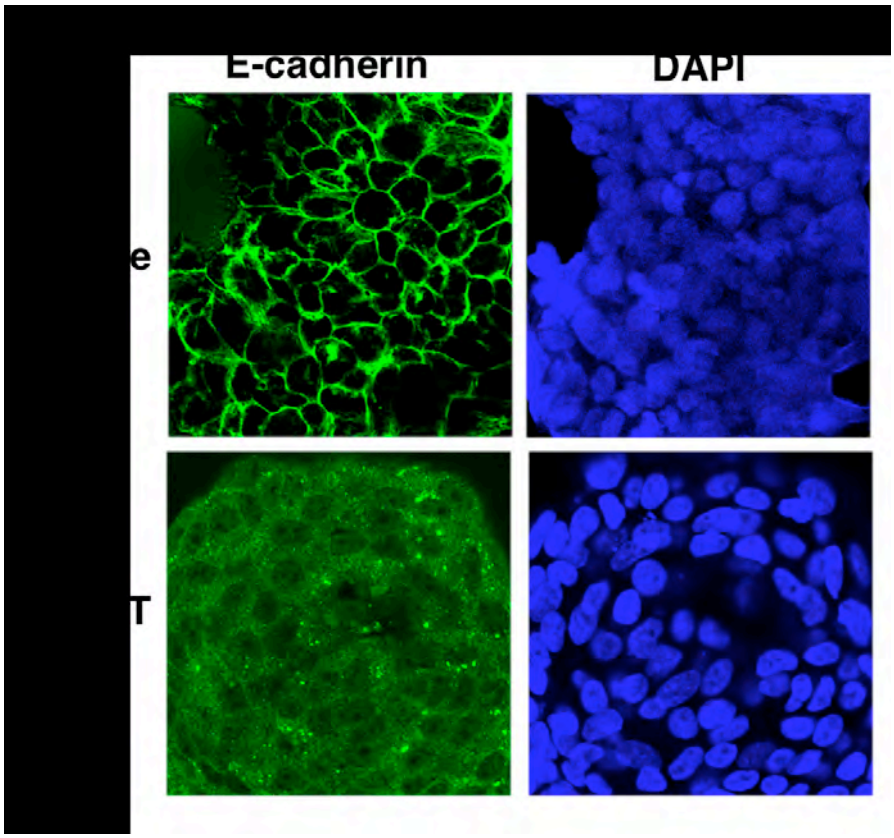

Figure 2.5 | TGF-β/TNF-α alters E-cadherin localization in LIM 1863. LIM 1863 organoids were treated with TGF-β and TNF-α (T + T) or without (Vehicle) for 24 hours and the distribution pattern of E-cadherin was detected with anti-E-cadherin antibodies. Nuclei were stained with DAPI.

Figure 2.6

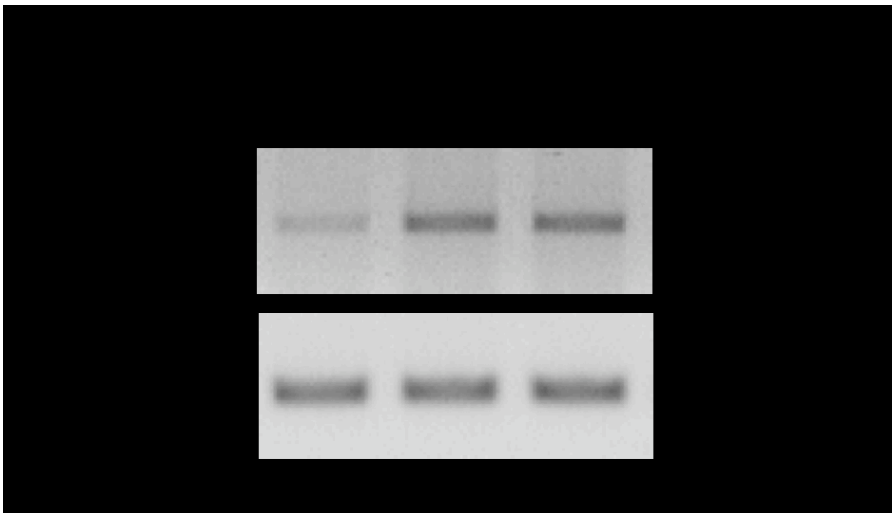

Figure 2.6 | IL-6 is upregulated by miR-21 and miR-31. LIM 1863 organoids were transfected with indicated miRNA precursors. After 48 hours, RNA was collected and reverse transcribed. Standard PCR was performed with the resultant cDNA to evaluate the expression of IL-6 using forward primer, 5'- ATGAACTCCTTCTCCACAAGCGC -3' and reverse primer, 5'- GAAGAGCCCTCAGGCTGGACTG -3'. GAPDH levels were determined as an internal reference using forward primer 5'-AACAGCCTCAAGATCAGCAA-3' and reverse primer, 5'-CAGTCTGGGTGGCAGTGAT-3'.

Figure 2.7

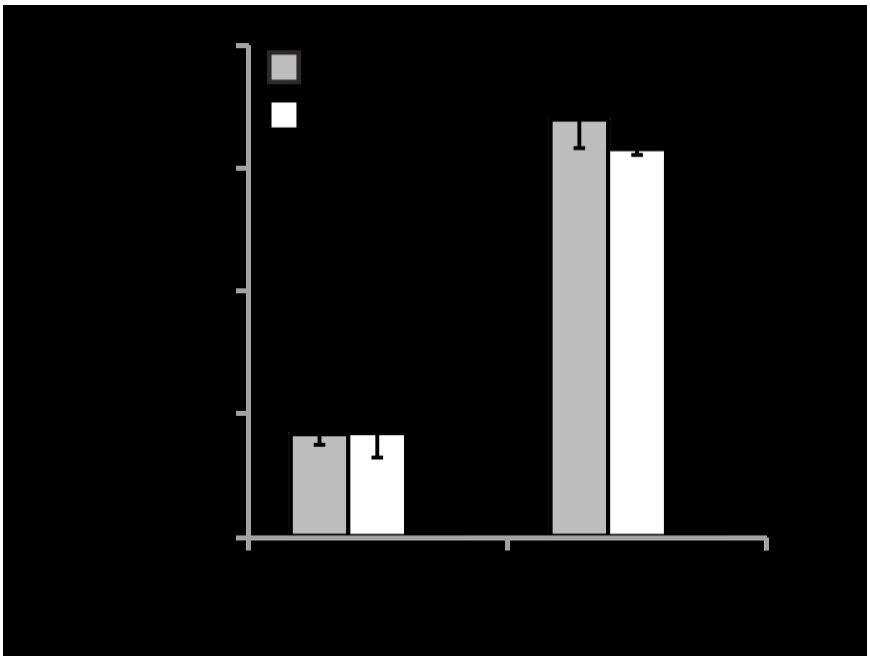

Figure 2.7 | TGF-β regulation of Smad7 is not affected by miR-21 or miR-31. LIM 1863 organoids transfected with indicated miRNA precursors were stimulated with TGF-β for 24 h and Smad7 expression was measured by quantitative real-time PCR (mean + S.D, n>3), using U6 snRNA as an internal standard.

We further carried out loss-of-function studies to evaluate the importance of miR-21 and miR-31 in TGF-β/TNF-α-induced EMT of LIM 1863 cells. Indeed, when miR-21 or miR-31 activity was neutralized by antisense 2'O-methyl RNA oligonucleotides, TGF-β/TNF-α induction of the mesenchymal markers LAMC2 and MMP7 was substantially decreased (Fig. 2.3D). This further supported the requirement of miR-21 and miR-31 in EMT-associated morphological changes induced by TGF-β/TNF-α in LIM 1863 cells.

miR-21 and miR-31 Regulate the Migration and Invasion of LIM 1863 cells

EMT is often linked to a gain in the migratory and invasive properties of the cell. Even though the LIM 1863 organoids hardly migrated in the standard transwell assay, we found that if cells were immediately plated after dissociation of the organoids by trypsin, there was migration in the transwell assay with 10% FBS and media from NIH 3T3 culture as the chemoattractant (Fig. 2.8A). The same was observed in the Matrigel invasion assay (Fig. 2.8B). When LIM 1863 cells transfected with miR-21 or miR-31 precursors were tested in these assays, they exhibited significantly enhanced migratory and invasive properties compared to cells transfected with a control miRNA precursor (Figs. 2.8A and 2.8B). Interestingly, corroborating the results in Matrigel invasion assays, we noticed that when plated onto the Matrigel filter, LIM 1863 cells transfected with miR-21 or miR-31 readily adhered and spread out, whereas cells transfected with a control miRNA precursor did not show such characteristics (Fig. 2.8B, phase contrast images). These observations support the notion that an increase in miR-21 or miR-31 enhances the migration and invasion properties of LIM 1863 cells.

Figure 2.8

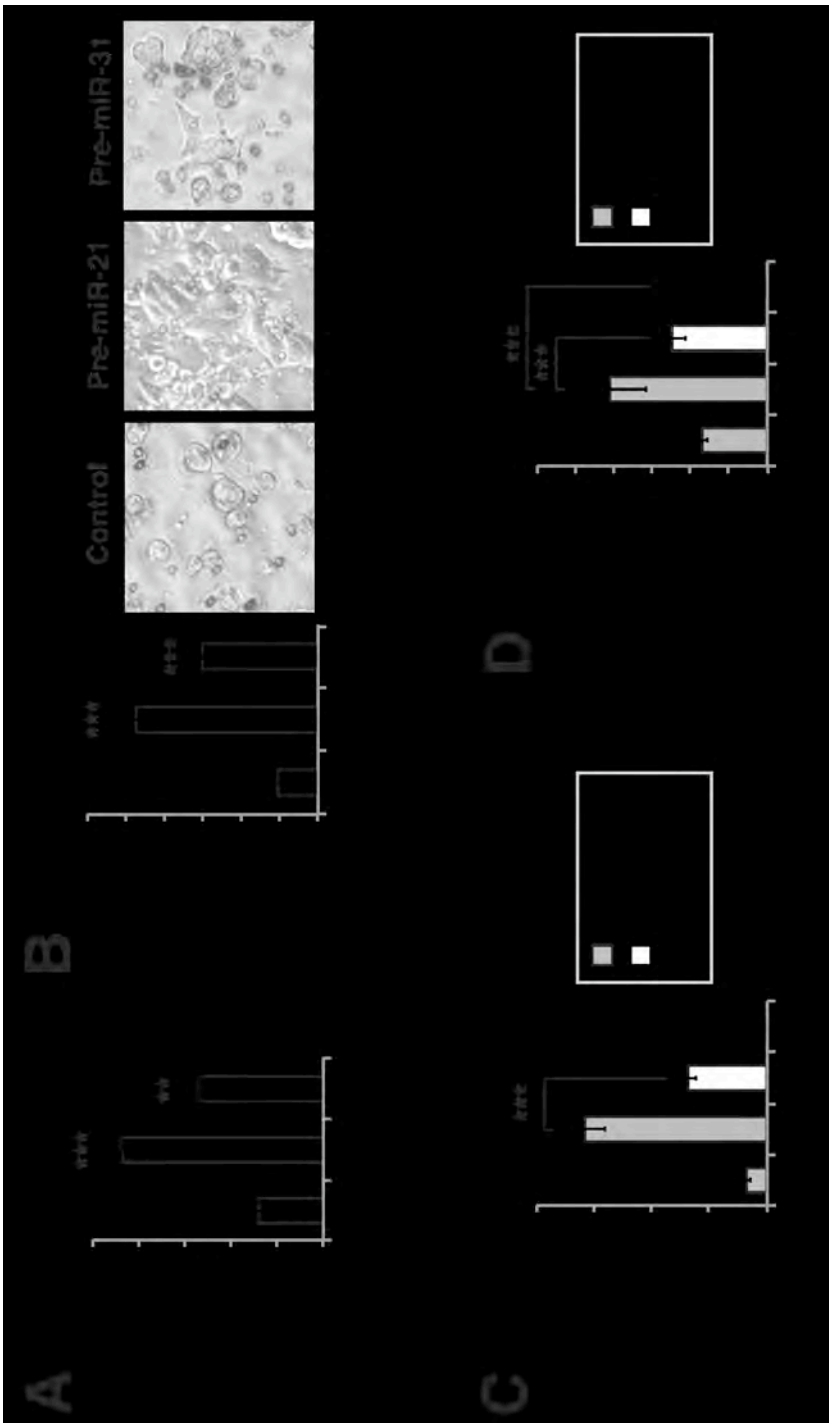

Figure 2.8 | miR-21 and miR-31 regulate LIM 1863 migration and invasion. (A and B) LIM 1863 organoids were transfected with 100 nM miR-21 or miR-31 precursors or a negative precursor control (C). Forty-eight hours later, the organoids were dissociated by trypsin and 1 x 10^5 cells were seeded into the upper wells of transwell chambers coated without **(A)** or with Matrigel **(B)**. After 72 h, cells that migrated to the lower chambers were counted (mean $\pm$ S.D., 8 fields per filter were examined per experiment). LIM 1863 cells transfected with miR-21 and miR-31 precursors spontaneously attached and spread on Matrigel-coated filters (phase contrast images in *B*, right). **(C and D)**, Inhibition of miR-21 and miR-31 activities affects TGF-β//TNF-α-induced LIM 1863 cell migration and invasion. LIM 1863 cells were transfected with 250 nM 2'O-Methyl RNA inhibitors of miR-21, miR-31 or control. Eight hours post-transfection, cells (5 x 10^5 for migration and 1x10^6 for invasion assay) were seeded into the upper wells of transwell chambers coated without **(C)** or with Matrigel **(D)** and treated with TGF-β//TNF-α (T+T) or without. After 72 h, the number of cells that migrated to the lower chamber was counted (mean $\pm$ S.D., 8 fields per filter were examined). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

If the dissociated LIM 1863 cells were treated with TGF-β/TNF-α upon plating into the transwells, the motility was markedly increased (Fig. 2.8C). Importantly, when miR-21 function was inhibited by the 2'*O*-Methyl anti-miR-21 oligonucleotide, TGF-β/TNF-α-induction of LIM 1863 motility was significantly reduced (Fig. 2.8C). This suggests that miR-21 is required for TGF-β/TNF-α-regulation of LIM 1863 cell migration. In contrast, we observed little effect with anti-miR-31, suggesting miR-31 is not as rate-limiting as miR-21 is in regulating LIM 1863 motility. In Matrigel assays, TGF-β/TNF-α also stimulated invasion of the LIM 1863 cells (Fig. 2.8D). Interestingly, inhibition of either miR-21 or miR-31 significantly decreased LIM 1863 invasion in response to TGF-β/TNF-α, so both of these two miRNAs have non-overlapping targets that are important for LIM 1863 to invade through the extracellular matrix (Fig. 2.8D). From these gain- and loss- of function studies, we conclude that both miR-21 and miR-31 positively regulate migration and invasion properties of the LIM 1863 cancer cells.

Pro-migration and Pro-invasion Functions of miR-21 and miR-31 in Other Colon Cancer Cell Lines

To determine if the activities of miR-21/miR-31 we observed so far are unique to LIM 1863 cells, we overexpressed these two miRNAs in SW480 and DLD1 colon cancer cell lines. Since in our hands these two cell lines do not undergo EMT in response to TGF-β, we only evaluated their motility and invasion. Indeed in both cell lines, transfection of miR-21 and miR-31 precursors resulted in a marked increase in cell migration and invasion (Fig. 2.9A and 2.9B). Given that SW480 cells do not express SMAD4 and experiments in Figure 2.9 were all done without TGF-β stimulation, our observations also suggest that the pro-metastasis activities of miR-21 and miR-31 do not depend on a functional TGF-β/SMAD pathway. Interestingly, under exactly the same conditions, miR-31 overexpression substantially suppressed motility and invasion of a breast cancer cell line MDA-MB-231 (Fig. 2.9C), consistent with previous reports of an anti-metastasis function of miR-31 in breast cancer (Valastyan et al., 2009; Valastyan et al., 2010). Therefore, the impact of miR-31 on cancer cell migratory properties is dependent on cell context.

All of the above evidence points to pro-cancer invasion and metastasis functions of miR-21 and miR-31 in multiple colon cancer cell lines. These results also agree with previous studies of colon cancer tissues in which higher levels of miR-21 and miR-31 have been linked to colon cancer progression to a late stage and metastasis (Bandrés et al., 2006; Slaby et al., 2008).

Figure 2.9

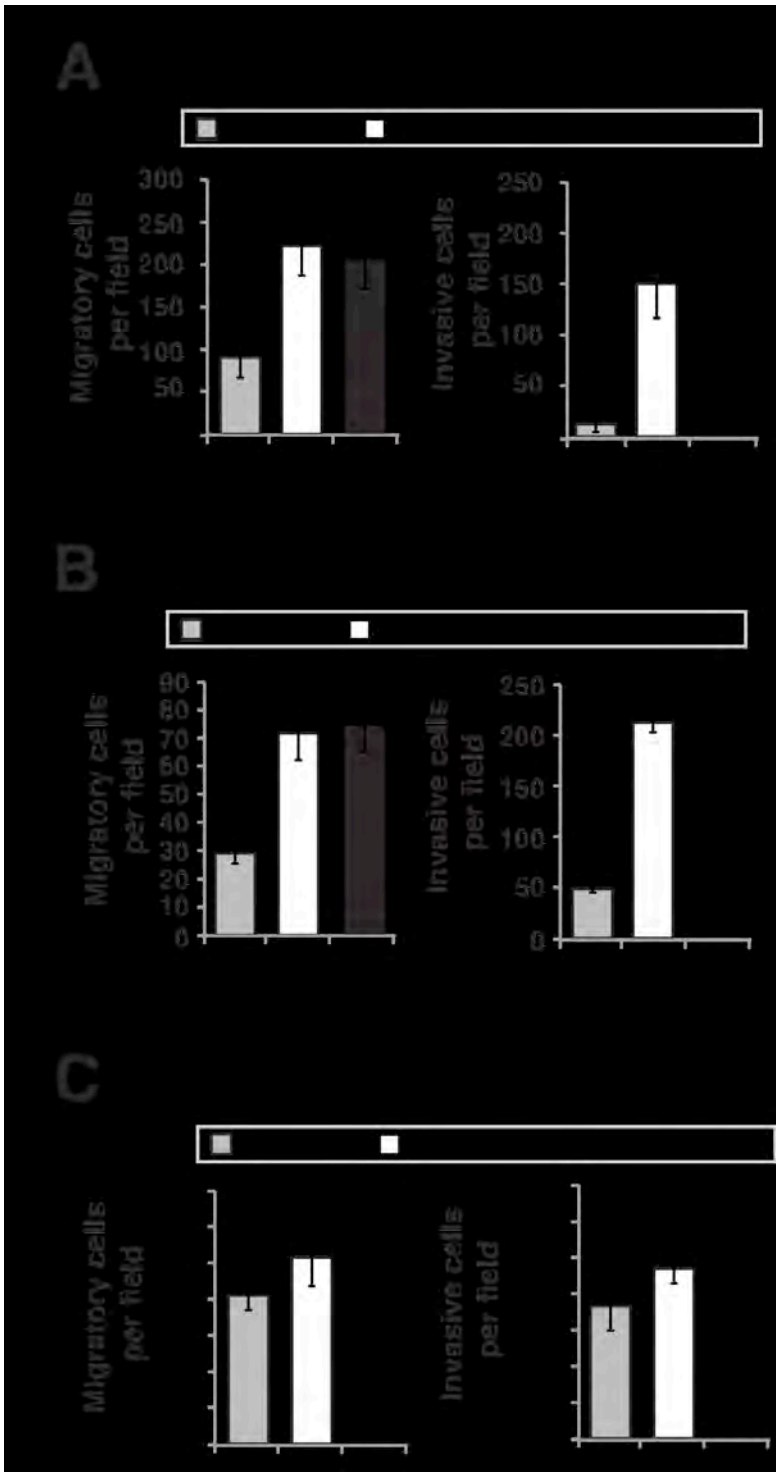
A
Migratory cells per field
300
250
200
150
100
50
Invasive cells per field
250
200
150
100
50
B
Migratory cells per field
90
80
70
60
50
40
30
20
10
0
Invasive cells per field
250
200
150
100
50
0
C
Migratory cells per field
Invasive cells per field

Figure 2.9 | Context-dependent pro-migration and pro-invasion activities of miR-21 and miR-31 in colon and breast cancer cells. (A-C), Control, miR-21 or miR-31 precursors (100 nM) were transfected into DLD1 **(A)**, SW480 **(B)** or MDA-MB-231 **(C)** cells. The motility and invasiveness of these cells were analyzed by transwell migration and invasion assays as in Fig. 2.8A and 2.8B. Plotted is the number of cells that migrated to the lower chamber was counted (mean $\pm$ S.D., >3 fields per filter were examined, **, $p < 0.01$; ***, $p < 0.001$).

TIAM1 is a Target for Both miR-21 and miR-31

One critical question is the downstream targets of miR-21 and/or miR-31 that contribute to the cellular impact of these two miRNAs. Using TargetScan to search for 3'-UTR sequences with 7 nucleotide matches to the seed region of miR-21 or miR-31, TIAM1 (T lymphoma invasion and metastasis 1) emerged as a possible target for miR-21 (Lewis et al., 2005). Upon visual inspection we found weak recognition sites for miR-31 (e.g. a 6 nucleotide match) in the TIAM1 3'-UTR as well. TIAM1 is a guanine nucleotide exchange factor (GEF) of Rac, and has been implicated in regulating cell migration, invasion and tumor progression (Habets et al., 1994; Mertens et al., 2003; Minard et al., 2006). Indeed, treating the LIM 1863 organoids with TGF-β/TNF-α substantially reduced the protein level of TIAM1 (Fig. 2.10A) and this downregulation of TIAM1 by TGF-β/TNF-α was not observed when organoids were transfected with inhibitors targeting both miR-21 and miR-31 (Fig. 2.11). These observations further prompted us to investigate TIAM1 as a possible target of miR-21 and/or miR-31. Transfection of LIM 1863 cells with precursors for either miR-21 or miR-31 resulted in markedly decreased abundance of TIAM1 protein when compared to the non-targeting miRNA precursor control (Fig. 2.10B). Quantitative real-time PCR revealed no change in the mRNA level of TIAM1 by either miR-21 or miR-31 (Fig. 2.12). Thus miR-21 and miR-31 appear to control TIAM1 expression mainly through repression of the protein translation, not degradation of the mRNA. Consistent with the functional data in Figure 2.9, miR-21 and miR-31 also downregulated TIAM1 in DLD1 and SW480 cells (Fig. 2.10C).

Figure 2.10

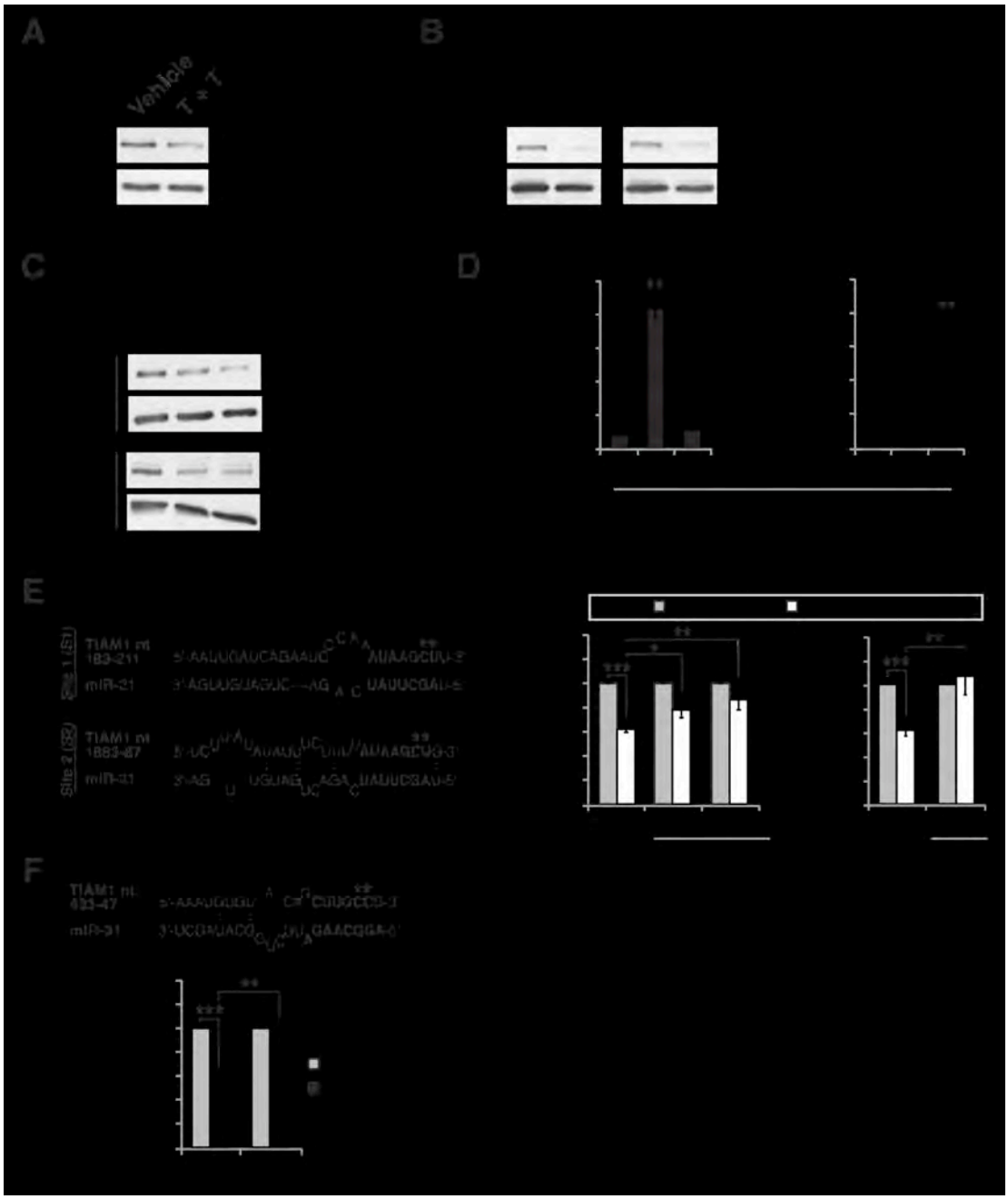

Figure 2.10 | TIAM1 is an endogenous target of both miR-21 and miR-31. (A) LIM 1863 organoids were treated with TGF-β/TNF-α (T+T) for 24 h and Western blotting was performed to determine TIAM1 levels (C16, Santa Cruz), with β-tubulin as a loading control. *B-C*, miR-21 and miR-31 downregulate TIAM1 protein abundance in multiple colon cancer cell lines. LIM 1863 **(B)**, DLD1 **(C)** or SW480 **(C)** cells were transfected with 100 nM miR-21 or miR-31 precursors, or a negative control precursor. After 72 h, TIAM1 protein level was measured by Western blotting. **(D)**, LIM 1863 cells were transfected with 100 nM indicated miRNA precursors and 48 h later, the levels of mature miR-21 and miR-31 were determined by the TaqMan miRNA assay (mean $\pm$ S.D., n>3). **(E)**, (*left*) Alignment of TIAM1 3'-UTR with the miR-21 sequence. The numbering starts from the first residue after the stop codon. Asterisks indicate the nucleotides that were mutated (CT->TG for both site 1 and site 2 mutations). (*right*), miR-21 directly targets the 3'-UTR of TIAM1. LIM 1863 cells were transfected first with 100 nM of miR-21 precursor or a control. Eighteen hours later the same cells were transfected with a *Renilla* luciferase reporter containing the wild type or mutant TIAM1 3'UTR (e.g. S1: site 1 mutation; S2: site 2 mutation; S1/2: double mutation). A constitutively active firefly luciferase reporter was used as the internal control. The luciferase activities were measured and plotted (mean $\pm$ S.D., n>3). *, $p < 0.05$.**(F)**, Similar experiments as in (*E*), analyzing the inhibitory impact of miR-31 on the 3'UTR of TIAM1. The mutated TIAM1 3'-UTR residues are marked by "*" (CC->TT), the numbering is the same as in (*E*). **, $p < 0.05$; ***, $p < 0.001$.

Importantly, we found that transfection of miR-21 did not cause a significant change in mature miR-31 level, and vice versa (Fig. 2.10D). Therefore, miR-21 and miR-31 do not influence the expression levels of each other and likely impact the expression of TIAM1 independently.

To further validate whether TIAM1 is a direct target of miR-21 or miR-31, we engineered a luciferase reporter construct containing the 1970-bp 3'-UTR of the TIAM1 gene. Indeed the miR-21 precursor significantly reduced the reporter expression (Fig. 2.10E). We identified two potential miR-21 target sites (matching 7 nt of the miR-21 seed sequence) in the TIAM1 3'-UTR (Fig. 2.10E, left) that are conserved across seven species (Fig. 2.13). Mutation of either one of the two potential miR-21 target sites in the TIAM1 3'UTR reduced, but did not fully abolish the suppression by miR-21 (Fig. 2.10E, left). Only when both sites were mutated did the miR-21 precursor completely fail to repress the reporter expression (Fig. 2.10F, right). These data strongly support the hypothesis that TIAM1 is a direct target of miR-21 in LIM 1863 cells. Transfection of miR-31 precursor also significantly reduced the TIAM1 3'-UTR reporter (Fig. 2.10F). However, we could only find one stretch of 6 nucleotides complementing the miR-31 seed sequence (Fig. 2.10F), which is poorly conserved (Fig. 2.13). Nevertheless, mutation of this sequence significantly alleviated the repression by miR-31 (Fig. 2.10F). This strongly suggests that miR-31 also directly represses TIAM1 translation. The remaining inhibitory effect of miR-31 may be due to additional cryptic sites within the TIAM1 3'-UTR or other indirect mechanisms.

Figure 2.11

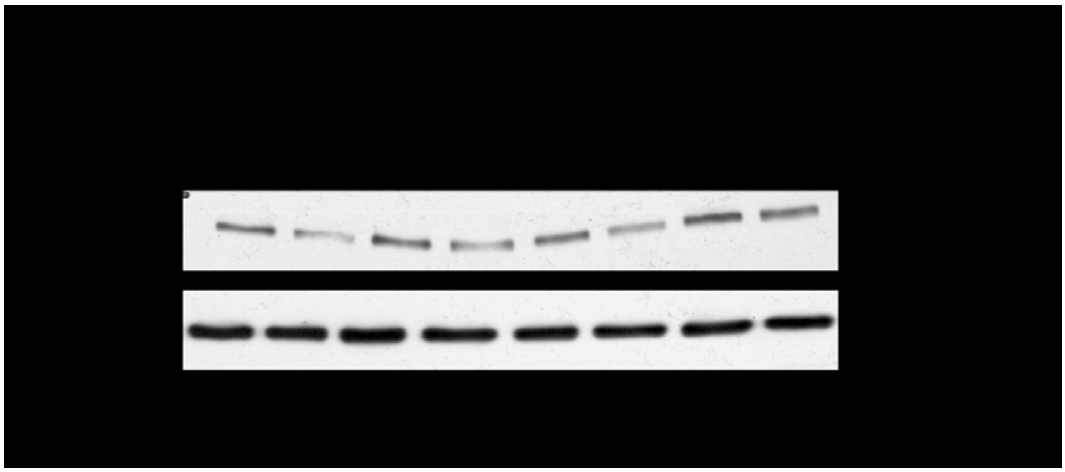

Figure 2.11 | Inhibition of both miR-21 and miR-31 negate TGF-β/TNF-α downregulation of TIAM1. LIM 1863 cells were transfected with 125 nM 2'O-Methyl RNA inhibitors (2'OMe) targeting miR-21, miR-31 or a negative control that targets eGFP. Eighteen hours later, cells were treated with TGF-β/TNF-α (T + T). After 24 h, protein lysates were made and western blotting was performed using antibodies to detect TIAM1. β-Tubulin expression was evaluated as a loading control.

Figure 2.12

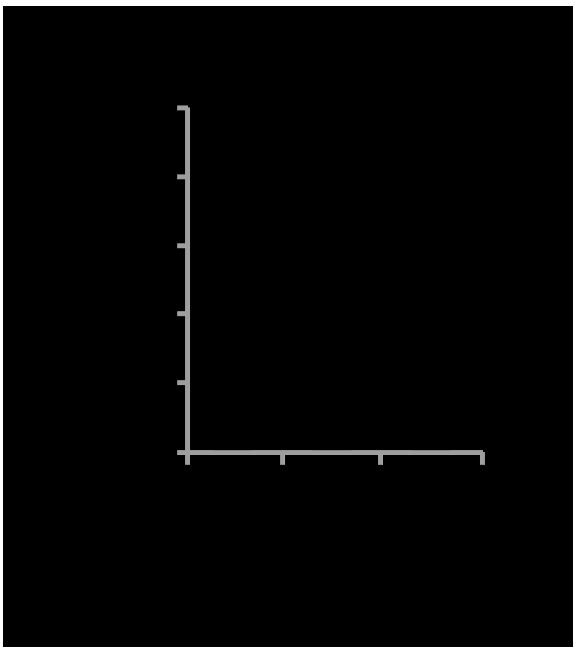

Figure 2.12 | miR-21 and miR-31 have no effect on TIAM1 mRNA expression. miR-21 and miR-31 has no effect on TIAM1 mRNA expression. Total RNA was isolated from LIM 1863 cells (as in Fig 3B) and the relative expression of TIAM1 was determined by real-time PCR, using U6 snRNA as an internal reference (mean + S.D., data represents 4 experiments).

Figure 2.13

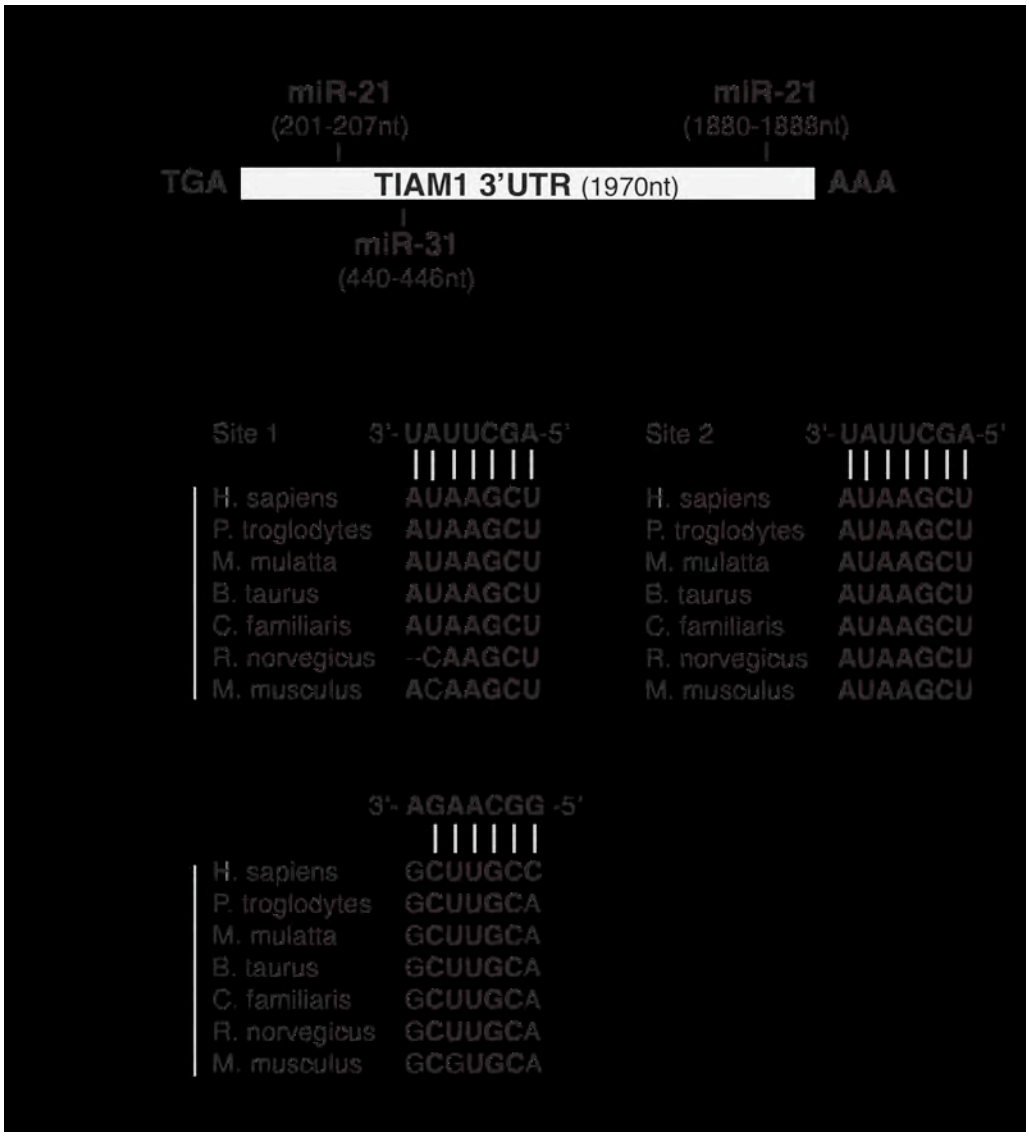

Figure 2.13 | Species conservation of the miR-21 and miR-31 sites in the TIAM1 3'UTR. *A,* Schematic of the human TIAM1 3'UTR. The relative location of the miR-21 and miR-31 binding sites are noted. Alignment of binding sites for miR-21, *B,* and miR-31, *C,* in the TIAM1 3'UTR in human, chimpanzee, rhesus macaque, cow, dog, rat and mouse.

Repression of TIAM1 Expression is Important for Pro-metastasis Functions of miR-21 and miR-31 in LIM 1863 cells

In order to validate whether TIAM1 is a relevant factor in LIM 1863 morphological changes in response to TGF-β/TNF-α, we introduced exogenous TIAM1 through a lentiviral vector (Fig. 2.14A, left) in order to override its suppression by miR-21 and miR-31. Ectopic TIAM1 expression did not cause any noticeable changes in the morphology of LIM 1863 organoids at basal state (Fig. 2.14A, right). However, upon TGF-β/TNF-α treatment, TIAM1-overexpressing LIM 1863 organoids completely failed to undergo morphological changes as the control cells did (Fig. 2.14B). Moreover, overexpression of TIAM1 also prevented TGF-β/TNF-α from enhancing motility and invasiveness of LIM 1863 cells (Figs. 2.14C and 2.14D). Therefore, a low level of TIAM1 is important for TGF-β/TNF-α to promote LIM 1863 EMT, migration and invasion.

Next we more directly tested whether the pro-migration and –invasion activities of miR-21 and miR-31 are also dependent on suppression of TIAM1. Indeed, miR-21/miR-31 precursors were no longer capable of enhancing motility and invasiveness of LIM 1863 cells overexpressing TIAM1 (Figs. 2.14E and 2.14F). These data further substantiate our model that repression of TIAM1 is a critical component in miR-21/miR-31 regulation of migratory and invasive properties of LIM 1863 cells.

Figure 2.14

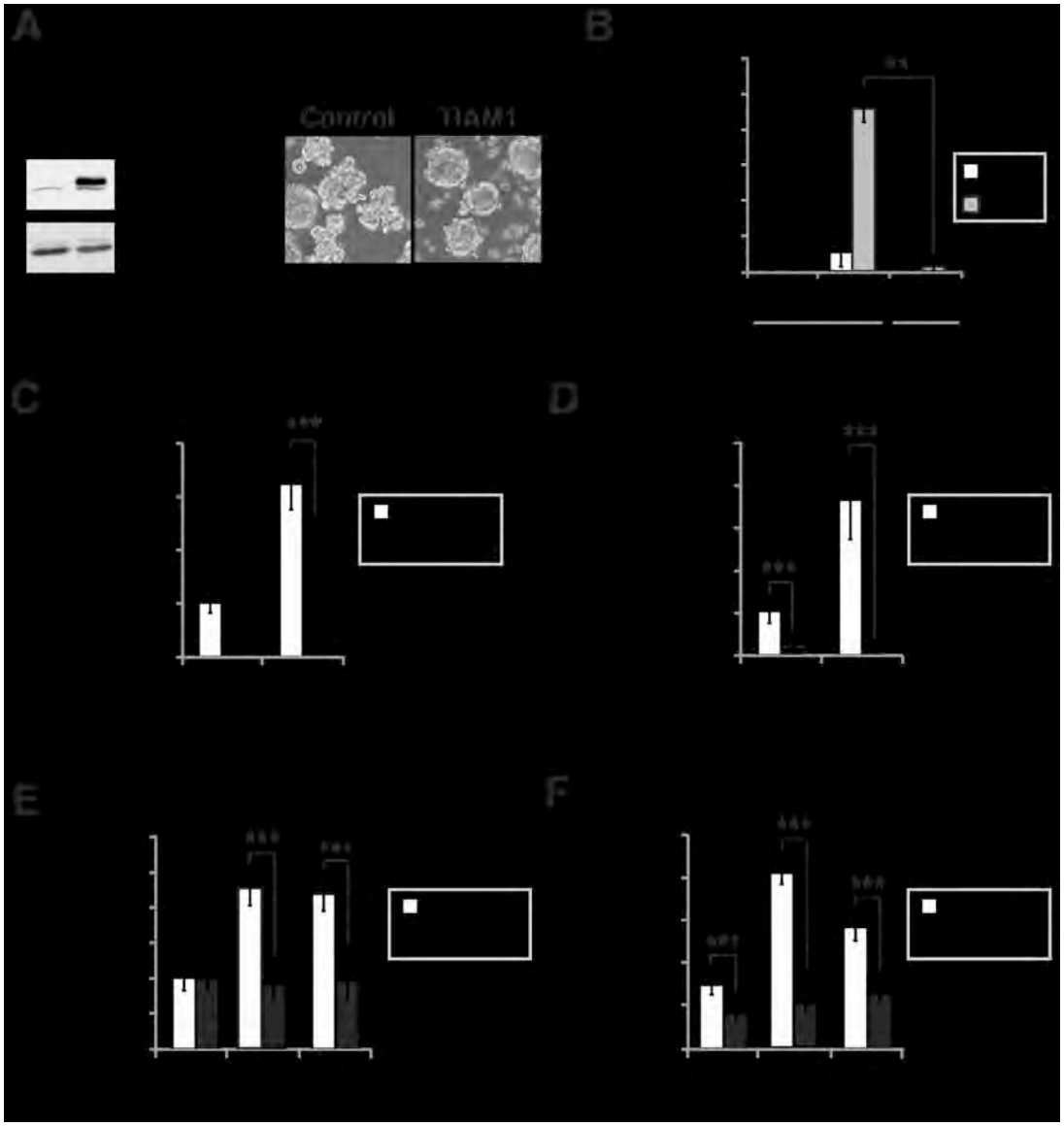

Figure 2.14 | Elevated TIAM1 level antagonizes LIM 1863 morphological changes, motility and invasion in response to TGF-β/TNF-α and miR-21 and miR-31. (A) Phase contrast images showing no obvious morphological changes in LIM 1863 organoids with or without TIAM1 overexpression. **(B)**, TIAM1 overexpression greatly reduced the ability of LIM 1863 cells to undergo EMT. Cells transduced with TIAM1 or empty vector (control) were stimulated with TGF-β/TNF-α (T+T). The morphology of the organoids (n > 75 per group) was scored as "spreading" or "not spreading" as in Fig. 2.3B at 6 h and 24 h time points (mean ± S.D., >75 organoids were counted in each experiment, data represent >3 experiments). **, $p < 0.01$. **(C and D)**, Transwell migration **(C)** and Matrigel invasion **(D)** assays measuring LIM 1863 cell motility and invasiveness, respectively. LIM 1863 cells were transduced with lentiviral vectors encoding TIAM1 or control. Cells (5×10^5 for the migration assay and 1×10^6 for the invasion assay) were then seeded into the upper chambers and treated with vehicle or TGF-β/TNF-α (T+T) as indicated. After 72 h, the number of cells that migrated to the lower chambers was counted (mean ± S.D., 8 fields per filter were examined). ***, $p < 0.001$. **(E and F)**, LIM 1863 cells transduced with TIAM1-expressing or control vectors were further transfected with miR-21 (21), miR-31 (31) or control (C) precursors as indicated. Cells were then analyzed for motility **(E)** and invasion **(F)** (mean ± S.D., 8 fields per filter were examined). ***, $p < 0.001$.

Summary

TGF-β is implicated in advancing carcinomas to malignancy. Our data provides strong evidence for the contribution of miR-21 and miR-31 to the metastasis-promoting utility of TGF-β. In this study, we examined LIM 1863, a 3D organoid culture derived from colon carcinoma and undergoes EMT in response to TGF-β. We observed upregulation of miR-21 and miR-31 during EMT of LIM 1863 organoid. Overexpression as well as inhibition experiments support the contributions of both miR-21 and miR-31 not only in the morphological changes of LIM 1863 in response to TGF-β, but also in cell motility and invasion. Furthermore, we show that TIAM1 (T lymphoma and metastasis gene 1) is a direct target of both miR-21 and miR-31, and that the suppression of TIAM1 is important for the pro-migration and –invasion activities of miR-21 and miR-31. Therefore, we have identified miR-21 and miR-31 as positive regulators of colon carcinoma migratory and invasive properties.

Experimental Procedures

Cell Culture and Cytokine Treatment – LIM 1863 cells and DLD1 were maintained in RPMI 1640 supplemented with 5% fetal or 10% bovine serum (FBS), respectively. 293T, MDA-MB-231 and SW480 cells were maintained in Dulbecco's Modifed Eagle's (DME) media supplemented with 10% FBS. All cell culture media also contained penicillin (100 units/ ml) and streptomycin (100 units/ ml) (Invitrogen). For cytokine treatment, human TGF-β1 (R&D Systems) and human TNF-α (R&D Systems) were used at a final concentration of 2.5 ng/ml and 10 ng/ml, respectively. Cycloheximide (Sigma), was used at a final concentration of 15 μg/ml for 30 min.

miRNA Microarray – Total RNA was extracted using the mirVana^{TM} RNA Isolation Kit (Ambion) according to the manufacturer's instructions from LIM 1863 cells that were treated with both TGF-β and TNF-α media only for 24 h. Total RNA was submitted to Exiqon (Vebaek, Denmark) for miRNA microarray profiling services, in which RNA was labeled with Hy3- and Hy5 fluorophores and hybridized to a miRCURY LNA microRNA Array (version 8.1). All subsequent data analysis was performed by Exiqon.

Northern Blotting – Total RNA was extracted using the mirVana^{TM} RNA Isolation Kit according to manufacturer's instructions (Ambion). Twenty micrograms of total RNA was resolved on denaturing agarose gels and transferred to nylon membranes. Following UV-crosslinking, membranes were incubated with random-primed miR-21 or GAPDH

cDNA probes in Church's hybridization buffer (0.5 M $NaHPO_4$, pH 7.2; 1 mM EDTA; 7% SDS) at 42 ˚C for 18 h. For detecting the miR-21 primary transcript, the probe encompassed 250 bp on both sides of the mature miR-21 sequence. Membranes were washed three times in 2X SSC, 0.1% SDS at room temperature. To detect precursor and mature miRNA species, 20-40 μg of total RNA was resolved on 20% denaturing polyacrylamide gels, transferred to nylon membranes, UV-crosslinked and probed with 5'-end labeled DNA oligonucleotide probes to detect miR-21 (5'-TCAACATCAGTCTGA TAAGCTA-3'), miR-31 (5'-CAGCTATGC CAGCATCTTGCC-3'), or 5S rRNA (5'-TTAGCTTCCGAGATCA-3') in Church's hybridization buffer at 37^0C for 18 h. Membranes were washed as above, except at 37^0C. All membranes were exposed to phosphor imaging screens, and scanned with a Storm 860 PhosphorImager (Molecular Dynamics).

Overexpression and Inhibition of miRNA Function – Human miRNA21 and miRNA31 precursors (AM17100) and a Cy3-labeled pre-miR negative control (AM17120) were purchased from Ambion. miRNA activity was inhibited using chemically synthesized 2'O-Methyl-modified RNA oligonucleotides (Dharmacon) that were antisense to miRNA21 (5'-mUmCmAmAmCmAmUmC mAmG mUmCmUmGm-AmGmCmUmA-3') or miRNA31 (5'-mCmAmGmCmUmAmUmGmCmC mAmGmCmAmUmCmUmUmGmCmC-3'). A 5'-Cy3 labeled RNA oligonucleotide (Integrated DNA Technologies) targeting eGFP (5'-Cy3mA mAmGmGmCmAm-AmGmCmUmGmAmC mCmCmUmGmAmAmGmU-3') was used as a negative control.

All transfections were performed utilizing Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol. LIM 1863 organoids were resuspended in RPMI 1640 supplemented with 5% FBS and transfected with 100 nM of miRNA precursors in 24-well tissue culture plates. For migration and invasion assays, LIM 1863 organoids were trypsinized and dissociated before transfection. In 24-well tissue culture plates, 3×10^5 cells were transfected with 100 nM miRNA precursors or 250 nM miRNA inhibitors.

Real-Time RT-PCR – Total RNA was isolated using the mirVana™ RNA Isolation Kit following manufacturer's instructions (Ambion). One microgram of RNA was reverse transcribed using the iScript cDNA synthesis kit (Bio-Rad, Richmond, CA). SYBR-green real-time quantitative PCR was performed using a Bio-Rad MyiQ PCR detection system with the following gene-specific primers: Fibronectin-1 (FN1), forward 5'-GAGCCATGTGTCT-TACCATT-3' and reverse 5'- AGTATTTCTGGTCCTGCTCA-3'; Interleukin-8 (IL8), forward 5'- ATGACTTC CAAGCTGGCCGTGGCT-3' and reverse 5'- TCTCAGCC-CTCTTCAAAAAACTTCTC-3'; Laminin-γ-2 (LAMC2), forward 5'- CTGCAGGT GGACAACA-GAAA-3' and reverse 5'-TCTGCTGTCACATTGGCTTC-3'; Matrix metalloproteinase-7 (MMP7), forward 5'-CATGAGTGAGCTACAGTGGG-A-3' and reverse 5'-CTAT-GACGCGGGAGTTTAACAT-3'; T-lymphoma invasion and metastasis-1 (TIAM1), forward 5'- AAGACGTACTCAGGC CATGTCC – 3' and reverse 5'- GACCCAA-

ATGTCGCAGTCAG -3'; and U6 snRNA, forward 5'-CTCGCTTCGGCAGCACA-3' and reverse 5'- AACGCTTCACGAATTTGCGT-3'.

To measure mature miR-21 and miR-31 levels by quantitative real-time PCR, 10 ng of total RNA was reverse-transcribed using the TaqMan miRNA reverse transcription kit and RT primers for miR-21, miR-31 and U6 snRNA (Applied Biosystems). The cDNAs were then analyzed by real-time PCR using TaqMan probes for miR-21, miR-31 and U6 snRNA (Applied Biosystems).

Cell Migration and Invasion Assay – Uncoated or Matrigel-coated transwells containing 8 μm pores were used for the assays (BD Biosciences). Cells were seeded into the upper chamber in serum-free RPMI 1640 media. Conditioned DME media from NIH 3T3 cells containing 10% FBS was added to the lower chamber. Cells were fixed in 100% methanol 72 h later and stained with a 1:5 dilution of Giemsa (Sigma) for 40 min at room temperature. Cells remaining on the upper side of the filter were removed with a cotton swab. The filters were then mounted onto cover slips and images were taken at 10X magnification. From these images, the number of migratory or invasive cells was counted.

Dual Luciferase Reporter Assay – The full-length TIAM1 3'UTR (1970 nt) was cloned into Xba I and Not I sites of a modified pRL-TK vector (Promega) immediately downstream of the *Renilla* luciferase stop codon and designated pRL-TIAM1. The

putative miR-21 and miR-31 recognition elements (MRE) in the TIAM1 3'UTR were mutated by site-directed mutagenesis (Stratagene). For the dual luciferase assay, LIM 1863 cells were transfected with 100 nM of pre-miR-21, pre-miR-31 or a negative precursor control using Lipofectamine 2000 (Invitrogen). After 24 h, cells were co-transfected with 200 ng of pRL-TIAM1 and 100 ng of pGL3-luc as the internal control. Cell extracts were prepared 48 h later and the Dual-Glo luciferase reporter assay (Promega) was performed according to the manufacturer's protocol.

Lentiviral Transduction – To generate lentiviral particles, 293T cells in 10 cm plate were transfected with 12 μg of pLenti-CMV-puro (empty vector or containing TIAM1-FLAG), 8 μg of pCMV-dR8.74, and 4 μg of pMD2-VSVG. Forty-eight hours later the media was harvested, cleared by a 0.45 μm filter, mixed with polybrene and applied to dissociated LIM 1863 cells. After overnight incubation, the virus-containing media was replaced with fresh media.

Acknowledgements

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CHAPTER III

IDENTIFICATION OF TGF-β REGULATORY MECHANISMS IN THE EXPRESSION OF miR-21 AND miR-31

Introduction

MicroRNAs (miRNAs) are short, non-coding RNAs that regulate gene expression by targeting mRNAs for translational repression or degradation (Lim et al., 2005; Selbach et al., 2008; Guo et al., 2010). It is apparent that miRNAs are intimately involved in the regulation of fundamental cell processes including differentiation, cell proliferation, apoptosis and migration (Gusev et al., 2007). Aberrant miRNA expression is linked to the development and progression of cancer (Garzon et al., 2009). Extensive profiling studies on human samples revealed that miRNA expression patterns are drastically different between normal and cancerous tissues. (Lu et al., 2005; Volinia et al., 2006). The downregulation of certain miRNAs (*e.g.* miR-15a/miR-16-1, let-7) and upregulation of others (*e.g.* miR-17-92 cluster and miR-21) have been shown to correlate with tumorigenesis, and many miRNAs function to regulate many aspects of cancer cell biology, from cell proliferation and apoptosis to metastasis. These findings have led to the notion that miRNAs could function as oncogenes or tumor suppressors (Garzon et al., 2009). Thus understanding how miRNA expression is controlled is an important topic in cancer biology.

A growing number of signaling pathways that are deregulated in cancer, such as p53, c-Myc and TGF-β, are implicated in the regulation of miRNA expression (Garzon et al., 2009; Heldin et al., 2009). TGF-β regulates of a multitude of miRNAs, such as miR-155, miR-192 and the miR-200 family (Heldin et al., 2009). In Chapter II, we added miR-21

and miR-31 to the growing list of miRNAs that are regulated by TGF-β signaling. The biological impact of TGF-β is mediated mostly by the Smad family of transcription factors (Massagué et al., 2005). Receptor Smads (R-Smads, including Smads 1, 2, 3, 5 and 8) are phosphorylated in response to TGF-β cytokines, and consequently associate with Smad4. Such R-Smad/Smad4 complexes, in conjunction with additional transcription factors, are responsible for transcriptional regulation of a large number of genes upon TGF-β stimulation (Massagué et al., 2005). Interestingly, in a study carried out in smooth muscle cells, Davis *et al.* reported that rather than their usual function as transcriptional activators, R-Smads act to enhance the Drosha-mediated processing of pri-miR-21 by interacting with the Microprocessor component p68 (Davis et al., 2008). More recently, such pro-miRNA processing function of R-Smads was expanded to a number of other miRNAs, all of which share with pri-miR-21 a 5'-CAGAC-3' RNA motif in the stem region that is directly bound by R-Smads (Davis et al., 2010). Interestingly, such pro-miRNA processing function was not observed for Smad4 (Davis et al., 2008).

We recently observed that TGF-β and TNF-α increased levels of miR-21 and miR-31 in colon cancer cells (Chapter II). In this study, we sought out to determine the nature by which TGF-β/TNF-α upregulates miR-21 and miR-31. Here we show that TGF-β/TNF-α signaling induces the synthesis of new factors to increase the levels of miR-21 and miR-31. Further studies provide evidence in a number of cell lines that miR-21 is regulated by TGF-β primarily at the transcription level, not at the miRNA processing

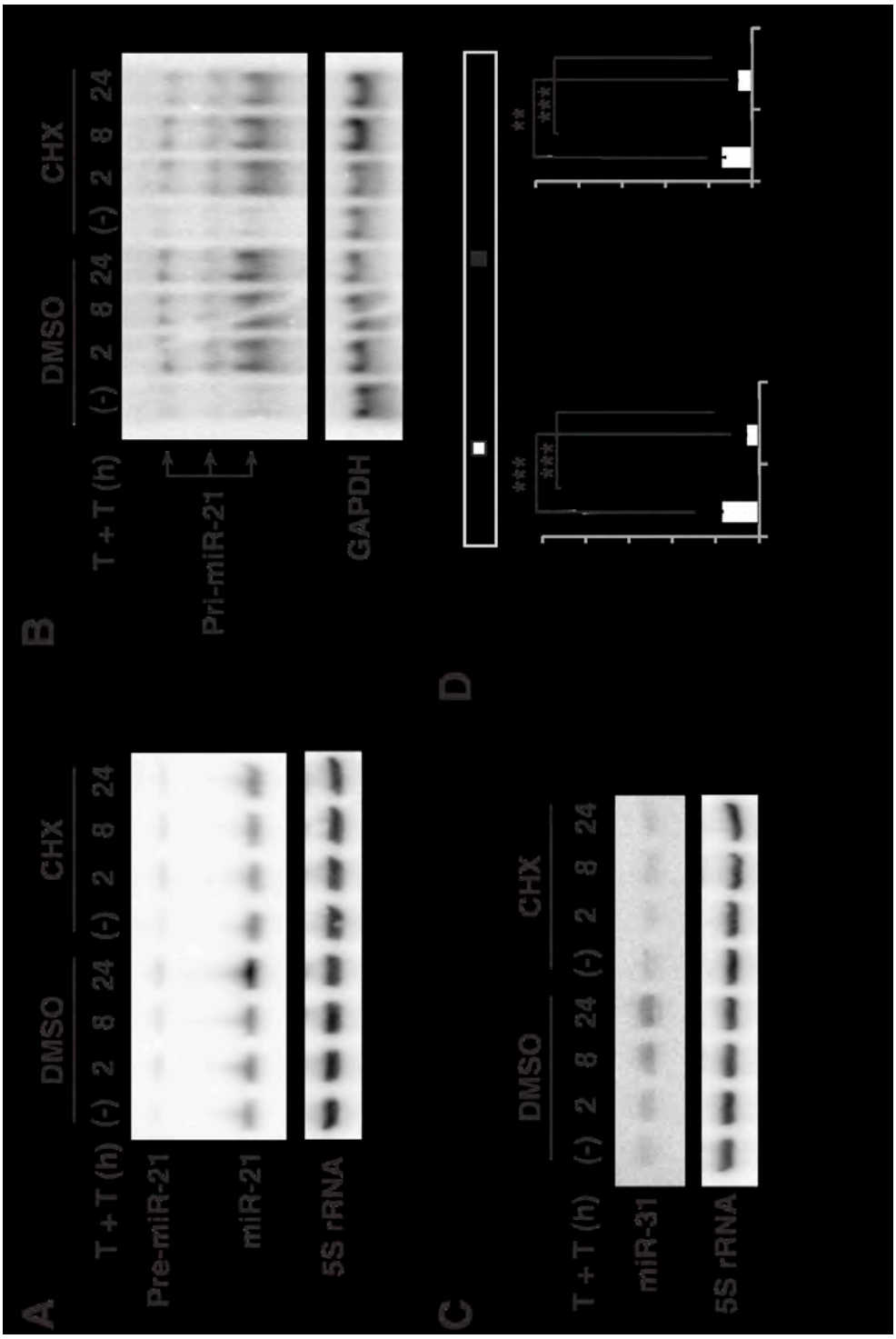
steps. Both loss- and gain-of function analyses suggest that Smad4 is essential for TGF-β transactivation of the gene coding miR-21. We mapped a promoter element critical for the upregulation of *miR-21* gene transcription by TGF-β and show that Smad4 binds to this region *in vivo*. Therefore, Smad4 acts as a classic transcription factor in mediating the increase in miR-21 expression in response to TGF-β. Smad4 was previously shown to enhance the processing, but not transcription of miR-21 in response to TGF-β in smooth muscle cells. Therefore, depending on cellular contexts, Smad4 may act on different steps of miR-21 biosynthesis to promote the expression level of this important miRNA.

Results

TGF-β/TNF-α Upregulate miR-21/miR-31 at the Transcription and Processing Levels

TGF-β and TNF-α synergize to induce EMT in the colorectal carcinoma cell line LIM1863 (Bates and Mercurio, 2003). In this accelerated model of EMT, we previously observed a robust increase in the mature forms of miR-21 and miR-31 (Chapter II). Here, we sought out to further investigate how TGF-β signaling regulates miR-21 and miR-31. In a time course analysis, we found that while the miR-21 precursor (a ~ 70 nt stem-loop processing intermediate) in LIM 1863 was rapidly increased and reached a plateau 2 hours after TGF-β/TNF-α stimulation, the increase in the mature miR-21 was delayed and did not reach its peak until 24 hours later (Fig. 3.1A). While this observation suggests activation of miR-21 gene transcription by TGF-β/TNF-α, it is also clear that the processing from precursor to mature miR-21 is the more rate-limiting step in TGF-β/TNF-α induction of this miRNA. Interestingly, pre-treating the LIM 1863 organoids with cycloheximide effectively abrogated the increase in mature miR-21 by TGF-β/TNF-α, and yet had no effect on the increase in miR-21 precursor (Fig. 3.1A). This suggests that TGF-β/TNF-α enhances the Argonaute-mediated processing of miR-21 precursor through an indirect mechanism, requiring the synthesis of unknown factors. Northern blotting of total RNA revealed three primary transcripts of the miR-21 gene, ranging in size from 1 to 4 kb (Fig. 3.1B). All three transcripts rapidly increased two hours after TGF-β/TNF-α treatment, which was not affected by cycloheximide (Fig. 3.1B). Therefore, the level of miR-21 is regulated by TGF-β/TNF-α at the initial transcription

Figure 3.1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(-)
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8
24
Pre-miR-21
miR-21
5S rRNA
B
DMSO
CHX
T + T (h)
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Pri-miR-21
GAPDH
C
DMSO
CHX
T + T (h)
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2
8
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8
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miR-31
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Figure 3.1 | TGF-β/TNF-α regulates miR-21 and miR-31 abundance at the transcription and processing steps. (A) TGF-β/TNF-α (T + T) induction of mature miR-21 was abrogated by cycloheximide (CHX). LIM 1863 organoids were incubated with DMSO or 15 μg/ml cycloheximide for 30 min followed by treatment with both TGF-β and TNF-a (T + T) for the indicated times. Shown are Northern blotting analysis of miR-21 precursor (pre-miR-21, ~70 nt) and mature miR-21 (~21 nt), with 5S rRNA as the loading control. **(B)** TGF-β/TNF-α induced a rapid increase in the miR-21 primary transcript (pri-miR-21), which was not affected by cycloheximide. Northern blotting of total RNA shows three prominent miR-21 primary transcripts with ~ 1 Kb, ~2 Kb and ~ 4 Kb in size (arrows). GAPDH served as the loading control. **(C)** Similar experiment as in (A) showing the increase in mature miR-31 in response to TGF-β/TNF-α was affected by cycloheximide. **(D)** TGF-β/TNF-α -induction of miR-21 and miR-31 is disrupted by Drosha knock-down. LIM 1863 cells that were stably transduced with a Drosha-targeting or control (Con) shRNA were treated with TGF-β/TNF-α (T + T) for 36 h after which mature miR-21 and miR-31 expression was evaluated by real-time quantitative RT-PCR (mean + S.D., **, p <0.01; ***, p < 0.001). U6 snRNA was used as the internal standard.

and the processing steps, and only miR-21 gene transcription is a direct downstream target of TGF-β/TNF-α signaling.

The increase in mature miR-31 by TGF-β/TNF-α became noticeable 2 h after the cytokine treatment, and continued to accumulate until 24 h later (Fig. 3.1C). Cycloheximide also effectively inhibited such upregulation of mature miR-31 by TGF-β/TNF-α (Fig. 3.1C). However, neither the primary nor the precursor forms of miR-31 were detectable by Northern blotting, so we could not determine whether miR-31 is also regulated at the primary transcription level like in the case of miR-21. Therefore, for both miR-21 and miR-31, the upregulation by TGF-β/TNF-α appears to be indirect and requires new synthesis of unknown factors.

Furthermore, when we knocked down the essential miRNA processing factor Drosha using a previously validated shRNA, TGF-β/TNF-α failed to elevate the level of mature miR-21 and miR-31 (Fig. 3.1D and 3.2) (Kumar et al., 2007). This observation suggested that the increase in miR-21/miR-31 upon TGF-β/TNF-α stimulation is due to new biosynthesis and/or processing of miRNAs.

Figure 3.2

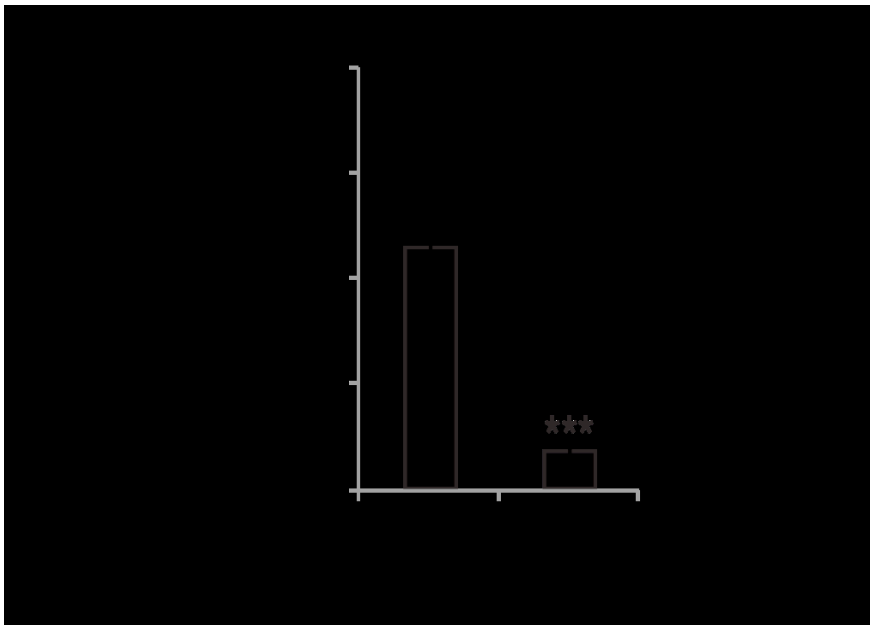


Figure 3.2 | Efficiency of Drosha knockdown by shRNA. Knockdown efficiency of Drosha shRNA construct. Total RNA was isolated from LIM 1863 cells that were stably transduced with a Drosha-targeting or control (Con) shRNA vector. Drosha expression was measured via quantitative real-time RT-PCR (mean + S.D., ***, $p < 0.001$). U6 snRNA was used as the internal standard.

The miR-21 primary transcript level is increased by TGF-β/TNF-α treatment

Next we aimed to determine whether TGF-β induction of miR-21 expression was mainly at the transcription level. Consistent with results in Fig. 3.1B, treatment of LIM 1863 cells with both TGF-β/TNF-α caused a rapid (< 1 h) and marked upregulation of pri-miR-21, which remained well over basal levels for at least 24 hours, as determined by real-time quantitative PCR (Fig. 3.3A). Moreover, either of these two cytokines alone was sufficient to induce the pri-miR-21 level in LIM 1863 cells (Fig. 3.3B). But the combined treatment with both TGF-β and TNF-α resulted in much higher pri-miR-21 than that achieved after treatment with TGF-β or TNF-α alone (Fig. 3.3B).

Effect of actinomycin D on miR-21 biogenesis

To determine if the upregulation of pri-miR-21 by TGF-β/TNF-α is mainly due to enhanced transcription, we employed actinomycin D, a potent inhibitor of transcription (Sobell, 1985). Treatment of LIM 1863 cells with actinomycin D prior to TGF-β/TNF-α stimulation abolished the ability of these cytokines to upregulate pri-miR-21 (Figs. 3.4A and 3.3B). In the presence of actinomycin D alone, the basal level of pri-miR-21 steadily declined over a 24-hour period (Figure 3.4C). This observation confirmed that active transcription is critical in the upregulation of pri-miR-21 by TGF-β/TNF-α signaling. Although we observed a sharp increase in the miR-21 primary transcript, it was not until at least 24 hours later that we saw elevated mature miR-21 levels (Figure 3.4D). This observation suggested that there is a lag between transcriptional activation of the *miR-21* gene and complete maturation of miR-21.

Figure 3.3

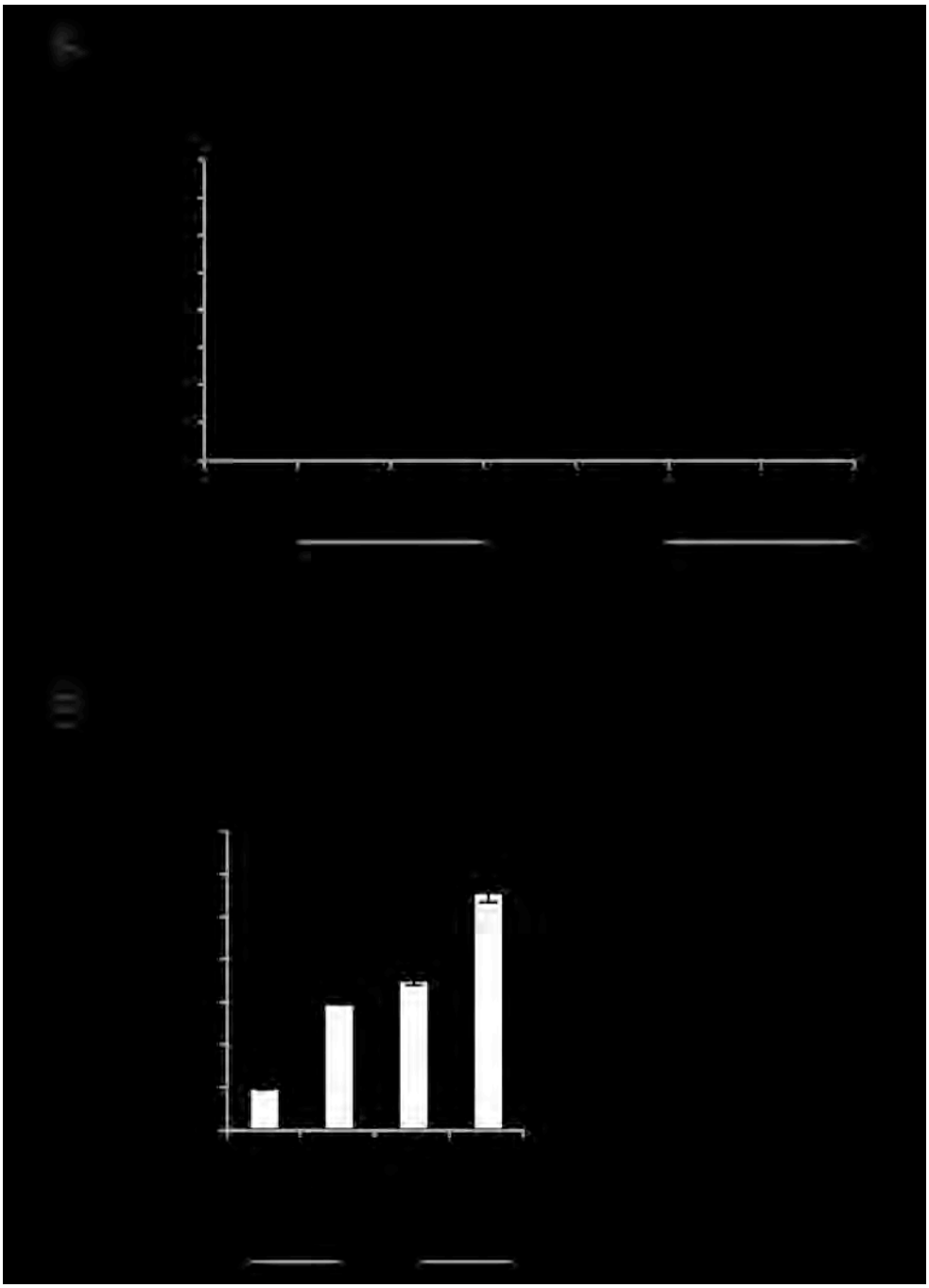


Figure 3.3 | TGF-β/TNF-α increases the miR-21 primary transcript level. (A) Real-time quantitative PCR analysis of pri-miR-21 level in LIM 1863 cells treated with both TGF-β and TNF-α (T + T) or with media only (Vehicle) as indicated. **(B)** After treatment of LIM 1863 cells with TNF-α, TGF-β, or TGF-β and TNF-α combined (T + T) for 2 hours, the pri-miR-21 level was measured by real-time quantitative PCR. For all experiments, U6 snRNA expression was determined as an internal reference (mean $\pm$ S.D, n>3). ***, $p < 0.001$.

Figure 3.4

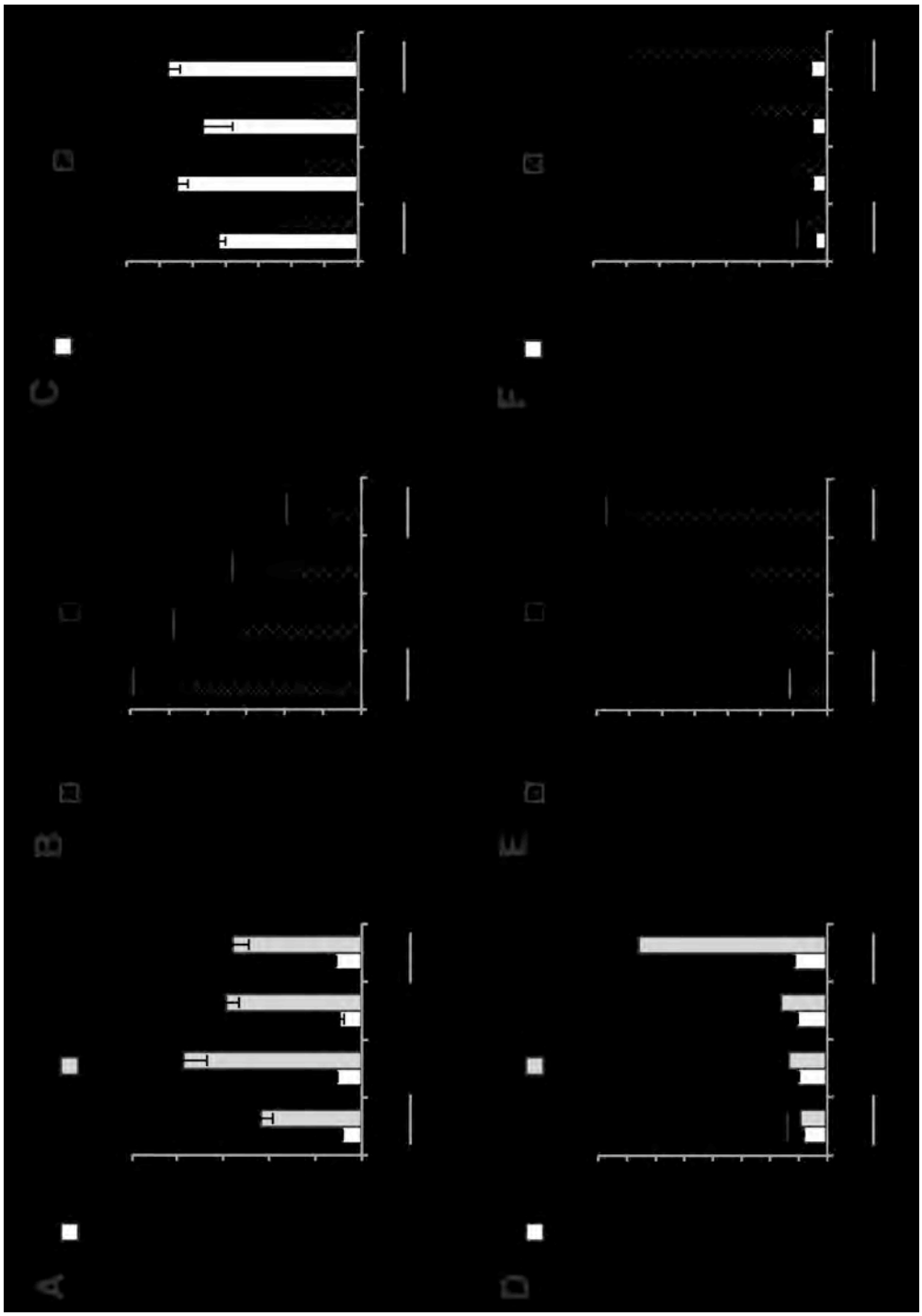


Figure 3.4 | *miR-21* is transcriptionally regulated by TGF-β/TNF-α. LIM 1863 cells were pretreated with **(A)** DMSO or **(B)** actinomycin D (ActD) for 30 min followed by treatment with TGF-β/TNF-α (T + T) or media as indicated. The level of pri-miR-21 was measured using real-time quantitative PCR. **(C)** The data in (A) and (B) were re-plotted to emphasize the effect of ActD alone on pri-miR-21 expression. **(D and E)** The same experiments were performed as in (A and B), with the exception that the level of mature miR-21 was measured. **(F)** A new graph of the data from (D and E) was generated to highlight the effect of ActD alone on the level of mature miR-21. For all experiments, U6 snRNA expression was used as an internal reference (mean $\pm$ S.D, n>3). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Interestingly, we observed that actinomycin D treatment itself caused a substantial increase in mature miR-21 over time, through unknown mechanisms (Figs. 3.4E and 3.4F). Importantly, in cells treated with actinomycin D, TGF-β/TNF-α did not further induce the level of mature miR-21 (Fig. 3.4E). Together, these experiments support the hypothesis that the upregulation of mature miR-21 by TGF-β/TNF-α in LIM 1863 cells is primarily at the level of transcriptional activation of the *miR-21* gene.

TGF-β upregulates the miR-21 primary transcript in HaCaT cells

Next, we aimed to determine whether TGF-β/TNF-α induction of *miR-21* transcription was a unique response of LIM 1863 cells. We tested HaCaT, an immortalized human keratinocyte cell line that is highly responsive to TGF-β (Hannon and Beach, 1994; Reynisdóttir et al., 1995). Northern blot analysis confirmed that treatment of HaCaT cells with TGF-β/TNF-α for 2 hours upregulated the miR-21 primary transcript nearly 8-fold and this level persisted for at least 24 hours (Fig. 3.4A), similar to results observed in LIM 1863 cells (Fig. 3.2). Like in LIM 1863 cells, pri-miR-21 was robustly induced within 2 hours of TGF-β treatment in HaCaT cells (Fig. 3.4B). However, in contrast to LIM 1863 cells, TNF-α did not induce pri-miR-21 expression in HaCaT cells, which could be due differences in TNF-α signaling components in these cell lines (Fig. 3.4B). Moreover, TGF-β treatment of HaCaT cells for 24 hours yielded nearly four times more mature miR-21 than in untreated cells (Fig. 3.4C), consistent with published data (Zavadil et al., 2007). In all, these results show that in both HaCaT and LIM 1863, TGF-β alone is sufficient to activate transcription of *miR-21*.

Figure 3.5

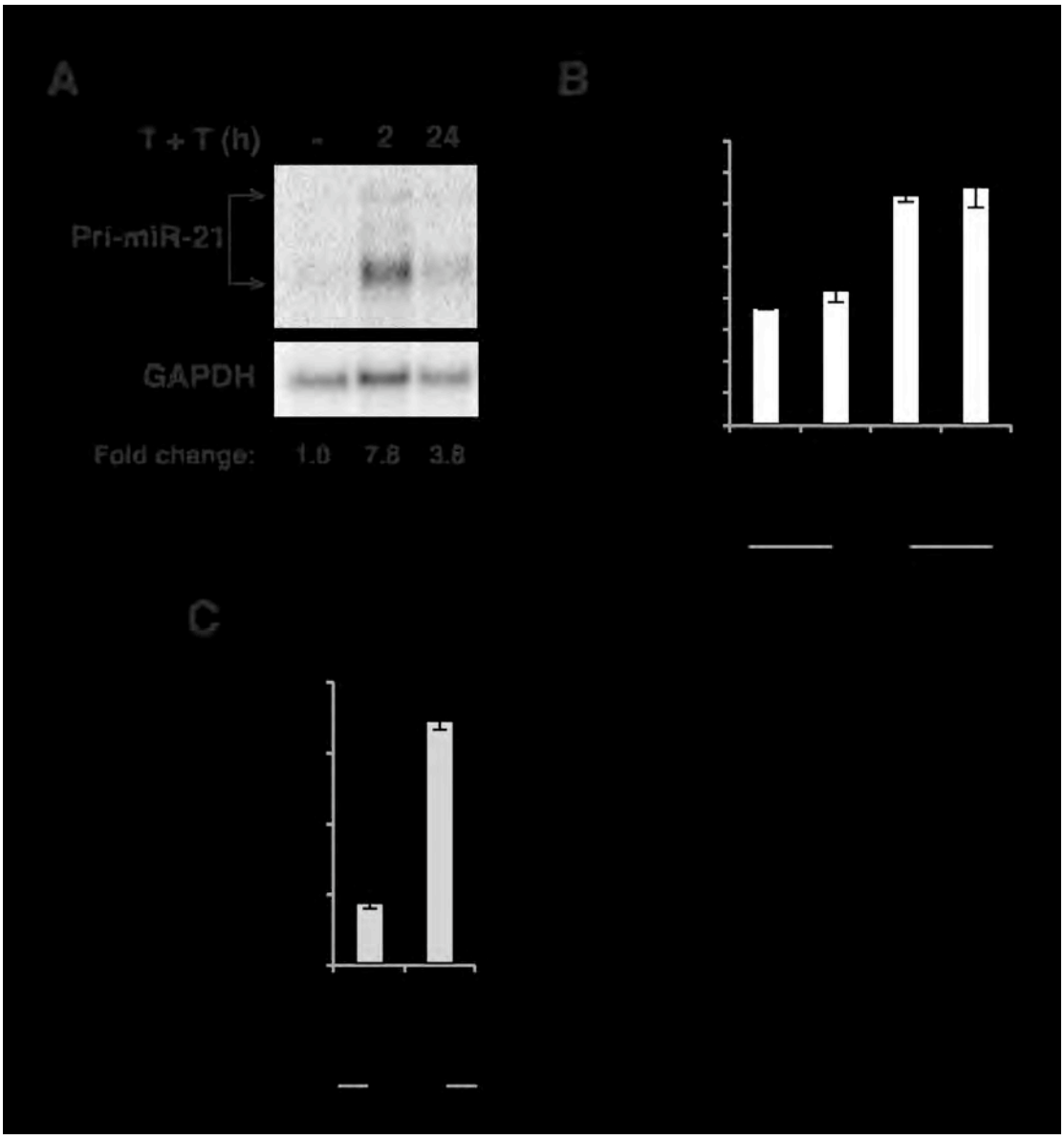
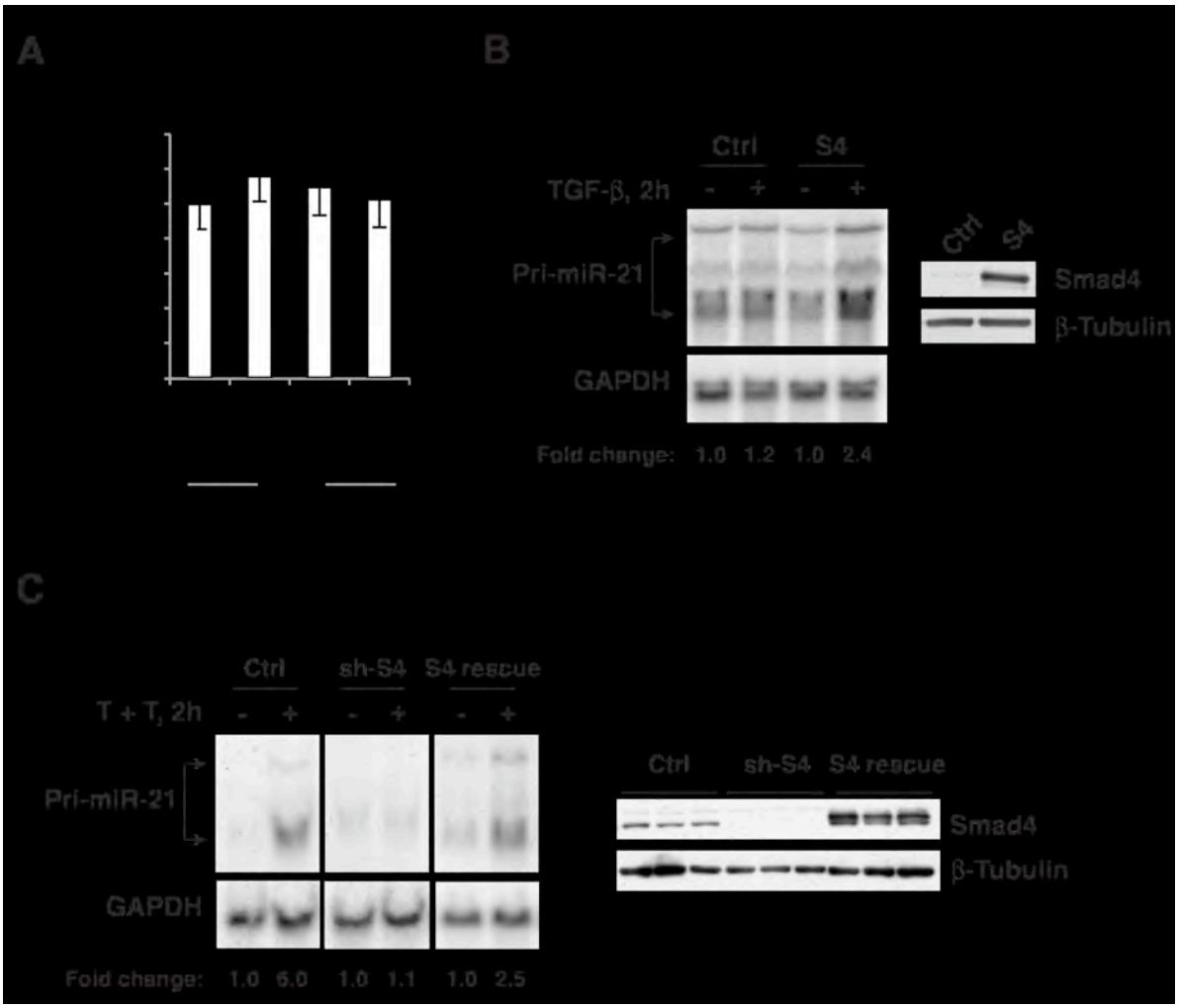


Figure 3.5 | TGF-β induces *miR-21* gene transcription in HaCaT cells. (A) Northern blot analysis of pri-miR-21 expression in HaCaT cells after treatment with TGF-β/TNF-α (T + T) or media only (-) as indicated. GAPDH was the loading control. **(B)** Pri-miR-21 expression in HaCaT after a 2 hour treatment with TNF-α, TGF-β, TGF-β/TNF-α (T + T) or media only (Vehicle), as determined by real-time quantitative PCR. **(C)** The mature miR-21 level in HaCaT cells following 24 hours of TGF-β or media alone (Vehicle) was measured by real-time quantitative PCR. U6 snRNA expression was determined as the internal standard for real-time quantitative PCR experiments and data represent the mean ($\pm$ S.D, n>3). ***, $p < 0.001$.

Transcriptional activation of miR-21 is dependent on Smad4

Smads are the primary intercellular effectors of TGF-β-induced transcriptional responses (Massagué, 1998; Massagué et al., 2005). Thus, we next tested if the common Smad, Smad4, was required for transcriptional activation of the *miR-21* gene by TGF-β. We first examined SW480 cells, a Smad4-deficient colon carcinoma cell line (Zhang et al., 1996). When SW480 cells were treated with TGF-β, TNF-α, or both for 2 hours, no increase in pri-miR-21 expression was detected (Fig. 3.6A). However, after we introduced a Smad4 expression vector into SW480 cells, pri-miR-21 was rapidly (< 2 h) induced by TGF-β, supporting the importance of Smad4 in the transcriptional regulation of *miR-21* (Fig. 3.6B). Furthermore, we carried out a loss-of-function analysis to study the role of Smad4 in *miR-21* gene transcription. The human breast carcinoma cell line MDA-MB-231 expresses functional Smad4 and pri-miR-21 was substantially induced within 2 hours of TGF-β/TNF-α treatment (Fig. 3.6C). Such upregulation of the pri-miR-21 level was abolished when the endogenous Smad4 was depleted by a previously validated shRNA construct (Fig. 3.6C) (Kang et al., 2005). Importantly, upon expression of an shRNA-resistant Smad4, the induction of pri-miR-21 by TGF-β/TNF-α was largely restored (Fig. 3.6C) (Kang et al., 2005). Together these data strongly suggest that Smad4 plays a critical role in transcriptional activation of the *miR-21* gene in response to TGF-β/TNF-α. This conclusion is different from the observations in smooth muscle cells where TGF-β-induction of miR-21 production was shown to be independent of Smad4 (Davis et al., 2008).

Figure 3.6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Smad4
β-Tubulin
C
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sh-S4
S4 rescue
T + T, 2h
- + - + - +
Pri-miR-21
GAPDH
Fold change: 1.0 6.0 1.0 1.1 1.0 2.5
Ctrl
sh-S4
S4 rescue
Smad4
β-Tubulin

Figure 3.6 | Smad4 is required for TGF-β induction of *miR-21* transcription. (A) SW480 cells were treated for 2 hours with TNF-α, TGF-β, combined TGF-β and TNF-a (T + T) or media only (Vehicle) and pri-miR-21 level was measured by real-time quantitative PCR. U6 snRNA was used as the standard (mean ± S.D, n>3). **(B)** Northern blot analysis of pri-miR-21 expression in parental SW480 cells (Ctrl) or those expressing Smad4 (S4) after TGF-β treatment as indicated (left panel). Western blot analysis to detect Smad4 in the indicated SW480 cell lines; β-Tubulin level served as a loading control (right panel). **(C)** *Left*, MDA-MB-231 parental cells (Ctrl) or those in which endogenous Smad4 was depleted by shRNA (sh-S4) and then restored by a shRNA-insensitive Smad4 expression vector (S4 rescue) were treated with TGF-β/TNF-α (T + T) or media only (Vehicle) for 2 hours and pri-miR-21 expression was evaluated with Northern blotting. As a loading control, GAPDH expression was determined. *Right*, Western blot to detect Smad4 expression in MDA-MB-231 cell lines. β-Tubulin expression was used a loading control.

Identifying TGF-β response elements in the promoter of the miR-21 gene

We next investigated the promoter elements responsible for transcriptional regulation of *miR-21* by TGF-β/TNF-α. To do this, we first evaluated the location of the *miR-21* promoter. According to miRBase: the microRNA database (version 10), miR-21 transcripts overlapped with those of VMP-1 (also known as TMEM49), a TGF-β target gene (Hill et al., 2005; Griffiths-Jones et al., 2008). Based on this information, we surmised that elements in the VMP-1 promoter were sensitive to TGF-β/TNF-α signaling and, as a result, gave rise to increased levels of transcripts containing miR-21. However the expression pattern of transcripts containing VMP-1 and miR-21 did not coincide following treatment of LIM 1863 cells with either TNF-α, TGF-β, or both cytokines (Fig. 3.7). This observation suggested that that miR-21 did not arise from VMP-1-containing transcripts and that miR-21 is an independently expressed gene, an idea that was independently validated (Fujita et al., 2008).

We examined regions within 5 kb of the miR-21 stem loop to locate the miR-21 promoter. We generated a series of luciferase reporter constructs and tested them in LIM 1863 organoids (Fig. 3.8A). We started with a 2.7 kb promoter fragment, which was activated by either TGF-β or TNF-α (Fig. 3.8B). More importantly, the 2.7 kb construct also recapitulated synergistic activation by TGF-β/TNF-α stimulation (Fig. 3.8B). By serial deletions, we identified an 812 bp minimal region that conferred TGF-β inducibility in LIM 1863 cells (Fig. 3.8B). Combined TGF-β/TNF-α treatment resulted in further enhancement of the reporter expression, while TNF-α alone elicited only a

moderate effect (Fig. 3.8B). To better define the TGF-β sensitive element, 200-bp deletions were made from either the 5'- or 3'- end of the 812-bp luciferase reporter. When these reporters were tested for responsiveness to TGF-β/TNF-α, all of those lacking the 3'-200 bp showed no increase in luciferase activity (Fig. 3.8C), whereas the reporters that retained this 3'-200 bp region were induced to similar levels as the 812-bp reporter (Fig. 3.8C). This suggested that this 200-bp region contained a TGF-β sensitive element and prompted us to search for possible sequences that might confer such a response to TGF-β. Within this 200-bp region are Smad-binding elements and AP-1 consensus sequences (Fig. 3.8D). Mutation of these elements revealed that the most 3' AP-1 site reduced luciferase activity nearly to basal level in response to TGF-β/TNF-α (Figs 3.8D and 3.8E) or TGF-β alone; TNF-α did not induce luciferase activity (Fig. 3.8E). Therefore, we have identified the promoter region responsible for TGF-β-upregulation of miR-21 gene transcription.

Next, we tested the ability of Smad4 to bind to this TGF-β-responsive region *in vivo* by chromatin immunoprecipitation (ChIP). Since the strongest reporter activity was observed when cells were treated with both TGF-β and TNF-α (Fig. 3.8), we decided to perform ChIP under this condition. Indeed the ChIP analysis showed increased binding of endogenous Smad4 to the TGF-β-response region in the *miR-21* promoter after TGF-β/TNF-α stimulation (Fig. 3.9). This result supports the notion that Smad4 acts as a conventional transcription factor to activate transcription of the *miR-21* gene upon TGF-β stimulation.

Figure 3.7

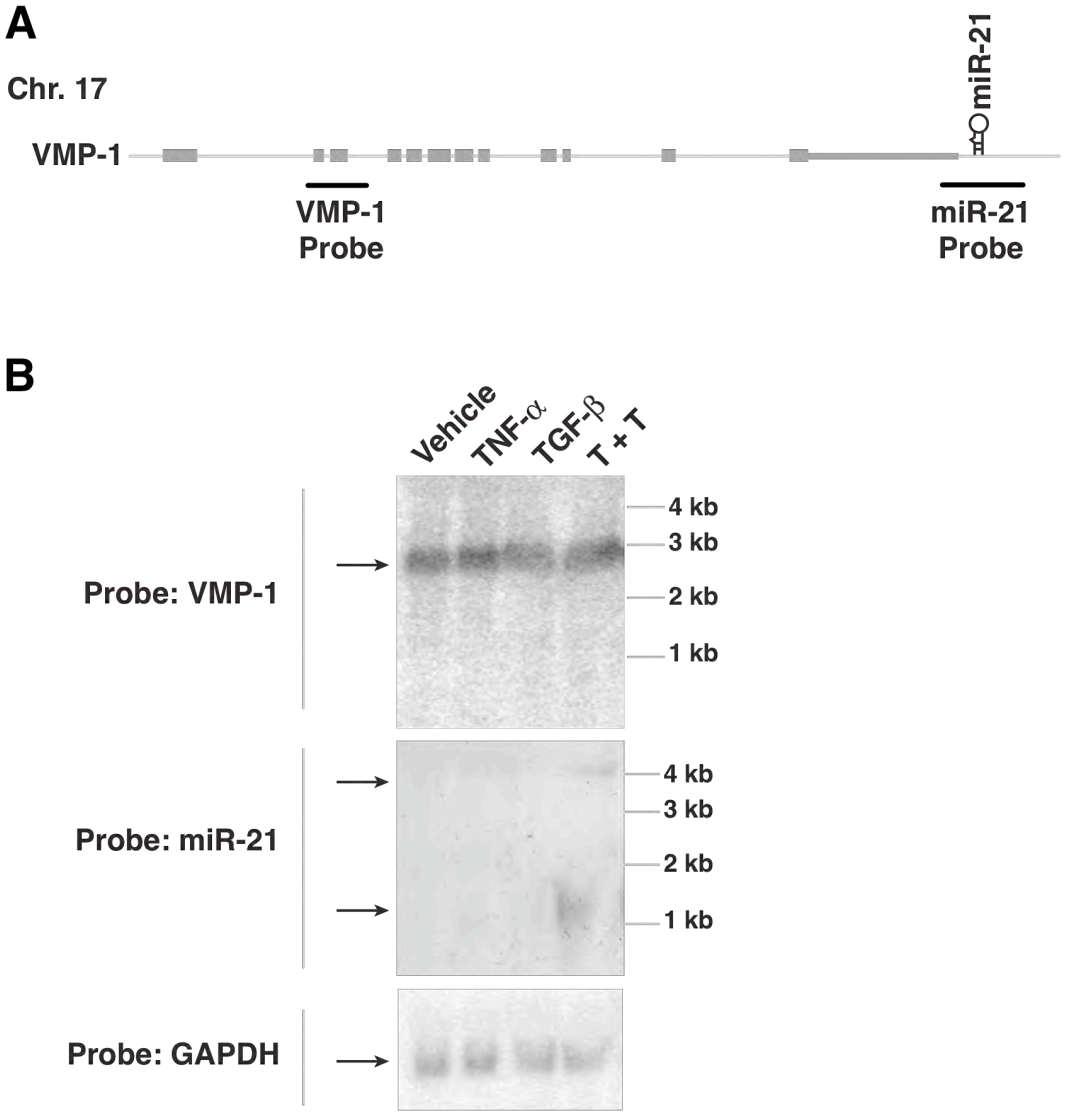
A
Chr. 17
VMP-1
miR-21
VMP-1
Probe
miR-21
Probe
B
Vehicle
TNF-α
TGF-β
T + T
Probe: VMP-1
4 kb
3 kb
2 kb
1 kb
Probe: miR-21
4 kb
3 kb
2 kb
1 kb
Probe: GAPDH

Figure 3.7 | *VMP-1* and *miR-21* are independently regulated genes. (A) Schematic of *VMP-1* and *miR-21* on chromosome 17 in the human genome. **(B)** The expression patterns for transcripts containing VMP-1 and miR-21 are different. LIM 1863 organoids were treated as indicated for 24 h followed by Northern blot analysis with probes depicted in (A). GAPDH expression was determined as a control.

Figure 3.8

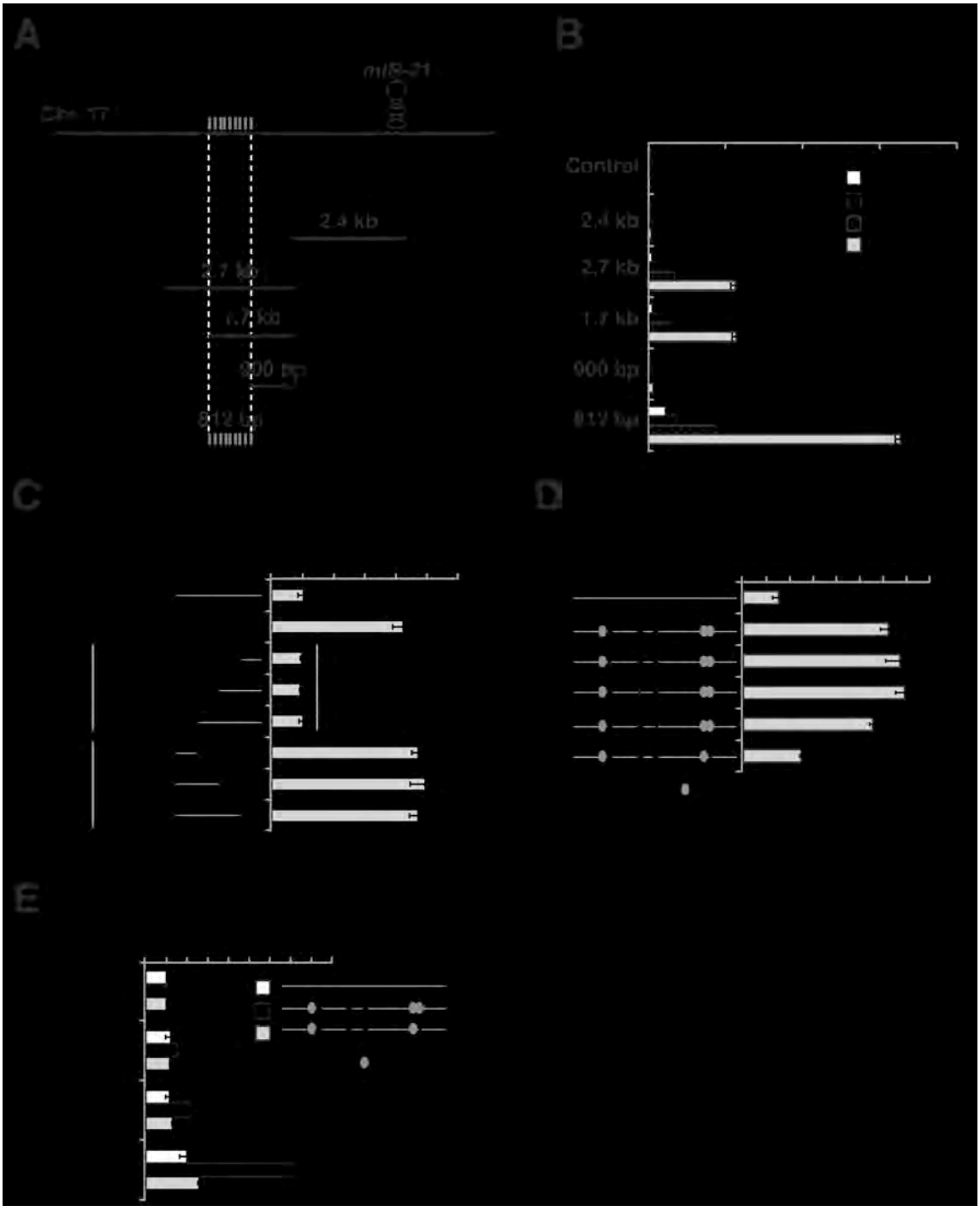

Figure 3.8 | The *miR-21* gene contains a TGF-β-responsive region. (A) Schematic view of the *miR-21* locus in the human genome. Solid bars indicate the DNA fragments that were cloned into firefly luciferase reporters. Dashed bars correspond to the DNA region bearing TGF-β response elements. **(B)** Luciferase reporter activities after treatment of LIM 1863 cells with TNF-α, TGF-β, TGF-β/TNF-α (T + T) or media only (Vehicle) for 24 hours. **(C)** Luciferase reporter activities after treatment of LIM 1863 cells with TGF-β/TNF-α or media only (Vehicle) for 24 hours of reporters with 200-bp deletions from either the 5'- or 3'-end of the 812-bp region **(D)** Luciferase reporter activities from constructs bearing mutations in Smad and AP-1 sites after treatment of LIM 1863 cells with TGF-β/TNF-α or media only (Vehicle) for 24 hours **(E)** Luciferase reporter activities after treatment of LIM 1863 cells with TNF-α, TGF-β, TGF-β/TNF-α (T + T) or media only (Vehicle) for 24 hours of the indicated wild type and mutant 200-bp reporter. As an internal control for all reporter experiments, each reporter was co-transfected with a *Renilla* luciferase construct (mean $\pm$ S.D, n>3). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Figure 3.9

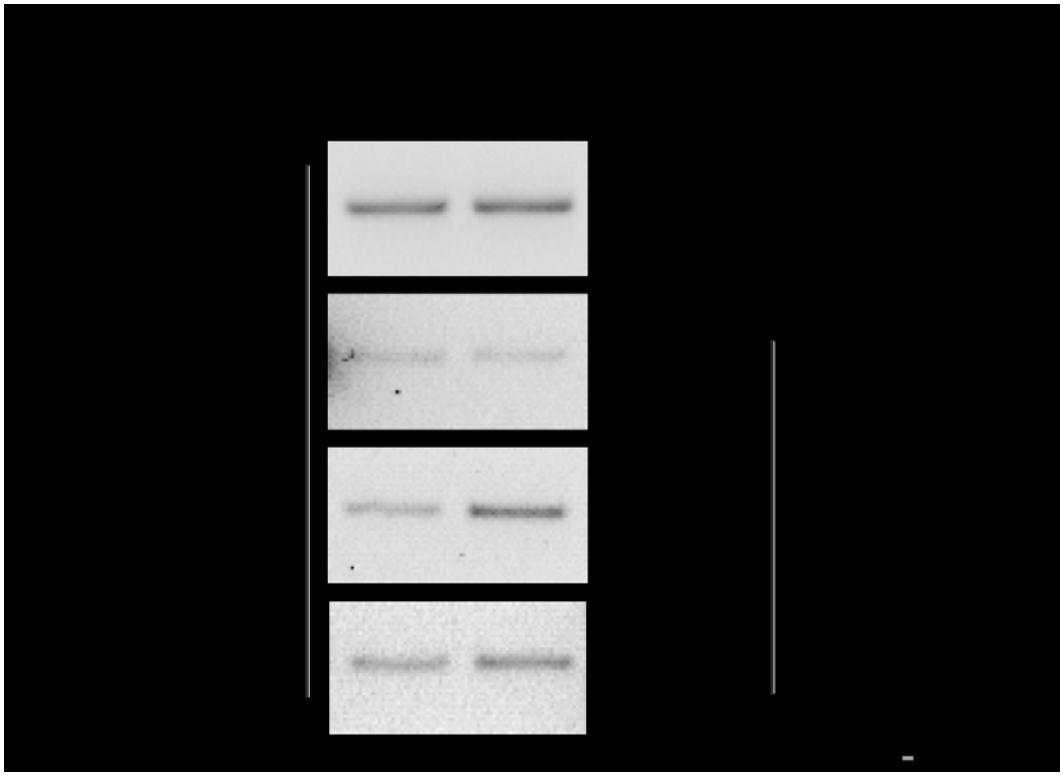

Figure 3.9 | Smad4 binds to the mapped TGF-β response element in the *miR-21* locus. After treatment of LIM 1863 cells with TGF-α/TNF-β (T + T) or media alone (Vehicle) for 2 hours, chromatin immunoprecipitation (ChIP) was performed using antibodies that recognize Smad4 or c-Jun as described in the Experimental Procedures.

Summary

Increased levels of miR-21 and miR-31 correlate with advanced tumor stage in patients with colorectal carcinoma, emphasizing the need to uncover factors involved in the metabolism and function of these two miRNAs. In Chapter II we observed increased levels of miR-21 and miR-31 in response to TGF-β/TNF-α signaling in LIM 1863 colorectal carcinoma cells, and this contributes to enhanced cancer cell motility and invasiveness. In this study we investigated the mechanism by which TGF-β and TNF-α regulate miR-21 and miR-31. We found that higher levels of the mature forms of miR-21 and miR-31 induced by TGF-β/TNF-α signaling required the synthesis of new proteins. Furthermore in the case of miR-21, we found that TGF-β appeared to enhance the transcription of the gene encoding miR-21, contrasting to findings in smooth muscle cells where TGF-β enhanced mainly the processing of miR-21 (Davis et al., 2008; Davis et al., 2010). Our data strongly suggest that in a number of cell lines TGF-β activates the transcription of the miR-21 gene through Smad4. Therefore, in a cell-context-dependent manner, TGF-β can regulate the biogenesis of miR-21 through different mechanisms.

Experimental Procedures

Cell culture and cytokine treatment

LIM 1863 cells were maintained in RPMI 1640 supplemented with 5% fetal bovine serum (FBS). HaCaT and MDA-MB-231 cell lines were maintained in Dulbecco's Modified Eagle (DME) media supplemented with 10% FBS. All culture media also contained penicillin and streptomycin (100 units/ml), both from Invitrogen. For experiments using cytokines, cells were treated with 2.5 ng/ml TGF-β (R&D Systems) and 10 ng/ml TNF-a (R&D Systems). Actinomycin D (Sigma) was used at a final concentration of 1 ug/ml.

Northern blotting

Northern analysis was performed as previously described (Cottonham et al., 2010). Briefly, total RNA was separated on denaturing agarose gels and cross-linked to nylon membranes. Membranes were probed with 5'-end-labeled radiolabeled probes to detect transcripts containing VMP-1, miR-21 or GAPDH. Radioactive signals were quantified using the ImageQuant software (Molecular Probes) or ImageJ software (Abramoff et al., 2004).

Real-time quantitative PCR

Total RNA was isolated using Trizol (Invitrogen) and 1 ug was reverse-transcribed with the iScript cDNA synthesis kit (Bio-Rad). Real-time quantitative PCR was performed

using the following gene-specific primers: miR-21 primary transcript (pri-miR-21) forward, 5'-TCCGTTTTCTTGAGCGTTTT-3' and reverse, 5'-AGTATGCAGCAGCCCAGTTT-3'; and U6 snRNA forward, 5'-CTCGCTTCGGCAGCACA-3' and reverse 5'-AACGCTTCACGAATTTGCGT-3'. Mature miR-21 levels were determined using the TaqMan miRNA assay with primers to detect miR-21 and U6 snRNA provided by the manufacturer (Applied Biosystems).

Luciferase reporter assay

Genomic DNA was isolated from LIM 1863 cells and used as a template to clone regions upstream of *miR-21* into the MluI and XhoI sites of pGL3-promoter (Promega). The 2.7 kb reporter was cloned using primers forward, 5'-TTAAAAGTCAGGGCCAGGAG-3' and reverse, 5'-CTGTGCTTCCCTCGAGTTTC-3' and the 2.4 kb reporter was cloned using forward primer, 5'-GACTACGCGTGTCTTTTCTGTAAACGATTCTGAGG-3' and reverse primer, 5'-GACTGCTAGCAGGTGGTACAGCCATGGAGATG-3'. Deletion constructs (i.e. the 1.8 kb, 0.9 kb, 0.8 kb, etc.) of the miR-21 gene promoter depicted in Figure 3.8 were further generated by PCR cloning.

LIM 1863 cells were transfected with 200 ng of firefly reporter vector and 250 ng of the control *Renilla* luciferase reporter vector using Lipofectamine 2000 (Invitrogen). Eighteen hours later, cells were treated with TNF-α, TGF-β, TGF-β/TNF-α (T + T) or media only. After 24 hours of cytokine treatment, firefly and *Renilla* luciferase activities were measured using the Dual Luciferase Reporter Assay (Promega).

Chromatin immunoprecipitation

Approximately 300 mg of LIM 1863 cells were treated with TGF-β/TNF-α (T + T) or media (vehicle) for 2 h and then fixed with 1% formaldehyde for 15 min at 37 ^{0}C. Cross-linking was quenched with 0.125 M glycine for 5 min at room temperature. Extracts were prepared and sonicated to yield DNA fragments of approximately 500 bp. The chromatin was adjusted to 1X ChIP buffer (10 mM Tris, pH 8; 1% Triton X-100; 0.1% deoxycholate; 140 mM NaCl; 1 mM EDTA, pH 8; 0.5 mM EGTA, pH 8; 1 mM PMSF). Chromatin was pre-cleared for 2.5 h with Dyna Protein A magnetic beads (Invitrogen) that had been pre-blocked with 1 mg/ml herring sperm DNA and 1 mg/ml BSA. Pre-cleared chromatin was incubated with 4 μg of antibodies that recognize Smad4 or c-Jun (Santa Cruz) or rabbit IgG (negative control) at 4 ^{0}C for 16 h. Immune complexes were recovered by incubating samples with blocked Dyna Protein A magnetic beads for 1 hr at 4 ^{0}C. The beads were washed three times with 1X ChIP buffer and resuspended in 0.5% SDS, 200 μg/ml proteinase K in TE buffer. Cross-links were reversed by incubating the samples at 55 ^{0}C for 3 h and then at 65 ^{0}C for 6 h. DNA was phenol-chloroform extracted followed by ethanol precipitation. Standard PCR was performed to detect the *miR-21* promoter region using forward primer, 5'-GACTACGCGTTCCATGTATTCTGGGTAAGAAGG-3' and reverse primer, 5'-GACTGCTAGCGAGGCACCTCCCACTAGTCA-3'.

Acknowledgements

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CHAPTER IV

GENERAL DISCUSSION

Overview

TGF-β is linked to the advancement of late-stage tumors towards malignancy (Padua and Massagué, 2009). Previous studies to elucidate molecular determinants involved in the pro-metastasis function of TGF-β focused on its transcriptional regulation of protein-coding genes. However, mounting evidence shows miRNAs significantly regulate gene expression at the level of translation. Importantly, miRNAs function in key cellular processes and deregulation of miRNA activity is involved with several diseases, including cancer. At the commencement of this thesis research, little knowledge existed about the function of miRNAs in TGF-β signaling, especially in the context of cancer. Thus, this body of work aimed to increase our understanding of the role of miRNAs in the metastasis-promoting function of TGF-β.

MiR-21 and miR-31 as Effectors of TGF-β Signaling

We conclude that miR-21 and miR-31 are novel downstream effectors of TGF-β and TNF-α signaling, and directly regulate the motility and invasiveness of colon carcinoma cells. Although these two miRNAs are likely to have many different direct targets, they converge on TIAM1, a protein known to regulate migration and invasion of various cancer cells (Habets et al., 1994; Mertens et al., 2003; Minard et al., 2006). Therefore, we have uncovered a novel miRNA-mediated mechanism through which TGF-β, in conjunction with TNF-α, promotes invasion and metastasis of colon cancer. Our data from Chapter II corroborate previous clinical studies which associate elevated miR-21 and miR-31 levels to late stage colon cancer progression and metastasis (Bandrés et al.,

2006; Slaby et al., 2007). With this new mechanistic understanding of miR-21 and miR-31 function in colon cancer cell biology, we suggest a possible utility of miR-21 and miR-31 as molecular markers or therapeutic targets of colon cancer.

Interestingly, although there have been several reports that downregulation of the miR-200 family is a common element in EMT, we did not find evidence of a change in miR-200 expression during EMT in LIM 1863 organoids (Burk et al., 2008b; Burk et al., 2008a; Gregory et al., 2008a; Gregory et al., 2008b; Korpal et al., 2008b; Korpal et al., 2008a; Park et al., 2008a; Park et al., 2008b). This could suggest that either the miR-200 level is already low in LIM 1863 or that ZEB1/2, the miR-200 target, is not rate-limiting in this system. Unlike miR-200 that suppresses an upstream master regulator of the EMT program such as ZEB1/2, miR-21 and miR-31 may impact on more downstream events such as TIAM1. Therefore, overexpression of miR-21 or miR-31 may not be sufficient to initiate the EMT program, but rather play a facilitating role.

In Chapter II of this study we found that both miR-21 and miR-31 target TIAM1 in LIM 1863 cells. Functional assays further confirmed the biological relevance of TIAM1 downregulation in miR-21/miR-31 regulation of LIM 1863 motility and invasiveness. However, it is important to point out that miR-21 and miR-31 functions are not limited to suppressing TIAM1. These two miRNAs likely have other different targets, in addition to TIAM1, that may also be important for cell migration and invasion. Such a scenario could explain how in anti-miR-21-treated LIM 1863 cells, even though TIAM1 would

still be repressed by miR-31 upon TGF-β/TNF-α treatment, the motility of LIM 1863 cells was inhibited (Fig. 2.8C). Furthermore, the anti-miR experiments also suggested that while miR-31 is not as critical as miR-21 in TGF-β/TNF-α-induced enhancement of migration, in terms of invasiveness, miR-21 and miR-31 play non-redundant roles and both are indispensable (Fig. 2.8D). This finding highlights the need to identify a comprehensive cohort of genes regulated by miR-21 and miR-31. Recent technological advances and an increased understanding of the biochemistry underlying miRNA target recognition make it possible to use high-throughput methods (i.e. deep-sequencing or proteomics) to identify these targets and test their functional relevance. Additionally, such data will prove useful in deciphering whether these miRNAs, either individually or in concert, target mRNAs with similar functions, thereby effecting specific biological pathways that are regulated by TGF-β, such as those pertaining to migratory and invasive cellular properties. Nevertheless, the smaller-scale efforts undertaken in this study identified TIAM1 as a biologically functional target of miR-21 and miR-31.

As a GEF for the Rho family of small G proteins, TIAM1 has been implicated in many aspects of cellular regulation, and its roles in several types of cancer have been documented (Mertens et al., 2003; Minard et al., 2004). However, the contribution of TIAM1 to tumor growth, invasion and metastasis is rather complicated and context-dependent. For example, whether TIAM1 stimulates migration or adhesion may depend on which G protein is specifically under TIAM1 regulation. A recent model suggests that in migratory cells, TIAM1 preferentially activates Rho, whereas in adherent cells TIAM1

preferentially activates Rac (Minard et al., 2004). Mediation of adherence by Rac is counterintuitive as Rac often plays a central role in migration by stimulating lamellipodia formation (Ridley, 2001). However, Rac is not required for migration and has been linked to the formation of cadherin-based adhesion structures (Ridley, 2001). Perhaps the more convincing evidence for the role of TIAM1 in migration and invasion comes from studies with the *Tiam* $^{-/-}$ mice. Interestingly, while ablation of the TIAM1 gene significantly inhibited tumorigenesis in a Ras-induced skin cancer model, the tumor that did form progressed to malignance more efficiently (Malliri et al., 2002). More relevant to our study, in an *APC* mutant Min mouse strain that develop intestinal tumors, TIAM1 deficiency reduced the incidence of polyps formation, but the tumors were more invasive (Malliri et al., 2006). Consistent with this, knockdown of TIAM1 by siRNA suppressed the proliferation of DLD1 colon cancer cells and reduced cell adhesion (Malliri et al., 2006). These all support our model that downregulation of TIAM1 by miR-21/miR-31 facilitates colon cancer invasion and metastasis.

There is likely more complexity regarding the roles of miR-21 and miR-31 in cancer biology, considering that each miRNA impacts on many targets (Bartel, 2004). From many miRNA profile studies of clinical samples, a high miR-21 level has emerged as a common molecular marker of several types of solid tumor including colon cancer (Cummins et al., 2006; Volinia et al., 2006). Several targets for miR-21 have been demonstrated in many cell lines, and it is evident that miR-21 is involved in multiple aspects of cancer development from initiation to metastasis (Krichevsky and Gabriely,

2009). Interestingly, opposite to our hypothesis in colon cancer cells, miR-31 appears to be a negative regulator of breast cancer metastasis (Valastyan et al., 2009; Valastyan et al., 2010). In breast cancer cells, the anti-metastasis impact of miR-31 was attributed to suppression of integrin α5, radixin and rhoA, and TIAM1 was not identified as a miR-31 target in that context (Valastyan et al., 2009; Valastyan et al., 2010). Whether the difference in biological outcomes could be attributed to distinct cohorts of mRNAs regulated by miR-31 in colon versus breast cancer cells still awaits investigation. Equally possible is that TIAM1 function could also be cancer cell-type specific, and indeed previous studies have found that heightened TIAM1 serves to enhance motility and invasion of breast cancer cells, in contrast to our observation here in colon cancer cells (Bourguignon et al., 2000; Moriarty et al., 2010). How TIAM1 may function in a cell context-dependent manner is another interesting question in understanding the molecular underpinnings of carcinoma metastasis.

Regulation of miR-21 and miR-31 by TGF-β and TNF-α

Many studies identified changes in miRNA levels that accompany normal development and disease processes (Bushati and Cohen, 2007). With elucidation of the biological functions of various miRNAs, how the expression levels of these miRNAs are regulated in normal and pathophysiological contexts become important questions. Conceivably, regulation of miRNA biogenesis can occur at several points, including transcription, posttranscriptional processing, nuclear export and RISC loading (Kim et al., 2009).

There are a number of examples in which miRNA gene transcription is activated by transcription factors that are also known to regulate protein-coding genes. For instance, the transcription factors myogenin and MyoD1 directly bind to the loci of *miR-1* and *miR-133* to upregulate expression of these miRNAs during skeletal muscle differentiation (Chen et al., 2006; Rao et al., 2006). Similarly, the tumor suppressor p53 transcriptionally activates the miR-34 and miR-107 families to enhance cell cycle arrest and apoptosis (He et al., 2007b).

An interesting observation presented in Chapter III is that the elevation of both miR-21 and miR-31 is not an immediate early signaling event downstream of TGF-β/TNF-α and requires *de novo* protein synthesis. Moreover, it is the processing from precursor to mature miR-21 that is more rate limiting. This agrees with a report by Davis *et al.* in which TGF-β upregulation of miR-21 in vascular smooth muscle cells (VSMC) was attributed primarily to miRNA maturation, not transcription (Davis et al., 2008). However, in LIM 1863 cells at least, transcription of the miR-21 gene was robustly activated by TGF-β/TNF-α, with a time course typical of an immediate early target gene. Therefore, the transcriptional regulation of miR-21 by TGF-β/TNF-α is cell context-dependent. In VSMC the processing of miR-21 was acutely activated by TGF-β (Davis et al., 2008), but in contrast the accumulation of mature miR-21 in LIM 1863 organoids was much delayed and dependent on new protein synthesis. Therefore, the underlying mechanism for TGF-β/TNF-α upregulation of mature miR-21 in LIM 1863 likely differs from that in VSMC.

Further investigations put forth in Chapter III, show that Smad4, acting as a transcription factor, is responsible for TGF-β-induced *miR-21* gene transcription. Interestingly, in primary smooth muscle cells TGF-β upregulates the level of miR-21 through a posttranscriptional mechanism that is independent of Smad4 (Davis et al., 2008). It was proposed that upon TGF-β treatment, the interaction between R-Smads and pri-miRNAs containing a 5'-CAGAC-3' motif in their stem region facilitates recruitment of the Drosha Microprocessor and enhances processing of pri-miRNA into precursor miRNA. Interestingly, Smad4 apparently lacks such ability to bind RNA or the Drosha/DGCR8/p68 complex and does not play a role in Drosha-mediated miRNA processing (Davis et al., 2008). An unresolved question is whether such R-Smad-mediated regulation of pri-miRNA processing is a common mechanism in other cell lines.

On the other hand, our study in Chapter III strongly supports the role of Smad4 as a classic transcription factor in activating the transcription of *miR-21* in response to TGF-β. We observed this function of Smad4 in a number of cell lines. Our experiments with actinomycin D indicated that TGF-β upregulates mature miR-21 level mainly through enhancing the transcription of the miR-21 gene. Therefore, R-Smads and Smad4 may act at different steps of the miRNA biosynthesis pathway, in a cell context-dependent manner, to regulate the level of specific miRNAs such as miR-21. Recent studies also suggested that miRNA precursor processing can be co-transcriptional (Morlando et al.,

2008; Pawlicki and Steitz, 2008). In this model, Drosha is recruited to the miRNA gene locus and such recruitment appears to require ongoing transcription. Thus, it is plausible that the function of Smad as a typical transcription factor and its role in miRNA precursor processing can be linked.

MiR-21 is emerging as a critical player in cancer biology. The level of miR-21 is elevated in several major types of solid tumor and could serve as a useful molecular marker of these cancers (Volinia et al., 2006). Recent work in mouse models further confirmed the general pro-tumorigenic function of miR-21 (Hatley et al., 2010; Medina et al., 2010). Besides our finding here that the TGF-β pathway directly activates transcription of the *miR-21* gene, transcription factors such as STAT3, AP1 and steroid hormone receptors have also been found to upregulate the transcription of *miR-21* (Loffler et al., 2007; Fujita et al., 2008; Ribas et al., 2009; Wickramasinghe et al., 2009). Therefore, the promoter region of *miR-21* appears to be responsive to a multitude of signaling pathways, consistent with the idea that this biologically important miRNA is under exquisite regulation.

Implications for the Role of miR-21, miR-31, and Stromal Factors in Carcinoma Metastasis

A provocative finding presented in Chapters II and III of this study is that both TGF-β and TNF-α act together to upregulate miR-21 and miR-31- a finding that hints at the interplay between tumor cells and their surrounding environment during malignant

conversion. In the tumor stroma, TGF-β and TNF-α are present and generate an environment permissive for tumor metastasis (Tlsty and Coussens, 2006). Tumor cells synthesize and secrete copious amounts of TGF-β (Derynck et al., 2001), which initiates alterations in cell physiology and the surrounding extracellular matrix to progress tumors toward malignancy (see Chapter I). Furthermore, elevated TGF-β levels in the tumor microenvironment attract macrophage precursors (Assoian et al., 1987). Infiltrating macrophages are stimulated by TGF-β to secrete a milieu of cytokines that includes TGF-β and TNF-α (Roberts et al., 1988; Tlsty and Coussens, 2006). Like TGF-β, chronic levels of TNF-α are also associated with tumor progression and metastasis, as this cytokine promotes cell migration, proliferation, ECM remodeling, stroma development and angiogenesis (Szlosarek et al., 2006; Tlsty and Coussens, 2006). Secretion of TGF-β and TNF-α by tumor cells and stromal cells, respectively, form a locally sustained source of these cytokines in the tumor microenvironment, which facilitates carcinoma malignancy.

In culture, stimulation of LIM 1863 organoids with TGF-β and TNF-α leads to EMT, autocrine production of TGF-β and TNF-α, (Bates and Mercurio, 2003; Bates et al., 2005) and increased levels of miR-21 and miR-31 (Chapters II and III). In LIM 1863 organoids, TNF-α is not sufficient to increase levels of either miR-21 or miR-31 (Chapter II) or drive EMT induction in this colon carcinoma model (Bates and Mercurio, 2003). However, TGF-β alone impacts miR-21 biogenesis at multiple levels (Chapters II and III). Furthermore, in LIM 1863 organoids TGF-β induces EMT, albeit it is a slow

process (5-7 days) in comparison to TGF-β/TNF-α stimulated EMT (24-48 hours). As demonstrated in Chapter II, the combined exposure of LIM 1863 cells to TGF-β/TNF-α not only accelerates EMT, but also intensifies the upregulation of both miR-21 and miR-31. Thus, it is feasible that a tumor microenvironment rich in TGF-β causes macrophage infiltration, TNF-α secretion and subsequent activation of signal cascades to yield increased levels of miR-21 and miR-31 as a means to facilitate tumor metastasis (Fig. 4.1).

Elevated levels of miR-21 and miR-31 facilitate metastatic conversion of colon carcinoma cells by altering tumor cell plasticity (Chapter II). It is also possible that miR-21 and miR-31 activities influence the tumor microenvironment. Support for this idea is provided by the finding in Chapter II that miR-21 and miR-31 function increases the expression of fibronectin (FN-1), laminin-γ5 (LAMC2), matrix metalloprotease-7 (MMP-7), and interleukin-8 (IL-8), which are all upregulated to a greater extent in the presence of TGF-β. These factors are all markers of EMT and metastasis and play roles in altering the tumor microenvironment (Bates et al., 2004; Vincan et al., 2007b). Continual deposition of extracellular matrix proteins such as FN-1 and LAMC2, and their degradation by proteases like matrix MMP-7, contribute to remodeling of the tumor microenvironment and enables tumor cells to migrate and invade (Stetler-Stevenson et al., 1993).

Figure 4.1

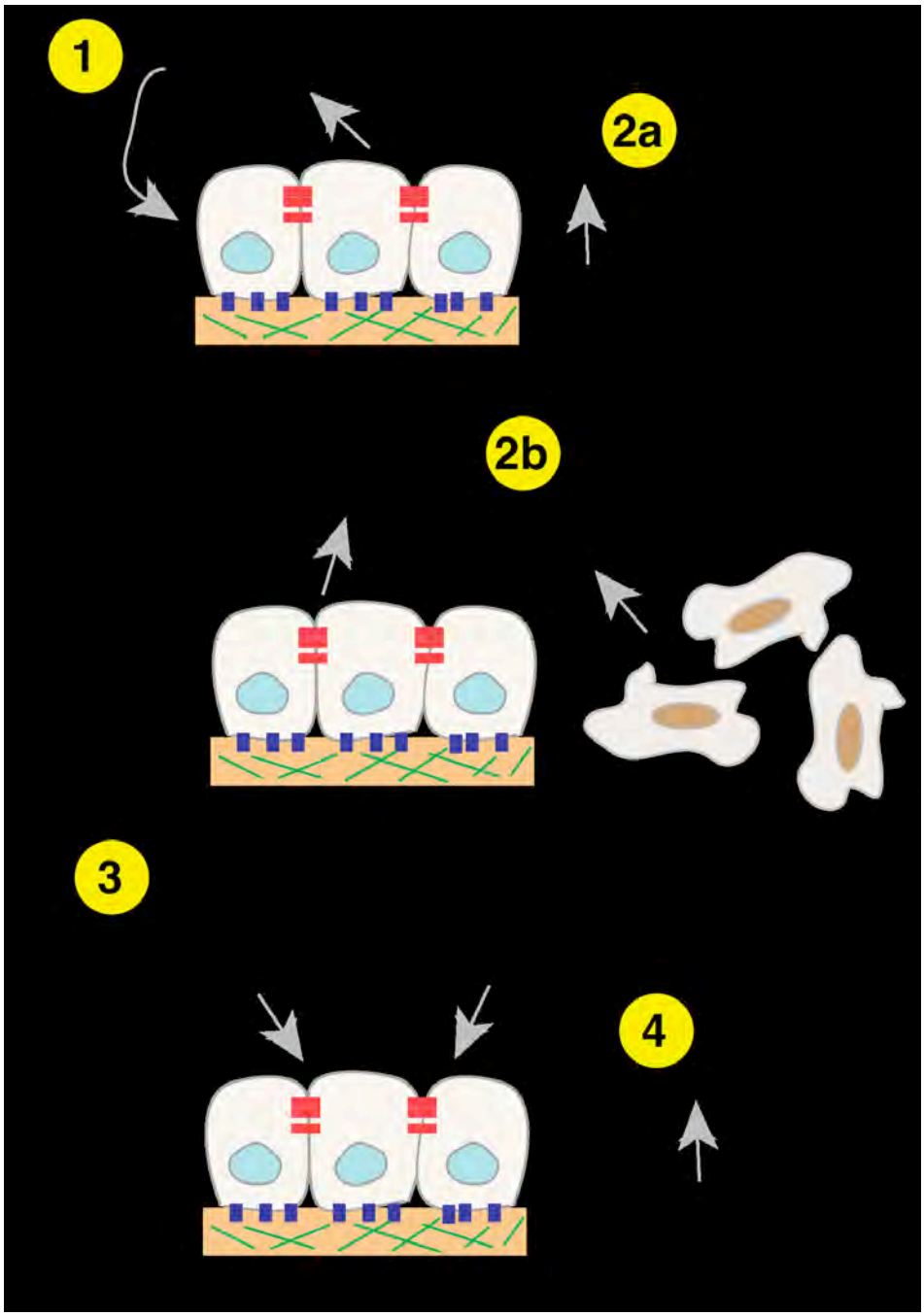

Figure 4.1 | Contribution of tumor cells and the microenvironment to the upregulation of miR-21 and miR-31. (1) Tumor cells secrete TGF-β in an autocrine fashion, which initiates alterations in tumor cell physiology, **(2a)** moderately upregulates *miR-21* and **(2a)** attracts macrophage precursors into the area surrounding the tumor. **(2b)** Infiltrating macrophages secrete TNF-α. **(3)** Increased levels of TGF-β and TNF-α act on tumor cells and lead to **(4)** significant upregulation of mature miR-21 and miR-31 levels.

Furthermore, upregulation of MMP7 expression in cancer cells activates pro-MMP9, which in turn is linked to the proteolytic activation of latent TGF-β (Yu and Stamenkovic, 2000; Ii et al., 2006). This suggests that miR-21 and miR-31 may cause increased biologically active TGF-β in the tumor stroma.

Increased levels of IL-8 are associated with invasive and malignant phenotypes in colon carcinoma (Li et al., 2001) and is secreted by LIM 1863 cells during EMT (Bates et al., 2004). Besides leukocyte attraction, IL-8 is also involved with angiogenesis, which facilitates tumor survival and spread of carcinoma cells to distant locations (Koch et al., 1992). Provocatively, miR-31 leads to the secretion of soluble factors that appear to be trophic in nature, as conditioned media from miR-31 overexpressing cells altered the morphology of control cells (Chapter II). The identity of these factors awaits further investigation. Taken together, it appears that the microenvironment of the tumor facilitates upregulation of miR-21 and miR-31, which then mediates the influence of TGF-β on metastasis.

Conclusions and Remaining Questions

Aberrant TGF-β signaling forms the basis of several diseases, especially cancer. In later stages of tumor progression, in which TGF-β is highly expressed, TGF-β signaling often promotes tumor invasion and metastasis (Massagué et al., 2000; Han, 2005). It is believed that cancerous cells gain metastatic capability by recapitulating EMT, which

TGF-β is known to potently induce (Thiery, 2002; Nawshad et al., 2005). Therefore elucidating the molecular underpinnings that enable carcinoma cells to respond to TGF-β with EMT and ultimately metastasis may yield novel points of regulation in the TGF-β signaling pathway. Most importantly, increased understanding of how TGF-β exerts its pro-metastasis function may aid in developing therapies to impede tumor progression. To that end, this research shows that miR-21 and miR-31 are components of a larger molecular program that TGF-β initiates to ultimately elicit EMT, migration and invasion in colorectal carcinoma (Fig. 4.2). This new knowledge impacts our understanding of the regulatory mechanisms involved with the metastasis-promoting utility of TGF-β.

Given the actions of miR-21 and miR-31 on TGF-β-mediated EMT, migration, and invasion in colon carcinoma, what can be inferred about the role of these miRNAs in the role of TGF-β in normal colonic physiology? As described in Chapter I, EMT and migration play important roles in tissue regeneration that is mediated by TGF-β (Kalluri and Weinberg, 2009). Furthermore, the colon epithelium suffers persistent injury from parasite, bacterial, and viral infections in addition to tissue damage caused by toxins absorbed through the diet. These injuries often lead to chronic states of inflammation and disorders such as irritable bowel syndrome, Crohn's disease and ulcerative colitis (Xavier and Podolsky, 2007). At these sites of tissue damage, both TGF-β and TNF-α are present due to similar mechanisms as those observed in the microenvironment of tumor cells. In the colon, tissue injury initiates a number of molecular events, such as activation of the NFκB pathway in immune and intestinal epithelial cells (Lin and Karin, 2007; Nenci et

al., 2007). NFkB signaling leads to upregulation and secretion of a milieu of pro-inflammatory cytokines, including TNF-α, to mediate inflammation as a component of the host immune response (Lin and Karin, 2007). To limit the duration of the inflammatory process, anti-inflammatory cytokines, such as TGF-β, are upregulated (Lin and Karin, 2007). TGF-β serves a dual role- both mediating the conclusion of inflammation and orchestrating the repair of the injured tissue. Thus, exposure of colonic tissue to pathogens can lead to increased levels of TGF-β and TNF-α, which then act on the colon epithelium, increasing levels of miR-21 and miR-31. Once upregulated, it is possible that these miRNAs mitigate the actions of TGF-β (i.e., stimulation of EMT and migration) during repair of the colonic mucosa. An equally provocative and unresolved question includes, how might miR-21 and miR-31 impact the role of TGF-β in the repair of other tissues, such as those of the kidney or liver? Do these miRNAs play a role in the pathophysiological counterpart of tissue repair, fibrosis? At present studies are underway in our laboratory to determine the contribution of miRNAs to kidney fibrosis. How miR-21 and miR-31 impact the role of TGF-β in renal fibrosis is currently an open question.

Figure 4.2

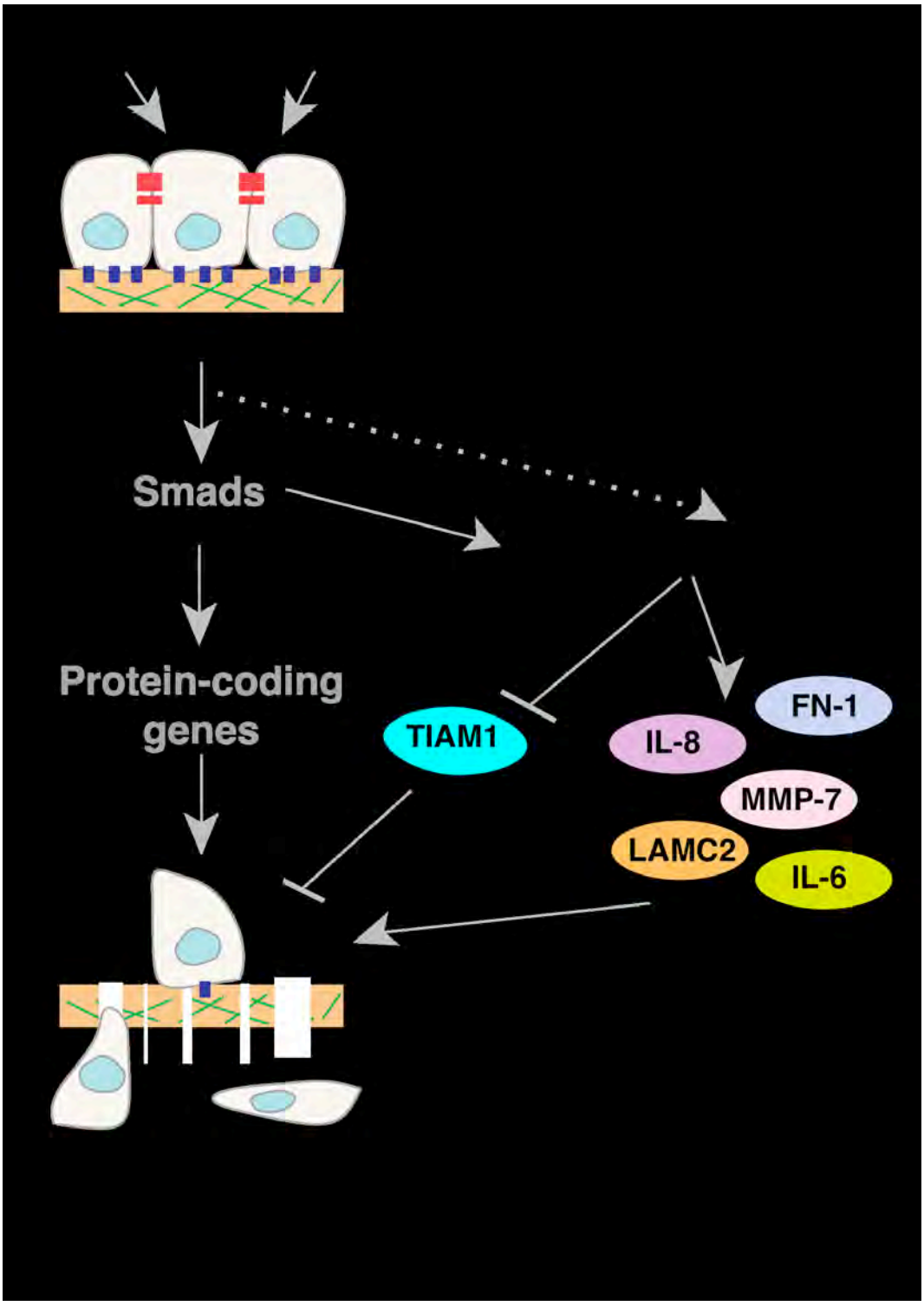

Figure 4.2 | The role of miR-21 and miR-31 in the pro-metastasis function of TGF-β in colorectal carcinoma. TGF-β cooperates with TNF-α to increase levels of miR-21 and miR-31, two miRNAs that facilitate the ability of TGF-β to induce EMT, migration, and invasion of colorectal carcinoma cells.

REFERENCES

Abramoff, M.D., Magelhaes, P.J. and Ram, S.J. Image processing with ImageJ. *Biophotonics International* **11** (2004), pp. 36-42.

Akhurst, R., Fee, F. and Balmaint, A. Localized production of TGF-β mRNA in tumour promoter-stimulated mouse epidermis. *Nature* **331** (1988), pp. 363-365.

Alexandrow, M.G. and Moses, H.L. Transforming growth factor-beta and cell cycle regulation. *Cancer Research* **55** (1995), pp. 1452-1457.

Assoian, R.K., Fleurdelys, B.E., Stevenson, H.C., Miller, P.J., Madtes, D.K., Raines, E.W., Ross, R. and Sporn, M.B. Expression and secretion of type beta transforming growth factor by activated human macrophages. *Proceedings of the National Academy of Sciences USA* **84** (1987), pp. 6020-6024.

Bandrés, E., Cubedo, E., Agirre, X., Malumbres, R., Zarate, R., Ramirez, N., Abajo, A., Navarro, A., Moreno, I. and Monzo, M. Identification by Real-time PCR of 13 mature microRNAs differentially expressed in colorectal cancer and non-tumoral tissues. *Molecular Cancer* **5** (2006), p. 29.

Bartel, D.P. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* **116** (2004), pp. 281-297.

Bartel, D.P. MicroRNAs: target recognition and regulatory functions. *Cell* **136** (2009), pp. 215-233.

Bates, R.C., Bellovin, D.I., Brown, C., Maynard, E., Wu, B., Kawakatsu, H., Sheppard, D., Oettgen, P. and Mercurio, A.M. Transcriptional activation of integrin beta6 during the epithelial-mesenchymal transition defines a novel prognostic indicator of aggressive colon carcinoma. *Journal Clinical Investigation* **115** (2005), pp. 339-347.

Bates, R.C., DeLeo III, M.J. and Mercurio, A.M. The epithelial-mesenchymal transition of colon carcinoma involves expression of IL-8 and CXCR-1-mediated chemotaxis. *Experimental Cell Research* **299** (2004), pp. 315-324.

Bates, R.C. and Mercurio, A.M. Tumor necrosis factor-alpha stimulates the epithelial-to-mesenchymal transition of human colonic organoids. *Molecular Biology of the Cell* **14** (2003), pp. 1790-1800.

Bierie, B. and Moses, H.L. TGF-β and cancer. *Cytokine & Growth Factor Reviews* **17** (2006a), pp. 29-40.

Bierie, B. and Moses, H.L. Tumour microenvironment: TGFβ: the molecular Jekyll and Hyde of cancer. *Nature Reviews Cancer* **6** (2006b), pp. 506-520.

Biswas, S., Guix, M., Rinehart, C., Dugger, T.C., Chytil, A., Moses, H.L., Freeman, M.L. and Arteaga, C.L. Inhibition of TGF-β with neutralizing antibodies prevents radiation-induced acceleration of metastatic cancer progression. *Journal of Clinical Investigation* **117** (2007), pp. 1305-1313.

Border, W. and Noble, N. Transforming growth factor β in tissue fibrosis. *New England Journal of Medicine* **331** (1994), pp. 1286-1292.

Border, W.A. and Ruoslahti, E. Transforming growth factor-β in disease: the dark side of tissue repair. *Journal of Clinical Investigation* **90** (1992), pp. 1-7.

Bourguignon, L.Y.W., Zhu, H., Shao, L. and Chen, Y.W. Ankyrin-Tiam1 interaction promotes Rac1 signaling and metastatic breast tumor cell invasion and migration. *The Journal of Cell Biology* **150** (2000), pp. 177-192.

Broadley, K.N., Aquino, A.M., Woodward, S.C., Buckley-Sturrock, A., Sato, Y., Rifkin, D.B. and Davidson, J.M. Monospecific antibodies implicate basic fibroblast growth factor in normal wound repair. *Laboratory investigation; a journal of technical methods and pathology* **61** (1989), pp. 571-575.

Buck, M.B., Fritz, P., Dippon, J., Zugmaier, G. and Knabbe, C. Prognostic significance of transforming growth factor beta receptor II in estrogen receptor-negative breast cancer patients. *Clinical Cancer Research* **10** (2004), pp. 491-498.

Burk, U., Schubert, J., Wellner, U., Schmalhofer, O., Vincan, E., Spaderna, S. and Brabletz, T. A reciprocal repression between ZEB1 and members of the miR-200 family promotes EMT and invasion in cancer cells. *EMBO Rep* **9** (2008a), pp. 582-9.

Burk, U., Schubert, J., Wellner, U., Schmalhofer, O., Vincan, E., Spaderna, S. and Brabletz, T. A reciprocal repression between ZEB1 and members of the miR-200 family promotes EMT and invasion in cancer cells. *EMBO reports* **9** (2008b), pp. 582-589.

Bushati, N. and Cohen, S.M. MicroRNA functions. *Annual Review of Cell and Developmental Biology* **23** (2007), pp. 175-205.

Cai, X., Hagedorn, C.H. and Cullen, B.R. Human microRNAs are processed from capped, polyadenylated transcripts that can also function as mRNAs. *RNA* **10** (2004), pp. 1957-1966.

Calin, G., Dumitru, C., Shimizu, M., Bichi, R., Zupo, S., Noch, E., Aldler, H., Rattan, S., Keating, M., Rai, K., Rassenti, L., Kipps, T., Negrini, M., Bullrich, F. and Croce, C.M. Frequent deletions and down-regulation of micro-RNA genes *miR15* and *miR16* at 13q14 in chronic lymphocytic leukemia. *Proceedings of the National Academy of Sciences USA* **99** (2002), pp. 15524-15529.

Chang, T.C., Wentzel, E.A., Kent, O., Ramachandran, K., Mullendore, M., Lee, K., Feldmann, G., Yamakuchi, M., Ferlito, M., Lowenstein, C.J., Arking, D.E., Beer, M.A., Maitra, A. and Mendell, J.T. Transactivation of miR-34a by p53 broadly influences gene expression and promotes apoptosis. *Molecular Cell* **26** (2007), pp. 745-752.

Chen, C.R., Kang, Y. and Massagué, J. Defective repression of c-myc in breast cancer cells: a loss at the core of the transforming growth factor beta growth arrest program. *Proceedings of the National Academy of Sciences USA* **98** (2001), pp. 992-999.

Chen, J.F., Mandel, E.M., Thomson, J.M., Wu, Q., Callis, T.E., Hammond, S.M., Conlon, F.L. and Wang, D.Z. The role of microRNA-1 and microRNA-133 in skeletal muscle proliferation and differentiation. *Nature Genetics* **38** (2006), pp. 228-233.

Chen, X. and Xu, L. Specific nucleoporin requirement for smad nuclear translocation. *Molecular and Cellular Biology* **30** (2010), pp. 4022-4034.

Cimmino, A., Calin, G., Fabbri, M., Iorio, M., Ferracin, M., Shimizu, M., Wojcik, S., Aqeilan, R., Zupo, S., Dono, M., Rassenti, L., Hansjuerg, A., Volinia, S., Liu, C., Kipps, T., Massimo, N. and Croce, C.M. *miR-15* and *miR-16* induce apoptosis by targeting BCL2. *Proceedings of the National Academy of Sciences USA* **102** (2005), p. 13944.

Costinean, S., Sandhu, S.K., Pedersen, I.M., Tili, E., Trotta, R., Perrotti, D., Ciarlariello, D., Neviani, P., Harb, J. and Kauffman, L.R. Src homology 2 domainñcontaining inositol-5-phosphatase and CCAAT enhancer-binding protein are targeted by miR-155 in B cells of E[m] -MiR-155 transgenic mice. *Blood* **114** (2009), pp. 1374-1382.

Costinean, S., Zanesi, N., Pekarsky, Y., Tili, E., Volinia, S., Heerema, N. and Croce, C.M. Pre-B cell proliferation and lymphoblastic leukemia/high-grade lymphoma in E[m]-miR155 transgenic mice. *Proceedings of the National Academy of Sciences USA* **103** (2006), pp. 7024-7029.

Cottonham, C.L., Kaneko, S. and Xu, L. miR-21 and miR-31 converge on TIAM1 to regulate migration and invasion of colon carcinoma cells. *Journal of Biological Chemistry* **285** (2010), pp. 35293-35302

Coussens, L.M. and Werb, Z. Inflammation and cancer. *Nature* **420** (2002), pp. 860-867.

Cui, W., Fowlis, D.J. and Bryson, S. TGF-β1 inhibits the formation of benign skin tumors, but enhances progression to invasive spindle carcinomas in transgenic mice. *Cell* **86** (1996), pp. 531-542.

Cummins, J.M., He, Y., Leary, R.J., Pagliarini, R., Diaz, L.A., Jr., Sjoblom, T., Barad, O., Bentwich, Z., Szafranska, A.E., Labourier, E., Raymond, C.K., Roberts, B.S., Juhl, H., Kinzler, K.W., Vogelstein, B. and Velculescu, V.E. The colorectal microRNAome. *Proc Natl Acad Sci U S A* **103** (2006), pp. 3687-92.

Dalal, B., Keown, P. and Greenberg, A. Immunocytochemical localization of secreted transforming growth factor-beta 1 to the advancing edges of primary tumors and to lymph node metastases of human mammary carcinoma. *The American Journal of Pathology* **143** (1993), pp. 381-389.

Datto, M.B., Li, Y., Panus, J.F., Howe, D.J., Xiong, Y. and Wang, X.F. Transforming growth factor beta induces the cyclin-dependent kinase inhibitor p21 through a p53-independent mechanism. *Proceedings of the National Academy of Sciences USA* **92** (1995), pp. 5545-5549.

Davis, B.N., Hilyard, A.C., Lagna, G. and Hata, A. SMAD proteins control DROSHA-mediated microRNA maturation. *Nature* **454** (2008), pp. 56-61.

Davis, B.N., Hilyard, A.C., Nguyen, P.H., Lagna, G. and Hata, A. Smad Proteins Bind a Conserved RNA Sequence to Promote MicroRNA Maturation by Drosha. *Molecular Cell* **39** (2010), pp. 373-384.

Denli, A.M., Tops, B.B., Plasterk, R.H., Ketting, R.F. and Hannon, G.J. Processing of primary microRNAs by the Microprocessor complex. *Nature* **432** (2004), pp. 231-235.

Derynck, R., Akhurst, R.J. and Balmain, A. TGF-β signaling in tumor suppression and cancer progression. *Nature Genetics* **29** (2001), pp. 117-129.

Derynck, R., Goeddel, D.V., Ullrich, A., Gutterman, J.U., Williams, R.D., Bringman, T.S. and Berger, W.H. Synthesis of messenger RNAs for transforming growth factors and and the epidermal growth factor receptor by human tumors. *Cancer Research* **47** (1987), pp. 707-712.

Derynck, R. and Zhang, Y.E. Smad-dependent and Smad-independent pathways in TGF-β family signalling. *Nature* **425** (2003), pp. 577-584.

Dickson, M., Martin, J., Cousins, F., Kulkarni, A., Karlsson, S. and Akhurst, R. Defective haematopoiesis and vasculogenesis in transforming growth factor-beta 1 knock out mice. *Development* **121** (1995), pp. 1845-1854.

Dickson, R.B., Kasid, A., Huff, K.K., Bates, S.E., Knabbe, C., Bronzert, D., Gelmann, E.P. and Lippman, M.E. Activation of growth factor secretion in tumorigenic states of breast cancer induced by 17β-estradiol or v-Ha-ras oncogene. *Proceedings of the National Academy of Sciences USA* **84** (1987), pp. 837-841.

Dvorak, H.F. Tumors: wounds that do not heal: similarities between tumor stroma generation and wound healing. *The New England Journal of Medicine* **315** (1986), pp. 1650-1659.

Elliott, R.L. and Blobe, G.C. Role of transforming growth factor-β in human cancer. *Journal of Clinical Oncology* **23** (2005), pp. 2078-2093.

Eppert, K., Scherer, S., Ozcelik, H., Pirone, R., Hoodless, P., Kim, H., Tsui, L., Bapat, B., Gallinger, S. and Andrulis, I. MADR2 maps to 18q21 and encodes a TGF β-regulated MAD-related protein that is functionally mutated in colorectal carcinoma. *Cell* **86** (1996), pp. 543-552.

Fabbri, M., Garzon, R., Cimmino, A., Liu, Z., Zanesi, N., Callegari, E., Liu, S., Alder, H., Costinean, S., Fernandez-Cymering, C., Volinia, S., Guler, G., Morrison, C.D., Chan, K.K., Marcucci, G., Calin, G.A., Huebner, K. and Croce, C.M. MicroRNA-29 family reverts aberrant methylation in lung cancer by targeting DNA methyltransferases 3A and 3B. *Proceedings of the National Academy of Sciences USA* **104** (2007), pp. 15805-15810.

Feng, X.H. and Derynck, R. Specificity and versatility in TGF-β signaling through Smads. *Annual Review of Cell and Developmental Biology* **21** (2005), pp. 659-693.

Fitchett, J.E. and Hay, E.D. Medial edge epithelium transforms to mesenchyme after embryonic palatal shelves fuse. *Developmental Biology* **131** (1989), pp. 455-474.

Fujita, S., Ito, T., Mizutani, T., Minoguchi, S., Yamamichi, N., Sakurai, K. and Iba, H. miR-21 Gene expression triggered by AP-1 is sustained through a double-negative feedback mechanism. *Journal of Molecular Biology* **378** (2008), pp. 492-504.

Garzon, R., Calin, G. and Croce, C. MicroRNAs in cancer. *Annual Review of Medicine* **60** (2009), pp. 167-179.

Garzon, R., Garofalo, M., Martelli, M.P., Briesewitz, R., Wang, L., Fernandez-Cymering, C., Volinia, S., Liu, C.-G., Schnittger, S., Haferlach, T., Liso, A., Diverio, D.,

Mancini, M., Meloni, G., Foa, R., Martelli, M.F., Mecucci, C., Croce, C.M. and Falini, B. Distinctive miRNA signature of acute myeloid leukemia bearing cytoplasmic mutated nucleophosmin. *Proceedings of the National Academy of Sciences USA* **105** (2008), pp. 3945-3950.

Garzon, R., Volinia, S., Liu, C.G., Fernandez-Cymering, C., Palumbo, T., Pichiorri, F., Fabbri, M., Coombes, K., Alder, H., Nakamura, T., Flomenberg, N., Marcucci, G., Calin, G.A., Kornblau, S.M., Kantarjian, H., Bloomfield, C.D., Andreff, M. and Croce, C.M. MicroRNA signatures associated with cytogenetics and prognosis in acute myeloid leukemia. *Blood* **111** (2005), pp. 3183-3189.

Geissmann, F., Revy, P., Regnault, A., Lepelletier, Y., Dy, M., Brousse, N., Amigorena, S., Hermine, O. and Durandy, A. TGF-β1 prevents the noncognate maturation of human dendritic Langerhans cells. *The Journal of Immunology* **162** (1999), pp. 4567-4575.

Goggins, M., Shekher, M., Turnacioglu, K., Yeo, C.J., Hruban, R.H. and Kern, S.E. Genetic alterations of the transforming growth factor β receptor genes in pancreatic and biliary adenocarcinomas. *Cancer Research* **58** (1998), pp. 5329-5332.

Greenburg, G. and Hay, E.D. Epithelia suspended in collagen gels can lose polarity and express characteristics of migrating mesenchymal cells. *The Journal of Cell Biology* **95** (1982), pp. 333-339.

Gregory, P., Bert, A., Paterson, E., Barry, S., Tsykin, A., Farshid, G., Vadas, M., Khew-Goodall, Y. and Goodall, G. The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and SIP1. *Nature Cell Biology* **10** (2008a), pp. 593-601.

Gregory, P.A., Bert, A.G., Paterson, E.L., Barry, S.C., Tsykin, A., Farshid, G., Vadas, M.A., Khew-Goodall, Y. and Goodall, G.J. The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and SIP1. *Nat Cell Biol* **10** (2008b), pp. 593-601.

Gregory, R., Yan, K.-p., Amuthan, G., Chendrimada, T., Doratotaj, B., Cooch, N. and Shiekhattar, R. The Microprocessor complex mediates the genesis of microRNAs. *Nature* **432** (2004), pp. 235-240.

Gregory, R.I., Chendrimada, T.P., Cooch, N. and Shiekhattar, R. Human RISC couples microRNA biogenesis and posttranscriptional gene silencing. *Cell* **123** (2005), pp. 631-640.

Griffiths-Jones, S., Saini, H.K., Van Dongen, S. and Enright, A.J. miRBase: tools for microRNA genomics. *Nucleic Acids Research* **36** (2008), pp. D154-D158.

Guo, H., Ingolia, N.T., Weissman, J.S. and Bartel, D.P. Mammalian microRNAs predominantly act to decrease target mRNA levels. *Nature* **466** (2010), pp. 835-840.

Gupta, A., Gartner, J., Sethupathy, P., Hatzigeorgiou, A. and Fraser, N. Anti-apoptotic function of a microRNA encoded by the HSV-1 latency-associated transcript. *Nature* **442** (2006), pp. 82-85.

Gusev, Y., Schmittgen, T., Lerner, M., Postier, R. and Brackett, D. Computational analysis of biological functions and pathways collectively targeted by co-expressed microRNAs in cancer. *BMC Bioinformatics* **8** (2007), p. S16.

Habets, G.G., Scholtes, E.H., Zuydgeest, D., van der Kammen, R.A., Stam, J.C., Berns, A. and Collard, J.G. Identification of an invasion-inducing gene, Tiam-1, that encodes a protein with homology to GDP-GTP exchangers for Rho-like proteins. *Cell* **77** (1994), pp. 537-49.

Haddow, A. Molecular repair, wound healing, and carcinogenesis: tumor production a possible overhealing. *Advanced Cancer Research* **16** (1972), pp. 181-234.

Hahn, S.A., Schutte, M., Hoque, A.T.M., Moskaluk, C.A., da Costa, L.T., Rozenblum, E., Weinstein, C.L., Fischer, A., Yeo, C.J., Hruban, R.H. and Kern, S.E. DPC4, a candidate tumor suppressor gene at human chromosome 18q21.1. *Science* **271** (1996), pp. 350-353.

Han, G. Distinct mechanisms of TGF-β1-mediated epithelial-to-mesenchymal transition and metastasis during skin carcinogenesis. *Journal of Clinical Investigation* **115** (2005), pp. 1714-1723.

Han, J., Lee, Y., Yeom, K.-H., Kim, Y.-K., Jin, H. and Kim, V.N. The Drosha-DGCR8 complex in primary microRNA processing. *Genes & Development* **18** (2004), pp. 3016-3027.

Han, S., Kim, H., Do Hwan Seong, Y., Park, Y., Bang, Y., Yang, H. and Kim, S. Loss of the Smad3 expression increases susceptibility to tumorigenicity in human gastric cancer. *Oncogene* **23** (2003), pp. 1333-1341.

Hanahan, D. and Weinberg, R.A. The hallmarks of cancer. *Cell* **100** (2000), pp. 57-70.

Hannon, G.J. and Beach, D. p15INK4B is a potentia| effector of TGF-beta-induced cell cycle arrest. *Nature* **371** (1994), pp. 257-261.

Hata, A., Lo, R.S., Wotton, D., Lagna, G. and Massagué, J. Mutations increasing autoinhibition inactivate tumour suppressors Smad2 and Smad4. *Nature* **388** (1997), pp. 82-87.

Hatley, M.E., Patrick, D.M., Garcia, M.R., Richardson, J.A., Bassel-Duby, R., van Rooij, E. and Olson, E.N. Modulation of K-Ras-dependent lung tumorigenesis by microRNA-21. *Cancer Cell* **18** (2010), pp. 282-293.

Hay, E.D. An overview of epithelio-mesenchymal transformation. *Cells Tissues Organs* **154** (1995), pp. 8-20.

Hayward, I., Whitehead, R., Ward, L., Gianello, R., Dempsey, P., Bates, R. and Burns, G. Effect of TGF-β on differentiated organoids of the colon carcinoma cell line LIM 1863. *Immunology and Cell Biology* **73** (1995), pp. 249-257.

Hayward, I.P. and Whitehead, R.H. Patterns of growth and differentiation in the colon carcinoma cell line LIM 1863. *International Journal of Cancer* **50** (1992), pp. 752-759.

He, L. A microRNA polycistron as a potential human oncogene. *Nature* **435** (2005), pp. 828-833.

He, L., He, X., Lim, L., de Stanchina, E., Xuan, Z., Liang, Y., Xue, W., Zender, L., Magnus, J., Ridzon, D., Jackson, A.L., Linsley, P.S., Chen, C.R., Lowe, S.W., Cleary, M.A. and Hannon, G.J. A microRNA component of the p53 tumour suppressor network. *Nature* **447** (2007a), pp. 1130-1134.

He, L., He, X., Lowe, S.W. and Hannon, G.J. microRNAs join the p53 network--another piece in the tumour-suppression puzzle. *Nature Reviews Cancer* **7** (2007b), pp. 819-822.

Heldin, C.H., Landström, M. and Moustakas, A. Mechanism of TGF-beta signaling to growth arrest, apoptosis, and epithelial-mesenchymal transition. *Current Opinion in Cell Biology* **21** (2009), pp. 166-176.

Hill, R., Song, Y., Cardiff, R.D. and Van Dyke, T. Selective evolution of stromal mesenchyme with p53 loss in response to epithelial tumorigenesis. *Cell* **123** (2005), pp. 1001-1011.

Howe, J., Roth, S., Ringold, J., Summers, R., Jarvinen, H., Sistonen, P., Tomlinson, I., Houlston, R., Bevan, S. and Mitros, F. Mutations in the SMAD4/DPC4 gene in juvenile polyposis. *Science* **280** (1998), pp. 1086-1088.

Hutvagner, G., McLachlan, J., Pasquinelli, A.E., Bálint, E., Tuschl, T. and Zamore, P.D. A cellular function for the RNA-interference enzyme Dicer in the maturation of the let-7 small temporal RNA. *Science* **293** (2001), pp. 834-838.

Ii, M., Yamamoto, H., Adachi, Y., Maruyama, Y. and Shinomura, Y. Role of matrix metalloproteinase-7 (matrilysin) in human cancer invasion, apoptosis, growth, and angiogenesis. *Experimental Biology and Medicine* **231** (2006), pp. 20-27.

Iwano, M., Plieth, D., Danoff, T., Xue, C., Okada, H. and Neilson, E. Evidence that fibroblasts derive from epithelium during tissue fibrosis. *Journal of Clinical Investigation* **110** (2002), pp. 341-350.

Janda, E., Lehmann, K., Killisch, I., Jechlinger, M., Herzig, M., Downward, J., Beug, H. and Gr¸nert, S. Ras and TGF-β cooperatively regulate epithelial cell plasticity and metastasis. *The Journal of Cell Biology* **156** (2002), pp. 299-313.

Jang, C.W., Chen, C.H., Chen, C.C., Chen, J.Y., Su, Y.H. and Chen, R.H. TGF-beta induces apoptosis through Smad-mediated expression of DAP-kinase. *Nature Cell Biology* **4** (2002), pp. 51-58.

Jechlinger, M., Grunert, S., Tamir, I.H., Janda, E., L¸demann, S., Waerner, T., Seither, P., Weith, A., Beug, H. and Kraut, N. Expression profiling of epithelial plasticity in tumor progression. *Oncogene* **22** (2003), pp. 7155-7169.

Jin, P., Alisch, R. and Warren, S. RNA and microRNAs in fragile X mental retardation. *Nature Cell Biology* **6** (2004), pp. 1048-1053.

Johnson, S., Grosshans, H., Shingara, J., Byrom, M., Jarvis, R., Cheng, A., Labourier, E., Reinert, K., Brown, D. and Slack, F. RAS is regulated by the let-7 microRNA family. *Cell* **120** (2005), pp. 635-647.

Jones, S., Zhang, X., Parsons, D.W., Lin, J.C., Leary, R.J., Angenendt, P., Mankoo, P., Carter, H., Kamiyama, H., Jimeno, A., Hong, S.M., Fu, B., Lin, M.T., Calhoun, E.S., Kamiyama, M., Walter, K., Nikolskaya, T., Nikolsky, Y., Hartigan, J., Smith, D.R., Hidalgo, M., Leach, S.D., Klein, A.P., Jaffee, E.M., Goggins, M., Maitra, A., Iacobuzio-Donahue, C., Eshleman, J.R., Kern, S.E., Hruban, R.H., Karchin, R., Papadopoulos, N., Parmigiani, G., Vogelstein, B., Velculescu, V.E. and Kinzler, K.W. Core signaling pathways in human pancreatic cancers revealed by global genomic analyses. *Science* **321** (2008), pp. 1801-6.

Kaartinen, V., Voncken, J.W., Shuler, C., Warburton, D., Bu, D., Heisterkamp, N. and Groffen, J. Abnormal lung development and cleft palate in mice lacking TGFñ 3 indicates defects of epithelial-mesenchymal interaction. *Nature Genetics* **11** (1995), pp. 415-421.

Kalluri, R. and Neilson, E. Epithelial-mesenchymal transition and its implications for fibrosis. *Journal of Clinical Investigation* **112** (2003), pp. 1776-1784.

Kalluri, R. and Weinberg, R.A. The basics of epithelial-mesenchymal transition. *The Journal of Clinical Investigation* **119** (2009), pp. 1420-1428.

Kang, Y. Pro-metastasis function of TGFβ mediated by the Smad pathway. *Journal of Cellular Biochemistry* **98** (2006), pp. 1380-1390.

Kang, Y., Chen, C.R. and Massagué, J. A self-enabling TGFbeta response coupled to stress signaling: Smad engages stress response factor ATF3 for Id1 repression in epithelial cells. *Molecular Cell* **11** (2003), pp. 915-926.

Kang, Y., He, W., Tulley, S., Gupta, G.P., Serganova, I., Chen, C.R., Manova-Todorova, K., Blasberg, R., Gerald, W.L. and Massagué, J. Breast cancer bone metastasis mediated by the Smad tumor suppressor pathway. *Proceedings of the National Academy of Sciences USA* **102** (2005), pp. 13909-13914.

Kim, I.Y., Ahn, H.J., Zelner, D.J., Shaw, J.W., Sensibar, J.A., Kim, J.H., Kato, M. and Lee, C. Genetic change in transforming growth factor beta (TGF-β) receptor type I gene correlates with insensitivity to TGF-β 1 in human prostate cancer cells. *Cancer Research* **56** (1996), pp. 44-48.

Kim, V.N., Han, J. and Siomi, M.C. Biogenesis of small RNAs in animals. *Nature Reviews Molecular Cell Biology* **10** (2009), pp. 126-139.

Kluiver, J., Poppema, S., de Jong, D., Blokzijl, T., Harms, G., Jacobs, S., Kroesen, B.J. and van den Berg, A. BIC and miR-155 are highly expressed in Hodgkin, primary mediastinal and diffuse large B cell lymphomas. *The Journal of Pathology* **207** (2005), pp. 243-249.

Knüpfer, H. and Preiss, R. Serum interleukin-6 levels in colorectal cancer patients--a summary of published results. *International Journal of Colorectal Disease* **25** (2010), pp. 135-140.

Koch, A.E., Polverini, P.J., Kunkel, S.L., Harlow, L.A., DiPietro, L.A., Elner, V.M., Elner, S.G. and Strieter, R.M. Interleukin-8 as a macrophage-derived mediator of angiogenesis. *Science* **258** (1992), pp. 1798-1801.

Kong, W., Yang, H., He, L., Zhao, J., Coppola, D., Dalton, W. and Cheng, J. MicroRNA-155 is regulated by the transforming growth factor β/Smad pathway and contributes to epithelial cell plasticity by targeting RhoA. *Molecular and Cellular Biology* **28** (2008), pp. 6773–6784.

Korpal, M., Lee, E.S., Hu, G. and Kang, Y. The miR-200 family inhibits epithelial-mesenchymal transition and cancer cell migration by direct targeting of E-cadherin transcriptional repressors ZEB1 and ZEB2. *J Biol Chem* **283** (2008a), pp. 14910-4.

Korpal, M., Lee, E.S., Hu, G. and Kang, Y. The miR-200 family inhibits epithelial-mesenchymal transition and cancer cell migration by direct targeting of E-cadherin transcriptional repressors ZEB1 and ZEB2. *Journal of Biological Chemistry* **283** (2008b), pp. 14910-14914.

Krichevsky, A.M. and Gabriely, G. miR 21: a small multi faceted RNA. *Journal of Cellular and Molecular Medicine* **13** (2009), pp. 39-53.

Krol, J., Loedige, I. and Filipowicz, W. The widespread regulation of microRNA biogenesis, function and decay. *Nature Reviews Genetics* **11** (2010), pp. 597-610.

Krupa, A., Jenkins, R., Luo, D., Lewis, A., Phillips, A. and Fraser, D. Loss of microRNA-192 promotes fibrogenesis in diabetic nephropathy. *Journal of the American Society of Nephrology* **21** (2010), pp. 438-447.

Kumar, M.S., Lu, J., Mercer, K.L., Golub, T.R. and Jacks, T. Impaired microRNA processing enhances cellular transformation and tumorigenesis. *Nat Genet* **39** (2007), pp. 673-7.

LaGamba, D., Nawshad, A. and Hay, E.D. Microarray analysis of gene expression during epithelial-mesenchymal transformation. *Developmental Dynamics* **234** (2005), pp. 132-142.

Lagna, G., Hata, A., Hemmati-Brivanlou, A. and Massagué, J. Partnership between DPC4 and SMAD proteins in TGF-β signalling pathways. *Nature* (1996), pp. 832-836.

Landthaler, M., Yalcin, A. and Tuschl, T. The human DiGeorge syndrome critical region gene 8 and its D. melanogaster homolog are required for miRNA biogenesis. *Current Biology* **14** (2004), pp. 2162-2167.

Lee, R.C., Feinbaum, R.L. and Ambros, V.T. C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell* **75** (1993), pp. 843-854.

Lee, Y. The nuclear RNase III Drosha initiates microRNA processing. *Nature* **425** (2003), pp. 415-419.

Lee, Y. and Dutta, A. The tumor suppressor microRNA let-7 represses the HMGA2 oncogene. *Genes & Development* **21** (2007), pp. 1025-1030.

Lee, Y., Kim, M., Han, J., Yeom, K., Lee, S., Baek, S. and Kim, V. MicroRNA genes are transcribed by RNA polymerase II. *EMBO Journal* **23** (2004), pp. 4051-4060.

Lehmann, K., Janda, E., Pierreux, C.E., Ryt^maa, M., Schulze, A., McMahon, M., Hill, C.S., Beug, H. and Downward, J. Raf induces TGF-β production while blocking its apoptotic but not invasive responses: a mechanism leading to increased malignancy in epithelial cells. *Genes & Development* **14** (2000), pp. 2610-2622.

Letterio, J.J., Geiser, A.G., Kulkarni, A.B., Roche, N.S., Sporn, M.B. and Roberts, A.B. Maternal rescue of transforming growth factor-beta 1 null mice. *Science* **264** (1994), pp. 1936-1938.

Lewis, B.P., Burge, C.B. and Bartel, D.P. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell* **120** (2005), pp. 15-20.

Li, A., Varney, M.L. and Singh, R.K. Expression of interleukin 8 and its receptors in human colon carcinoma cells with different metastatic potentials. *Clinical Cancer Research* **7** (2001), pp. 3298-3304.

Lim, L.P. Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. *Nature* **433** (2005), pp. 769-773.

Lim, L.P., Lau, N.C., Garrett-Engele, P., Grimson, A., Schelter, J.M., Castle, J., Bartel, D.P., Linsley, P.S. and Johnson, J.M. Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. *Nature* **433** (2005), pp. 769-773.

Loffler, D., Brocke-Heidrich, K., Pfeifer, G., Stocsits, C., Hackermüller, J., Kretzschmar, A.K., Burger, R., Gramatzki, M., Blumert, C., Bauer, K., Cvijic, H., Ullmann, A.K., Stadler, P.F. and F, H. Interleukin-6 dependent survival of multiple myeloma cells involves the Stat3-mediated induction of microRNA-21 through a highly conserved enhancer. *Blood* **110** (2007), pp. 1330-1333.

Lu, J., Getz, G., Miska, E., Alvarez-Saavedra, E., Lamb, J., Peck, D., Sweet-Cordero, A., Ebert, B., Mak, R. and Ferrando, A. MicroRNA expression profiles classify human cancers. *Nature* **435** (2005), pp. 834-838.

Lund, E., Güttinger, S., Calado, A., Dahlberg, J.E. and Kutay, U. Nuclear export of microRNA precursors. *Science* **303** (2004), pp. 95-98.

Ma, L., Teruya-Feldstein, J. and Weinberg, R.A. Tumour invasion and metastasis initiated by microRNA-10b in breast cancer. *Nature* **449** (2007), pp. 682-8.

Ma, L., Young, J., Prabhala, H., Pan, E., Mestdagh, P., Muth, D., Teruya-Feldstein, J., Reinhardt, F., Onder, T.T., Valastyan, S., Westermann, F., Speleman, F., Vandesompele, J. and Weinberg, R.A. miR-9, a MYC/MYCN-activated

microRNA, regulates E-cadherin and cancer metastasis. *Nature Cell Biology* **12** (2010), pp. 247-256.

Malliri, A., Rygiel, T.P., van der Kammen, R.A., Song, J.Y., Engers, R., Hurlstone, A.F.L., Clevers, H. and Collard, J.G. The rac activator Tiam1 is a Wnt-responsive gene that modifies intestinal tumor development. *Journal of Biological Chemistry* **281** (2006), pp. 543-548.

Malliri, A., van der Kammen, R.A., Clark, K., van der Valk, M., Michiels, F. and Collard, J.G. Mice deficient in the Rac activator Tiam1 are resistant to Ras-induced skin tumours. *Nature* **417** (2002), pp. 867-71.

Markowitz, S., Wang, J., Myeroff, L., Parsons, R., Sun, L., Lutterbaugh, J., Fan, R.S., Zborowska, E., Kinzler, K.W., Vogelstein, B., Brattain, M. and Willson, J.K. Inactivation of the type II TGF-β receptor in colon cancer cells with microsatellite instability. *Science* **268** (1995), pp. 1336-1338.

Markowitz, S.D. and Roberts, A.B. Tumor suppressor activity of the TGF-β pathway in human cancers. *Cytokine Growth Factor Rev* **7** (1996), pp. 93-102.

Martello, G., Zacchigna, L., Inui, M., Montagner, M., Adorno, M., Mamidi, A., Morsut, L., Soligo, S., Tran, U., Dupont, S., Cordenonsi, M., Wessely, O. and Piccolo, S. MicroRNA control of Nodal signalling. *Nature* **449** (2007), pp. 183-188.

Massague, J. TGFbeta in Cancer. *Cell* **134** (2008), pp. 215-30.

Massagué, J. TGF-beta signal transduction. *Annual Review of Biochemistry* **67** (1998), pp. 753-791.

Massagué, J., Blain, S. and Lo, R. TGF-beta signaling in growth control, cancer, and heritable disorders. *Cell* **103** (2000), pp. 295-310.

Massagué, J. and Gomis, R.R. The logic of TGFβ signaling. *FEBS Letters* **580** (2006), pp. 2811-2820.

Massagué, J., Seoane, J. and Wotton, D. Smad transcription factors. *Genes & Development* **19** (2005), pp. 2783-2810.

Medina, P.P., Nolde, M. and Slack, F.J. OncomiR addiction in an in vivo model of microRNA-21-induced pre-B-cell lymphoma. *Nature* **467** (2010), pp. 86-90.

Mendell, J.T. miRiad roles for the miR-17-92 cluster in development and disease. *Cell* **133** (2008), pp. 217-222.

Mercado-Pimentel, M.E. and Runyan, R.B. Multiple transforming growth factor-β isoforms and receptors function during epithelial-mesenchymal cell transformation in the embryonic heart. *Cells Tissues Organs* **185** (2007), pp. 146-156.

Mertens, A.E., Roovers, R.C. and Collard, J.G. Regulation of Tiam1-Rac signalling. *FEBS Lett* **546** (2003), pp. 11-6.

Metzler, M., Wilda, M., Busch, K., Viehmann, S. and Borkhardt, A. High expression of precursor microRNA 155/BIC RNA in children with Burkitt lymphoma. *Genes, Chromosomes and Cancer* **39** (2004), pp. 167-169.

Miettinen, P.J., Ebner, R., Lopez, A.R. and Derynck, R. TGF-β induced transdifferentiation of mammary epithelial cells to mesenchymal cells: involvement of type I receptors. *Journal of Cell Biology* **127** (1994), pp. 2021-2036.

Minard, M.E., Ellis, L.M. and Gallick, G.E. Tiam1 regulates cell adhesion, migration and apoptosis in colon tumor cells. *Clinical and Experimental Metastasis* **23** (2006), pp. 301-313.

Minard, M.E., Kim, L.S., Price, J.E. and Gallick, G.E. The role of the guanine nucleotide exchange factor Tiam1 in cellular migration, invasion, adhesion and tumor progression. *Breast Cancer Research and Treatment* **84** (2004), pp. 21-32.

Miyaki, M., Iijima, T., Konishi, M., Sakai, K., Ishii, A., Yasuno, M., Hishima, T., Koike, M., Shitara, N., Iwama, T., Utsunomiya, J., Kuroki, T. and Mori, T. Higher frequency of Smad4 gene mutation in human colorectal cancer with distant metastasis. *Oncogene* **18** (1999), pp. 3098-3103.

Moriarty, C.H., Pursell, B. and Mercurio, A.M. miR-10b targets Tiam1: Implications for Rac activation and carcinoma migration. *J Biol Chem* (2010).

Morlando, M., Ballarino, M., Gromak, N., Pagano, F., Bozzoni, I. and Proudfoot, N.J. Primary microRNA transcripts are processed co-transcriptionally. *Nature Structural & Molecular Biology* **15** (2008), pp. 902-909.

Moses, H.L., Yang, E.Y. and Pietenpol, J.A. TGF-β stimulation and inhibition of cell proliferation: new mechanistic insights. *Cell* **63** (1990), pp. 245-247.

Mott, J., Kobayashi, S., Bronk, S. and Gores, G. mir-29 regulates Mcl-1 protein expression and apoptosis. *Oncogene* **26** (2007), pp. 6133-6140.

Moustakas, A. and Heldin, C.H. Non-Smad TGF-β signals. *Journal of Cell Science* **118** (2005), pp. 3573-3584.

Moustakas, A. and Heldin, C.H. Signaling networks guiding epithelial-mesenchymal transitions during embryogenesis and cancer progression. *Cancer Sci* **98** (2007), pp. 1512-20.

Muraoka, R.S., Koh, Y., Roebuck, L.R., Sanders, M.E., Brantley-Sieders, D., Gorska, A.E., Moses, H.L. and Arteaga, C.L. Increased malignancy of Neu-induced mammary tumors overexpressing active transforming growth factor β1. *Molecular Cell Biology* **23** (2003), pp. 8691-8703.

Nawshad, A., LaGamba, D., Polad, A. and Hay, E.D. Transforming growth factor-β signaling during epithelial-mesenchymal transformation: implications for embryogenesis and tumor metastasis. *Cells Tissues Organs* **179** (2005), pp. 11-23.

Norton, J.D. ID helix-loop-helix proteins in cell growth, differentiation and tumorigenesis. *Journal of Cell Science* **113** (2000), pp. 3897–3905.

O'Connell, R.M., Chaudhuri, A.A., Rao, D.S. and Baltimore, D. Inositol phosphatase SHIP1 is a primary target of miR-155. *Proceedings of the National Academy of Sciences USA* **106** (2009), pp. 7113-7118.

O'Donnell, K.A., Wentzel, E.A., Zeller, K.I., Dang, C.V. and Mendell, J.T. c-Myc-regulated microRNAs modulate E2F1 expression. *Nature* **435** (2005), pp. 839-843.

Oft, M., Heider, K.H. and Beug, H. TGFβ signaling is necessary for carcinoma cell invasiveness and metastasis. *Current Biology* **8** (1998), pp. 1243-1252.

Oft, M., Peli, J., Rudaz, C., Schwarz, H., Beug, H. and Reichmann, E. TGF-β1 and Ha-Ras collaborate in modulating the phenotypic plasticity and invasiveness of epithelial tumor cells. *Genes & Development* **10** (1996), pp. 2462-2477.

Padua, D. and Massagué, J. Roles of TGFβ in metastasis. *Cell Research* **19** (2009), pp. 89-102.

Padua, D., Zhang, X.H., Wang, Q., Nadal, C., Gerald, W.L., Gomis, R.R. and Massagué, J. TGFβ primes breast tumors for lung metastasis seeding through angiopoietin-like 4. *Cell* **133** (2008), pp. 66-77.

Park, S.M., Gaur, A.B., Lengyel, E. and Peter, M.E. The miR-200 family determines the epithelial phenotype of cancer cells by targeting the E-cadherin repressors ZEB1 and ZEB2. *Genes & Development* **22** (2008a), pp. 894-907.

Park, S.M., Gaur, A.B., Lengyel, E. and Peter, M.E. The miR-200 family determines the epithelial phenotype of cancer cells by targeting the E-cadherin repressors ZEB1 and ZEB2. *Genes Dev* **22** (2008b), pp. 894-907.

Pasche, B., Kolachana, P., Nafa, K., Satagopan, J., Chen, Y.G., Lo, R.S., Brener, D., Yang, D., Kirsten, L., Oddoux, C., Ostrer, H., Vineis, P., Varesco, L., Jhanwar, S., Luzzatto, L., Massagué, J. and Offit, K. TβR-I(6A) is a candidate tumor susceptibility allele. *Cancer Research* **59** (1999), pp. 5678-5682.

Pawlicki, J.M. and Steitz, J.A. Primary microRNA transcript retention at sites of transcription leads to enhanced microRNA production. *Journal of Cell Biology* **182** (2008), pp. 61-76.

Pedersen, I.M., Otero, D., Kao, E., Miletic, A.V., Hother, C., Ralfkiaer, E., Rickert, R.C., Gronbaek, K. and David, M. Onco miR 155 targets SHIP1 to promote TNF dependent growth of B cell lymphomas. *EMBO Molecular Medicine* **1** (2009), pp. 288-295.

Pekarsky, Y., Santanam, U., Cimmino, A., Palamarchuk, A., Efanov, A., Maximov, V., Volinia, S., Alder, H., Liu, C., Rassenti, L., Calin, G.A., Hagan, J.P., Kipps, T. and Croce, C.M. Tcl1 expression in chronic lymphocytic leukemia is regulated by *miR-29* and *miR-181*. *Cancer Research* **66** (2006), p. 11590.

Petersen, O.W., Nielsen, H.L., Gudjonsson, T., Villadsen, R., Rank, F., Niebuhr, E., Bissell, M.J. and Ronnov-Jessen, L. Epithelial to mesenchymal transition in human breast cancer can provide a nonmalignant stroma. *American Journal of Pathology* **162** (2003), pp. 391-402.

Petrocca, F., Vecchione, A. and Croce, C. Emerging role of miR-106b-25/miR-17-92 clusters in the control of transforming growth factor signaling. *Cancer Research* **68** (2008a), pp. 8191-8194.

Petrocca, F., Visone, R., Onelli, M.R., Shah, M.H., Nicoloso, M.S., de Martino, I., Iliopoulos, D., Pilozzi, E., Liu, C.-G., Massimo, N., Cavazzini, L., Volinia, S., Alder, H., Ruco, L.P., Gustavo, B., Croce, C.M. and Vecchione, A. E2F1-regulated microRNAs impair TGFβ-dependent cell-cycle arrest and apoptosis in gastric cancer. *Cell* **13** (2008b), pp. 272-286.

Pfeffer, S., Zavolan, M., Grasser, F.A., Chien, M., Russo, J.J., Ju, J., John, B., Enright, A.J., Marks, D., Sander, C. and Tuschl, T. Identification of virus-encoded microRNAs. *Science* **304** (2004), pp. 734-736.

Potts, J. and Runyan, R. Epithelial-mesenchymal cell transformation in the embryonic heart can be mediated, in part, by transforming growth factor β. *Developmental Biology* **134** (1989), pp. 392-401.

Poy, M.N. A pancreatic islet-specific microRNA regulates insulin secretion. *Nature* **432** (2004), pp. 226-230.

Putz, E., Witter, K., Offner, S., Stosiek, P., Zippelius, A., Johnson, J., Zahn, R., Riethmüller, G. and Pantel, K. Phenotypic characteristics of cell lines derived from disseminated cancer cells in bone marrow of patients with solid epithelial tumors. *Cancer Research* **59** (1999), pp. 241-248.

Rao, P.K., Kumar, R.M., Farkhondeh, M., Baskerville, S. and Lodish, H.F. Myogenic factors that regulate expression of muscle-specific microRNAs. *Proceedings of the National Academy of Sciences USA* **103** (2006), pp. 8721-8726.

Raver-Shapira, N., Marciano, E., Meiri, E., Spector, Y., Rosenfeld, N., Moskovits, N., Bentwich, Z. and Oren, M. Transcriptional activation of miR-34a contributes to p53-mediated apoptosis. *Molecular Cell* **26** (2007), pp. 731-743.

Reynisdóttir, I., Polyak, K., Iavarone, A. and Massagué, J. Kip/Cip and Ink4 Cdk inhibitors cooperate to induce cell cycle arrest in response to TGF-beta. *Genes & Development* **9** (1995), pp. 1831-1845.

Ribas, J., Ni, X., Haffner, M., Wentzel, E.A., Salmasi, A.H., Chowdhury, W.H., Kudrolli, T.A., Yegnasubramanian, S., Luo, J., Rodriguez, R., Mendell, J.T. and Lupold, S.E. miR-21:an androgen receptor-regulated microRNA that promotes hormone-dependent and hormone-independent prostate cancer growth. *Cancer Research* **69** (2009), pp. 7165-7169.

Ridley, A.J. Rho GTPases and cell migration. *Journal of Cell Science* **114** (2001), pp. 2713-2720.

Roberts, A., Thompson, N., Heine, U., Flanders, C. and Sporn, M. Transforming growth factor-β: possible roles in carcinogenesis. *British Journal of Cancer* **57** (1988), pp. 594-600.

Saika, S., Kono-Saika, S., Ohnishi, Y., Sato, M., Muragaki, Y., Ooshima, A., Flanders, K.C., Yoo, J., Anzano, M. and Liu, C.Y. Smad3 signaling is required for epithelial-mesenchymal transition of lens epithelium after injury. *The American Journal of Pathology* **164** (2004), pp. 651-663.

Sampson, V., Rong, N., Han, J., Yang, Q., Aris, V., Soteropoulos, P., Petrelli, N., Dunn, S. and Krueger, L. MicroRNA let-7a down-regulates MYC and reverts MYC-induced growth in Burkitt lymphoma cells. *Cancer Research* **67** (2007), pp. 9762-9770.

Sanchez-Elsner, T., Botella, L., Velasco, B., Corbi, A., Attisano, L. and Bernabeu, C. Synergistic cooperation between hypoxia and transforming growth factor-beta pathways on human vascular endothelial growth factor gene expression. *Journal of Biological Chemistry* **276** (2001), pp. 38527-38535.

Sato, M., Muragaki, Y., Saika, S., Roberts, A.B. and Ooshima, A. Targeted disruption of TGF-beta1/Smad3 signaling protects against renal tubulointerstitial fibrosis induced by unilateral ureteral obstruction. *Journal of Clinical Investigation* **112** (2003), pp. 1486-1494.

Schutte, M., Hruban, R.H., Hedrick, L., Cho, K.R., Nadasdy, G.M., Weinstein, C.L., Bova, G.S., Isaacs, W.B., Cairns, P., Nawroz, H., Sidransky, D., Casero Jr., R.A., Meltzer, P.S., Hahn, S.A. and Kern, S.E. DPC4 gene in various tumor types. *Cancer Research* **56** (1996), pp. 2527-2530.

Selbach, M., Schwanhausser, B., Thierfelder, N., Fang, Z., Khanin, R. and Rajewsky, N. Widespread changes in protein synthesis induced by microRNAs. *Nature* **455** (2008), pp. 58-63.

Seoane, J., Le, H.V. and Massagué, J. Myc suppression of the p21(Cip1) Cdk inhibitor influences the outcome of the p53 response to DNA damage. *Nature* **419** (2002), pp. 729-734.

Seoane, J., Le, H.V., Shen, L., Anderson, S.A. and Massagué, J. Integration of Smad and forkhead pathways in the control of neuroepithelial and glioblastoma cell proliferation. *Cell* **117** (2004), pp. 211-223.

Shan, H., Zhang, Y., Lu, Y., Pan, Z., Cai, B., Wang, N., Li, X., Feng, T. and Hong, Y. Downregulation of miR-133 and miR-590 contributes to nicotine-induced atrial remodelling in canines. *Cardiovascular Research* **83** (2009), pp. 465-472.

Shi, Y. and Massagué, J. Mechanisms of TGF-β signaling from cell membrane to the nucleus. *Cell* **113** (2003), pp. 685-700.

Siegel, P.M. and Massagué, J. Cytostatic and apoptotic actions of TGF-beta in homeostasis and cancer. *Nature Reviews Cancer* **3** (2003), pp. 807-821.

Sirard, C., De La Pompa, J.L., Elia, A., Itie, A., Mirtsos, C., Cheung, A., Hahn, S., Wakeham, A., Schwartz, L. and Kern, S. The tumor suppressor gene Smad4/Dpc4 is required for gastrulation and later for anterior development of the mouse embryo. *Genes & Development* **12** (1998), pp. 107-119.

Slaby, O., Svoboda, M., Fabian, P., Smerdova, T., Knoflickova, D., Bednarikova, M., Nenutil, R. and Vyzula, R. Altered expression of miR-21, miR-31, miR-143 and miR-145 is related to clinicopathologic features of colorectal cancer. *Oncology* **72** (2007), pp. 397-402.

Slaby, O., Svoboda, M., Fabian, P., Smerdova, T., Knoflickova, D., Bednarikova, M., Nenutil, R. and Vyzula, R. Altered expression of miR-21, miR-31, miR-143 and

miR-145 is related to clinicopathologic features of colorectal cancer. *Oncology* **72** (2008), pp. 397-402.

Sobell, H.M. Actinomycin and DNA transcription. *Proceedings of the National Academy of Sciences USA* **82** (1985), pp. 5328-5231.

Stetler-Stevenson, W.G., Aznavoorian, S. and Liotta, L.A. Tumor cell interactions with the extracellular matrix during invasion and metastasis. *Annual Review of Cell Biology* **9** (1993), pp. 541-573.

Szlosarek, P., Charles, K.A. and Balkwill, F.R. Tumour necrosis factor-[alpha] as a tumour promoter. *European Journal of Cancer* **42** (2006), pp. 745-750.

Tachibana, I., Imoto, M., Adjei, P.N., Gores, G.J., Subramaniam, M., Spelsberg, T.C. and Urrutia, R. Overexpression of the TGFβ-regulated zinc finger encoding gene, TIEG, induces apoptosis in pancreatic epithelial cells. *Journal of Clinical Investigation* **99** (1997), pp. 2365-2374.

Tam, W. Identification and characterization of human BIC, a gene on chromosome 21 that encodes a noncoding RNA. *Gene* **274** (2001), pp. 157-167.

Tang, B., Bottinger, E.P., Jakowlew, S.B., Bagnall, K.M., Mariano, J., Anver, M.R., Letterio, J.J. and Wakefield, L.M. Transforming growth factor-β1 is a new form of tumor suppressor with true haploid insufficiency. *Nature Medicine* **4** (1998), pp. 802-807.

Tang, B., Vu, M., Booker, T., Santner, S.J., Miller, F.R., Anver, M.R. and Wakefield, L.M. TGF-β switches from tumor suppressor to prometastatic factor in a model of breast cancer progression. *Journal of Clinical Investigation* **112** (2003), pp. 1116-1124.

Tavazoie, S.F., Alarcon, C., Oskarsson, T., Padua, D., Wang, Q., Bos, P.D., Gerald, W.L. and Massague, J. Endogenous human microRNAs that suppress breast cancer metastasis. *Nature* **451** (2008), pp. 147-52.

Thiery, J.P. Epithelial-mesenchymal transitions in tumour progression. *Nature Reviews Cancer* **2** (2002), pp. 442-454.

Thiery, J.P. and Sleeman, J.P. Complex networks orchestrate epithelial-mesenchymal transitions. *Nat Rev Mol Cell Biol* **7** (2006), pp. 131-42.

Tlsty, T.D. and Coussens, L.M. Tumor stroma and regulation of cancer development. *Annual Review of Pathology: Mechanisms of Disease* **1** (2006), pp. 119-150.

Tsushima, H., Kawata, S. and Tamura, S. High levels of transforming growth factor β1 in patients with colorectal cancer: association with disease progression. *Gastroenterology* **110** (1996), pp. 375-382.

Uchida, K., Nagatake, M., Osada, H., Yatabe, Y., Kondo, M., Mitsudomi, T., Masuda, A. and Takahashi, T. Somatic in vivo alterations of the JV18-1 gene at 18q21 in human lung cancers. *Cancer Research* **56** (1996), pp. 5583-5585.

Valastyan, S., Chang, A., Benaich, N., Reinhardt, F. and Weinberg, R.A. Concurrent suppression of integrin alpha5, radixin, and RhoA phenocopies the effects of miR-31 on metastasis. *Cancer Res* **70** (2010), pp. 5147-54.

Valastyan, S., Reinhardt, F., Benaich, N., Calogrias, D., Szasz, A.M., Wang, Z.C., Brock, J.E., Richardson, A.L. and Weinberg, R.A. A pleiotropically acting microRNA, miR-31, inhibits breast cancer metastasis. *Cell* **137** (2009), pp. 1032-46.

Valcourt, U., Kowanetz, M., Niimi, H., Heldin, C.H. and Moustakas, A. TGF-β and the Smad signaling pathway support transcriptomic reprogramming during epithelial-mesenchymal cell transition. *Molecular Biology of the Cell* **16** (2005), pp. 1987-2002.

Valderrama-Carvajal, H., Cocolakis, E. and Lacerte, A. Activin/TGF-beta induce apoptosis through Smad-dependent expression of the lipid phosphatase SHIP. *Nature Cell Biology* **4** (2002), pp. 963-969.

Ventura, A. Targeted deletion reveals essential and overlapping functions of the miR-17 through 92 family of miRNA clusters. *Cell* **132** (2008), pp. 875-886.

Vincan, E., Brabletz, T., Faux, M.C. and Ramsay, R.G. A human three-dimensional cell line model allows the study of dynamic and reversible epithelial-mesenchymal and mesenchymal-epithelial transition that underpins colorectal carcinogenesis. *Cells Tissues Organs* **185** (2007a), pp. 20-8.

Vincan, E., Darcy, P.K., Farrelly, C.A., Faux, M.C., Brabletz, T. and Ramsay, R.G. Frizzled-7 dictates three-dimensional organization of colorectal cancer cell carcinoids. *Oncogene* **26** (2007b), pp. 2340-52.

Vincan, E., Whitehead, R.H. and Faux, M.C. Analysis of Wnt/FZD-mediated signalling in a cell line model of colorectal cancer morphogenesis. *Methods Mol Biol* **468** (2008), pp. 263-73.

Vogelstein, B., Fearon, E., Hamilton, S., Kern, S., Preisinger, A., Leppert, M., Nakamura, Y., White, R., Smits, A. and Bos, J. Genetic alterations during colorectal-tumor development. *The New England journal of medicine* **319** (1988), pp. 525-532.

Volinia, S., Calin, G.A., Liu, C.G., Ambs, S., Cimmino, A., Petrocca, F., Visone, R., Iorio, M., Roldo, C. and Ferracin, M. A microRNA expression signature of human solid tumors defines cancer gene targets. *Proceedings of the National Academy of Sciences USA* **103** (2006), pp. 2257-2261.

Voorhoeve, P.M., le Sage, C., Schrier, M., Gillis, A., Stoop, H., Nagel, R., Liu, Y., van Duijse, J., Drost, J. and Griekspoor, A. A genetic screen implicates miRNA-372 and miRNA-373 as oncogenes in testicular germ cell tumors. *Cell* **124** (2006), pp. 1169-1181.

Waldrip, W., Bikoff, E., Hoodless, P., Wrana, J. and Robertson, E. Smad2 signaling in extraembryonic tissues determines anterior-posterior polarity of the early mouse embryo. *Cell* **92** (1998), pp. 797-808.

Wang, D., Kanuma, T., Mizunuma, H., Takama, F., Ibuki, Y., Wake, N., Mogi, A., Shitara, Y. and Takenoshita, S. Analysis of specific gene mutations in the transforming growth factor-β signal transduction pathway in human ovarian cancer. *Cancer Research* **60** (2000), pp. 4507-4512.

Wang, J., Sun, L.Z., Myeroff, L., Wang, X., Gentry, L.E., Yang, J., Liang, J., Zborowska, E., Markowitz, S., Willson, J.K.V. and Brattain, M.G. Demonstration that mutation of the type II transforming growth factor receptor β inactivates its tumor suppressor activity in replication error-positive colon carcinoma cells. *Journal of Biological Chemistry* **270** (1995), pp. 22044-22049.

Wang, Q., Huang, Z., Xue, H., Jin, C., Ju, X., Han, J. and Chen, Y. MicroRNA miR-24 inhibits erythropoiesis by targeting activin type I receptor ALK4. *Blood* **111** (2008), p. 588.

Whitehead, R.H., Jones, J.K., Gabriel, A. and Lukies, R.E. A new colon carcinoma cell line (LIM1863) that grows as organoids with spontaneous differentiation into crypt-like structures in vitro. *Cancer Research* **47** (1987), pp. 2683-2689.

Wickramasinghe, N.S., Manavalan, T.T., Dougherty, S.M., Riggs, K.A., Li, Y. and Klinge, C.M. Estradiol downregulates miR-21 expression and increases miR-21 target gene expression in MCF-7 breast cancer cells. *Nucleic Acids Research* **37** (2009), pp. 2584-2595.

Wightman, B., Ha, I. and Ruvkun, G. Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in *C. elegans*. *Cell* **75** (1993), pp. 855-862.

Willis, B.C. and Borok, Z. TGF-β-induced EMT: mechanisms and implications for fibrotic lung disease. *American Journal of Physiology-Lung Cellular and Molecular Physiology* **293** (2007), pp. L525-L534.

Wolfraim, L., Fernandez, T., Mamura, M., Fuller, W., Kumar, R., Cole, D., Byfield, S., Felici, A., Flanders, K. and Walz, T. Loss of Smad3 in acute T-cell lymphoblastic leukemia. *New England Journal of Medicine* **351** (2004), pp. 552-559.

Wood, L.D., Parsons, D.W., Jones, S., Lin, J., Sjoblom, T., Leary, R.J., Shen, D., Boca, S.M., Barber, T., Ptak, J., Silliman, N., Szabo, S., Dezso, Z., Ustyanksky, V., Nikolskaya, T., Nikolsky, Y., Karchin, R., Wilson, P.A., Kaminker, J.S., Zhang, Z., Croshaw, R., Willis, J., Dawson, D., Shipitsin, M., Willson, J.K., Sukumar, S., Polyak, K., Park, B.H., Pethiyagoda, C.L., Pant, P.V., Ballinger, D.G., Sparks, A.B., Hartigan, J., Smith, D.R., Suh, E., Papadopoulos, N., Buckhaults, P., Markowitz, S.D., Parmigiani, G., Kinzler, K.W., Velculescu, V.E. and Vogelstein, B. The genomic landscapes of human breast and colorectal cancers. *Science* **318** (2007), pp. 1108-13.

Xu, J., Lamouille, S. and Derynck, R. TGF-β -induced epithelial to mesenchymal transition. *Cell Research* **19** (2009), pp. 156-172.

Xu, L., Kang, Y., Çöl, S. and Massagué, J. Smad2 nucleocytoplasmic shuttling by nucleoporins CAN/Nup214 and Nup153 feeds TGF-β signaling complexes in the cytoplasm and nucleus. *Molecular Cell* **10** (2002), pp. 271-282.

Xu, L., Yao, X., Chen, X., Lu, P., Zhang, B. and Ip, Y.T. Msk is required for nuclear import of TGF-β /BMP-activated Smads. *The Journal of Cell Biology* **178** (2007), pp. 981-994.

Xu, X., Brodie, S., Yang, X., Im, Y., Parks, W., Chen, L., Zhou, Y., Weinstein, M., Kim, S. and Deng, C. Haploid loss of the tumor suppressor Smad4/Dpc4 initiates gastric polyposis and cancer in mice. *Oncogene* **19** (2000), pp. 1868-1874.

Yang, Y.A., Dukhanina, O., Tang, B., Mamura, M., Letterio, J.J., MacGregor, J., Patel, S.C., Khozin, S., Liu, Z., Green, J., Anver, M.R., Merlino, G. and Wakefield, L.M. Lifetime exposure to a soluble TGF-beta antagonist protects mice against metastasis without adverse side effects. *Journal of Clinical Investigation* **109** (2002), pp. 1607-1615.

Yao, X., Chen, X., Cottonham, C. and Xu, L. Preferential utilization of Imp7/8 in nuclear import of Smads. *Journal of Biological Chemistry* **283** (2008), pp. 22867-22874.

Yi, R., Qin, Y., Macara, I.G. and Cullen, B.R. Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. *Genes & Development* **17** (2003), pp. 3011-3016.

Yu, Q. and Stamenkovic, I. Cell surface-localized matrix metalloproteinase-9 proteolytically activates TGF-β and promotes tumor invasion and angiogenesis. *Genes & Development* **14** (2000), pp. 163-176.

Yue, J. and Mulder, K.M. Transforming growth factor-β signal transduction in epithelial cells. *Pharmacology & Therapeutics* **91** (2001), pp. 1-34.

Zavadil, J., Bitzer, M., Liang, D., Yang, Y.C., Massimi, A., Kneitz, S., Piek, E. and Böttinger, E.P. Genetic programs of epithelial cell plasticity directed by transforming growth factor-β. *Proceedings of the National Academy of Sciences USA* **98** (2001), pp. 6686-6691.

Zavadil, J. and Bottinger, E.P. TGF-beta and epithelial-to-mesenchymal transitions. *Oncogene* **24** (2005), pp. 5764-74.

Zavadil, J., Narasimhan, M., Blumenberg, M. and Schneider, R.J. Transforming growth factor-beta and microRNA: mRNA regulatory networks in epithelial plasticity. *Cells Tissues Organs* **185** (2007), pp. 157-161.

Zhang, Y., Feng, X.-H., Wu, R.-Y. and Derynck, R. Receptor-associated Mad homologues synergize as effectors of the TGF-beta response. *Nature* **383** (1996), pp. 168-172.

Zhu, Y., Richardson, J., Parada, L. and Graff, J. Smad3 mutant mice develop metastatic colorectal cancer. *Cell* **94** (1998), pp. 703-714.

APPENDIX

Table A.1 | LNA miRNA microarray data for LIM 1863 cells treated with TGF-β/TNF-α.

Gene Id	Annotation	Median Ratios	Log Median Ratios	Median Hy3	Median Hy5	CV
17748	hsa-let-7a	1.04	0.05	3070	2965	2.29
17749	hsa-let-7b	1.16	0.21	1265	1084	1.12
19004	hsa-let-7c	1.06	0.08	2321	2195	4.10
17750	hsa-let-7d	0.92	-0.13	227	249	0.96
17751	hsa-let-7e	0.82	-0.29	128	156	3.78
17752	hsa-let-7f	0.80	-0.32	97	122	3.06
19602	hsa-let-7g	0.99	-0.01	170	171	2.95
19580	hsa-let-7i	1.18	0.24	464	384	3.47
10916	hsa-miR-1	1.30	0.38	110	84	6.81
19581	hsa-miR-100	NA	NA	NA	NA	NA
17615	hsa-miR-101	0.95	-0.08	92	98	5.83
10919	hsa-miR-103	0.94	-0.09	624	668	1.97
10920	hsa-miR-105	NA	NA	NA	NA	NA
19582	hsa-miR-106b	0.80	-0.31	192	239	2.23
10923	hsa-miR-107	1.21	0.28	867	713	1.29
13485	hsa-miR-10a	0.84	-0.25	939	1115	3.89
10925	hsa-miR-10b	0.74	-0.44	81	109	2.47
19583	hsa-miR-122a	1.11	0.15	72	64	1.80
14328	hsa-miR-124a	1.28	0.35	105	82	3.45
10928	hsa-miR-125a	0.98	-0.03	188	194	2.65
10929	hsa-miR-125b	1.06	0.09	166	154	6.49
4610	hsa-miR-126	1.00	0.00	74	74	4.17
10930	hsa-miR-126*	0.98	-0.03	69	71	48.88
10931	hsa-miR-127	0.81	-0.31	73	90	1.64
10932	hsa-miR-128a	1.00	0.00	91	91	4.94
19584	hsa-miR-128b	1.05	0.08	73	70	0.45
10934	hsa-miR-129	1.24	0.32	269	232	15.07
10935	hsa-miR-130a	1.05	0.08	74	69	4.24
10936	hsa-miR-130b	1.01	0.02	179	176	1.43
10937	hsa-miR-132	NA	NA	NA	NA	NA
10938	hsa-miR-133a-133b	0.90	-0.14	90	100	2.00
10940	hsa-miR-134	0.90	-0.16	82	92	2.81
10941	hsa-miR-135a	1.66	0.73	137	83	9.72
10942	hsa-miR-135b	1.19	0.25	111	93	7.25
10943	hsa-miR-136	1.09	0.12	100	91	0.41
10944	hsa-miR-137	1.45	0.53	121	84	5.21
13140	hsa-miR-138	1.10	0.14	101	90	2.97
10945	hsa-miR-139	1.18	0.24	85	72	4.34
4700	hsa-miR-140	1.09	0.13	84	77	18.57

10946	hsa-miR-141	1.08	0.11	155	143	1.97
10947	hsa-miR-142-3p	0.83	-0.27	1261	1498	6.93
19015	hsa-miR-142-5p	0.71	-0.50	311	430	3.27
13177	hsa-miR-143	0.98	-0.03	69	70	0.32
10950	hsa-miR-144	1.14	0.19	82	72	0.70
10951	hsa-miR-145	1.06	0.09	83	78	2.00
10952	hsa-miR-146a	1.07	0.10	89	83	4.37
10306	hsa-miR-146b	1.02	0.03	78	76	2.07
10954	hsa-miR-147	1.23	0.30	84	68	1.45
10955	hsa-miR-148a	1.16	0.21	134	117	3.78
19585	hsa-miR-148b	0.85	-0.23	111	131	2.01
19586	hsa-miR-149	NA	NA	NA	NA	NA
19587	hsa-miR-150	0.91	-0.14	76	84	0.87
17463	hsa-miR-151	0.96	-0.06	122	128	2.33
17676	hsa-miR-152	0.94	-0.09	81	86	1.21
10961	hsa-miR-153	1.08	0.11	71	66	2.95
10962	hsa-miR-154	1.16	0.21	106	92	2.81
10963	hsa-miR-154*	0.89	-0.17	76	85	6.37
10964	hsa-miR-155	1.16	0.21	103	89	2.94
10965	hsa-miR-15a	0.95	-0.08	115	121	1.68
17280	hsa-miR-15b	0.87	-0.19	432	495	3.01
10967	hsa-miR-16	0.86	-0.22	511	575	3.39
19588	hsa-miR-17-3p	1.00	0.00	238	241	4.54
17605	hsa-miR-17-5p-106a	0.70	-0.51	701	993	3.16
10971	hsa-miR-181a	1.08	0.11	78	72	6.57
11013	hsa-miR-181a*	1.03	0.04	73	71	2.27
10972	hsa-miR-181b	1.14	0.19	90	78	1.99
10973	hsa-miR-181c	1.91	0.94	156	81	3.30
10974	hsa-miR-181d	1.17	0.23	86	73	0.94
10975	hsa-miR-182	1.30	0.38	203	155	4.21
10976	hsa-miR-182*	1.17	0.22	111	95	4.38
10977	hsa-miR-183	0.93	-0.10	240	257	1.01
10978	hsa-miR-184	1.23	0.30	491	387	7.39
5560	hsa-miR-185	0.94	-0.09	1320	1339	5.38
10979	hsa-miR-186	1.07	0.09	103	96	7.85
17643	hsa-miR-187	1.00	0.00	69	69	2.28
19589	hsa-miR-188	1.10	0.14	75	68	1.18
10982	hsa-miR-189	0.91	-0.14	74	82	1.16
10983	hsa-miR-18a	0.91	-0.14	364	397	7.88
13178	hsa-miR-18a*	0.74	-0.44	77	104	3.11
13141	hsa-miR-18b	0.86	-0.22	278	332	2.21
10984	hsa-miR-190	1.28	0.35	98	77	2.09
10985	hsa-miR-191	0.97	-0.05	552	569	3.55
13126	hsa-miR-191*	1.14	0.19	90	79	2.29
17732	hsa-miR-192	0.93	-0.10	494	525	3.25
13172	hsa-miR-192	0.88	-0.19	73	82	3.66
10986	hsa-miR-193a	1.11	0.15	1570	1419	6.65
10987	hsa-miR-193b	1.05	0.07	105	100	3.90
10988	hsa-miR-194	1.00	0.00	254	255	3.43
13148	hsa-miR-195	0.74	-0.44	76	103	3.32

10990	hsa-miR-196a	1.12	0.16	144	127	5.33
10991	hsa-miR-196b	1.18	0.23	115	97	3.36
10992	hsa-miR-197	1.09	0.12	149	137	1.33
10993	hsa-miR-198	0.96	-0.06	371	380	4.16
19590	hsa-miR-199a	0.99	-0.01	69	70	1.30
10995	hsa-miR-199a*	1.03	0.05	69	67	1.80
19591	hsa-miR-199b	1.07	0.10	71	66	3.07
10997	hsa-miR-19a	1.00	0.00	187	184	4.57
10998	hsa-miR-19b	0.92	-0.11	143	154	4.07
11000	hsa-miR-200a	1.32	0.40	2886	2173	3.56
13127	hsa-miR-200a*	1.00	0.01	75	75	3.75
9578	hsa-miR-200b	1.01	0.02	664	654	2.41
17427	hsa-miR-200c	1.02	0.03	620	604	6.41
11003	hsa-miR-202	0.99	-0.02	617	624	3.39
10314	hsa-miR-202*	2.02	1.02	159	79	4.06
11004	hsa-miR-203	1.70	0.77	322	189	3.68
11005	hsa-miR-204	1.17	0.22	97	83	1.70
11006	hsa-miR-205	0.93	-0.10	75	80	3.59
11007	hsa-miR-206	1.04	0.06	87	83	3.80
5730	hsa-miR-208	0.95	-0.07	93	99	3.41
11008	hsa-miR-20a	0.77	-0.37	816	1058	3.37
10999	hsa-miR-20a	0.78	-0.35	697	899	3.35
11009	hsa-miR-20b	0.81	-0.30	135	168	1.66
5740	hsa-miR-21	2.80	1.48	2049	721	4.02
13511	hsa-miR-210	1.18	0.23	660	590	5.95
11011	hsa-miR-211	1.12	0.16	95	85	0.52
19592	hsa-miR-212	0.76	-0.39	173	226	2.86
11014	hsa-miR-214	1.02	0.02	539	520	4.32
11015	hsa-miR-215	0.92	-0.12	349	380	6.91
11016	hsa-miR-216	1.08	0.11	74	69	0.80
19016	hsa-miR-217	1.02	0.03	71	71	1.06
11018	hsa-miR-218	1.08	0.11	82	76	4.16
11019	hsa-miR-219	1.01	0.01	69	67	2.41
11020	hsa-miR-22	2.00	1.00	212	106	3.50
11021	hsa-miR-220	1.15	0.21	78	68	4.65
11022	hsa-miR-221	1.17	0.23	387	329	5.39
11023	hsa-miR-222	1.06	0.08	2089	1971	6.39
11024	hsa-miR-223	1.09	0.13	108	99	3.96
11025	hsa-miR-224	1.02	0.03	77	76	3.67
11026	hsa-miR-23a	1.98	0.98	932	471	3.89
11027	hsa-miR-23b	1.29	0.37	494	382	4.01
17506	hsa-miR-24	1.82	0.86	500	276	1.82
11029	hsa-miR-25	1.16	0.21	101	87	2.89
11030	hsa-miR-26a	1.31	0.39	346	267	1.74
11031	hsa-miR-26b	1.40	0.48	371	262	2.18
19593	hsa-miR-27a	1.87	0.91	251	134	2.57
13175	hsa-miR-27b	1.19	0.25	155	131	1.52
11034	hsa-miR-28	0.89	-0.17	77	87	3.54
19594	hsa-miR-296	1.24	0.31	228	183	4.86
11037	hsa-miR-299-3p	NA	NA	NA	NA	NA
11038	hsa-miR-299-5p	0.94	-0.10	79	83	3.49

11039	hsa-miR-29a	1.13	0.18	3781	3341	3.52
11040	hsa-miR-29b	1.22	0.28	329	267	4.99
11041	hsa-miR-29c	1.05	0.08	190	180	5.66
13143	hsa-miR-301	0.58	-0.79	65	111	5.81
11222	hsa-miR-302a	1.18	0.24	89	75	7.39
11042	hsa-miR-302a*	1.03	0.05	86	83	3.29
11043	hsa-miR-302b	1.04	0.06	74	70	2.39
5930	hsa-miR-302b*	1.09	0.13	83	76	6.04
11044	hsa-miR-302c	1.15	0.20	87	76	4.19
11045	hsa-miR-302c*	0.96	-0.06	125	130	0.86
11046	hsa-miR-302d	1.13	0.18	83	73	1.69
19595	hsa-miR-30a-3p	1.04	0.06	67	65	1.95
11048	hsa-miR-30a-5p	0.88	-0.19	112	128	2.99
17565	hsa-miR-30b	0.81	-0.31	329	407	0.68
17502	hsa-miR-30c	0.96	-0.05	231	241	0.82
19596	hsa-miR-30d	0.85	-0.23	104	122	2.20
11224	hsa-miR-30e-3p	0.91	-0.14	77	85	8.22
13174	hsa-miR-30e-5p	1.05	0.08	90	85	2.48
11052	hsa-miR-31	2.26	1.18	308	137	6.49
11053	hsa-miR-32	0.81	-0.30	80	97	2.92
11054	hsa-miR-320	1.12	0.16	2473	2213	6.18
11055	hsa-miR-323	1.05	0.07	77	74	6.19
11056	hsa-miR-324-3p	1.01	0.02	157	156	2.16
11057	hsa-miR-324-5p	0.96	-0.05	91	95	2.30
11058	hsa-miR-325	1.19	0.26	78	65	0.47
11059	hsa-miR-326	1.11	0.14	393	347	3.33
11060	hsa-miR-328	0.94	-0.10	75	80	2.40
11061	hsa-miR-329	1.04	0.06	71	68	0.73
11062	hsa-miR-33	1.02	0.02	81	80	0.46
11063	hsa-miR-330	0.83	-0.27	72	86	2.99
11064	hsa-miR-331	1.09	0.12	118	111	7.93
11065	hsa-miR-335	0.87	-0.20	92	107	4.95
11066	hsa-miR-337	1.20	0.27	83	70	4.87
11067	hsa-miR-338	1.48	0.56	209	142	1.62
19597	hsa-miR-339	0.82	-0.28	76	91	3.37
17294	hsa-miR-33b	1.18	0.24	98	83	1.76
13144	hsa-miR-340	0.97	-0.04	88	91	2.55
11069	hsa-miR-342	NA	NA	NA	NA	NA
11070	hsa-miR-345	0.92	-0.13	108	118	3.31
19018	hsa-miR-346	0.91	-0.13	166	183	1.67
11072	hsa-miR-34a	1.34	0.42	110	82	1.49
11073	hsa-miR-34b	1.01	0.01	66	67	1.71
11074	hsa-miR-34c	1.08	0.10	77	72	4.42
14301	hsa-miR-361	0.78	-0.35	108	139	2.82
14279	hsa-miR-362	1.09	0.12	70	65	1.92
11077	hsa-miR-363	1.09	0.13	75	69	0.76
11078	hsa-miR-365	1.03	0.04	140	137	4.16
14280	hsa-miR-367	1.14	0.19	73	64	0.87
19598	hsa-miR-368	1.02	0.03	63	62	3.14
11081	hsa-miR-369-3p	1.47	0.55	120	82	4.72
13145	hsa-miR-369-5p	NA	NA	NA	NA	NA

11082	hsa-miR-370	1.11	0.16	267	242	1.50
11083	hsa-miR-371	1.13	0.17	82	74	3.28
11084	hsa-miR-372	1.11	0.15	77	69	3.81
11085	hsa-miR-373	1.21	0.27	73	61	2.79
11086	hsa-miR-373*	1.42	0.51	680	487	3.24
11087	hsa-miR-374	1.06	0.08	79	75	3.97
11088	hsa-miR-375	1.09	0.12	120	108	3.46
11089	hsa-miR-376a	1.04	0.06	70	67	1.89
14268	hsa-miR-376a*	1.03	0.04	71	69	3.69
11090	hsa-miR-376b	1.14	0.19	82	72	3.32
11091	hsa-miR-377	1.15	0.20	90	80	1.96
11092	hsa-miR-378	0.96	-0.06	78	81	3.00
11093	hsa-miR-379	1.18	0.24	97	82	2.58
11094	hsa-miR-380-3p	1.14	0.19	73	63	2.14
13170	hsa-miR-380-5p	1.00	0.00	70	70	3.89
14306	hsa-miR-381	0.95	-0.08	274	292	2.70
11097	hsa-miR-382	1.06	0.09	187	174	1.99
11098	hsa-miR-383	1.10	0.14	71	66	2.12
11099	hsa-miR-384	1.14	0.19	84	73	4.69
11240	hsa-miR-409-3p	0.97	-0.05	77	79	3.38
14310	hsa-miR-409-5p	0.88	-0.19	80	91	2.96
11102	hsa-miR-410	0.99	-0.02	66	69	2.52
17482	hsa-miR-411	NA	NA	NA	NA	NA
11103	hsa-miR-412	1.04	0.06	76	72	4.43
17474	hsa-miR-421	0.82	-0.28	101	123	1.88
11104	hsa-miR-422a	1.17	0.22	83	72	2.72
11105	hsa-miR-422b	0.79	-0.35	177	229	5.19
11106	hsa-miR-423	0.85	-0.24	100	118	1.50
11107	hsa-miR-424	0.91	-0.13	84	92	2.39
11108	hsa-miR-425-3p	NA	NA	NA	NA	NA
17608	hsa-miR-425-5p	1.06	0.08	136	131	2.57
13171	hsa-miR-429	0.98	-0.03	137	140	5.12
11110	hsa-miR-431	1.06	0.08	72	68	4.41
11111	hsa-miR-432	1.27	0.34	88	70	1.26
13128	hsa-miR-432*	0.82	-0.29	92	113	3.61
11112	hsa-miR-433	1.06	0.09	79	74	1.94
11113	hsa-miR-448	1.10	0.13	78	71	3.31
11114	hsa-miR-449	1.16	0.22	89	77	1.88
17706	hsa-miR-449b	0.99	-0.01	74	75	2.90
17835	hsa-miR-450	0.98	-0.03	78	80	5.72
8538	hsa-miR-450	1.11	0.16	77	68	3.63
11248	hsa-miR-451	0.77	-0.37	82	105	0.79
11116	hsa-miR-452	0.94	-0.09	325	344	0.90
13129	hsa-miR-452*	NA	NA	NA	NA	NA
11117	hsa-miR-453	1.29	0.36	86	67	2.04
17450	hsa-miR-454-3p	1.10	0.14	72	66	2.55
13179	hsa-miR-455	1.03	0.04	71	69	3.12
13180	hsa-miR-483	2.30	1.20	331	144	1.38
13181	hsa-miR-484	1.73	0.79	182	106	2.09
11118	hsa-miR-485-3p	1.02	0.03	96	92	5.52
11119	hsa-miR-485-5p	1.12	0.17	74	67	3.05

13182	hsa-miR-486	1.57	0.65	149	95	5.21
13183	hsa-miR-487a	0.95	-0.07	83	87	5.02
14285	hsa-miR-487b	0.53	-0.91	154	290	4.04
11120	hsa-miR-488	NA	NA	NA	NA	NA
11121	hsa-miR-489	0.89	-0.16	74	83	2.87
11122	hsa-miR-490	0.93	-0.11	143	155	8.46
11123	hsa-miR-491	0.99	-0.02	84	85	2.31
11124	hsa-miR-492	1.93	0.95	2511	1306	5.01
14270	hsa-miR-493-3p	1.01	0.02	73	71	4.82
11125	hsa-miR-493-5p	1.35	0.43	116	86	4.76
14287	hsa-miR-494	0.61	-0.70	1719	2764	14.37
17348	hsa-miR-495	1.09	0.12	82	76	4.29
11128	hsa-miR-496	1.08	0.12	72	67	2.65
11129	hsa-miR-497	1.23	0.30	90	72	2.12
11130	hsa-miR-498	1.12	0.16	1757	1574	2.99
14313	hsa-miR-499	1.07	0.10	77	69	5.16
11132	hsa-miR-500	1.16	0.22	155	133	4.20
11133	hsa-miR-501	1.05	0.08	74	72	1.97
11134	hsa-miR-502	1.14	0.19	89	78	1.07
11135	hsa-miR-503	1.04	0.06	969	922	3.37
11136	hsa-miR-504	1.09	0.12	79	73	2.99
14314	hsa-miR-505	1.00	0.00	69	69	0.56
11138	hsa-miR-506	1.03	0.05	85	82	5.05
11139	hsa-miR-507	1.17	0.23	89	77	1.81
11140	hsa-miR-508	1.39	0.47	111	80	3.32
11141	hsa-miR-509	1.26	0.34	81	64	1.41
11142	hsa-miR-510	0.94	-0.09	141	155	6.45
11143	hsa-miR-511	1.12	0.16	76	69	3.61
11144	hsa-miR-512-3p	1.14	0.19	80	68	5.69
11145	hsa-miR-512-5p	0.98	-0.03	393	399	4.30
11146	hsa-miR-513	0.92	-0.12	1846	2011	3.31
11147	hsa-miR-514	1.17	0.23	80	69	1.33
11148	hsa-miR-515-3p	1.01	0.01	67	67	0.57
11149	hsa-miR-515-5p	1.08	0.11	77	72	2.47
11150	hsa-miR-516-3p	1.08	0.11	81	75	0.91
11151	hsa-miR-516-5p	0.86	-0.22	121	137	3.32
13130	hsa-miR-517*	1.89	0.92	160	85	4.77
11153	hsa-miR-517a-517b	1.16	0.22	87	73	2.59
11154	hsa-miR-517c	1.20	0.26	91	76	0.97
11155	hsa-miR-518a	1.13	0.17	83	74	0.64
11156	hsa-miR-518b	1.12	0.17	113	99	2.59
11157	hsa-miR-518c	1.17	0.23	79	68	3.94
13131	hsa-miR-518c*	0.89	-0.17	881	991	2.24
11158	hsa-miR-518d	1.12	0.16	83	73	2.92
11159	hsa-miR-518e	1.09	0.12	74	68	2.43
11160	hsa-miR-518f	1.10	0.14	78	72	4.19
10586	hsa-miR-518f*-526a	1.06	0.09	288	270	1.00
11161	hsa-miR-519a	1.04	0.05	71	68	1.93
11162	hsa-miR-519b	1.16	0.21	77	66	3.74

10482	hsa-miR-519c	1.07	0.09	68	63	3.06
11163	hsa-miR-519d	0.77	-0.38	117	152	2.60
11164	hsa-miR-519e	0.77	-0.38	80	103	3.40
13132	hsa-miR-519e*	0.97	-0.04	270	274	2.58
11165	hsa-miR-520a	1.09	0.12	74	68	0.96
13133	hsa-miR-520a*	0.83	-0.27	84	99	1.74
11166	hsa-miR-520c-520b	1.07	0.10	70	65	1.13
11168	hsa-miR-520d	1.12	0.17	75	66	2.13
13134	hsa-miR-520d*	0.79	-0.33	95	121	3.00
11169	hsa-miR-520e	1.62	0.69	130	80	3.12
11167	hsa-miR-520f-520c	1.19	0.25	77	65	1.95
13146	hsa-miR-520g-520h	1.14	0.19	73	64	0.26
11171	hsa-miR-521	1.14	0.19	80	71	5.85
11172	hsa-miR-522	1.21	0.28	82	68	6.07
11173	hsa-miR-523	1.02	0.03	78	77	5.10
10618	hsa-miR-524*	0.77	-0.38	77	100	7.25
11175	hsa-miR-525	0.82	-0.28	178	223	2.82
11174	hsa-miR-525*-524	1.08	0.11	83	76	2.76
11176	hsa-miR-526b	0.81	-0.31	91	113	1.46
13136	hsa-miR-526b*	1.46	0.54	119	81	5.40
13137	hsa-miR-526c	1.15	0.20	152	133	1.06
11177	hsa-miR-527	0.92	-0.12	156	172	2.00
17624	hsa-miR-532	0.83	-0.26	85	104	3.61
14271	hsa-miR-539	1.12	0.16	72	63	1.94
14315	hsa-miR-542-3p	1.10	0.14	72	66	2.20
14273	hsa-miR-542-5p	1.05	0.07	78	75	2.91
13712	hsa-miR-544	1.08	0.12	69	64	4.85
17846	hsa-miR-545	1.08	0.11	85	80	7.56
13721	hsa-miR-545	1.03	0.05	72	69	6.79
17535	hsa-miR-548a	1.09	0.12	80	73	4.64
17298	hsa-miR-548b	1.18	0.24	93	78	5.35
15313	hsa-miR-548c	1.11	0.15	81	72	2.64
17533	hsa-miR-548d	1.05	0.07	78	75	2.80
17370	hsa-miR-549	0.96	-0.05	76	79	3.62
17660	hsa-miR-550	0.84	-0.26	94	113	0.28
17272	hsa-miR-551a	1.24	0.31	183	148	1.97
17500	hsa-miR-551b	1.07	0.10	80	73	4.30
17668	hsa-miR-552	0.82	-0.28	75	90	3.55
17271	hsa-miR-553	1.12	0.16	77	70	2.04
17640	hsa-miR-554	0.96	-0.05	81	85	4.50
17612	hsa-miR-555	1.01	0.01	76	75	7.30
17426	hsa-miR-556	1.13	0.17	82	72	1.78
17376	hsa-miR-557	1.14	0.19	675	592	3.03
17652	hsa-miR-558	1.01	0.02	76	75	0.16
14755	hsa-miR-559	1.01	0.02	71	70	2.88
17456	hsa-miR-560	NA	NA	NA	NA	NA
14773	hsa-miR-561	1.19	0.25	92	78	1.34

17536	hsa-miR-562	1.04	0.06	76	73	4.25
17569	hsa-miR-563	1.04	0.05	69	67	4.16
17645	hsa-miR-564	1.16	0.21	103	89	1.08
17413	hsa-miR-565	1.04	0.05	151	148	5.88
17634	hsa-miR-567	1.02	0.03	67	67	1.88
17661	hsa-miR-568	1.00	0.01	79	79	3.27
14854	hsa-miR-569	1.07	0.10	77	73	2.47
14863	hsa-miR-570	0.98	-0.04	64	66	0.42
17490	hsa-miR-571	1.02	0.02	68	66	2.67
17551	hsa-miR-572	1.28	0.35	1913	1493	10.12
17641	hsa-miR-573	1.05	0.07	89	85	1.07
17662	hsa-miR-574	NA	NA	NA	NA	NA
17626	hsa-miR-575	1.44	0.53	133	93	7.03
17396	hsa-miR-576	0.92	-0.12	81	87	2.69
17420	hsa-miR-577	0.98	-0.02	69	70	2.87
17302	hsa-miR-578	1.13	0.17	80	71	2.04
17628	hsa-miR-579	1.04	0.06	71	68	3.07
17459	hsa-miR-580	1.05	0.07	77	72	3.03
14962	hsa-miR-581	1.21	0.27	99	81	3.50
17380	hsa-miR-582	1.09	0.12	73	66	2.43
17295	hsa-miR-583	0.96	-0.06	864	920	7.63
17423	hsa-miR-584	0.62	-0.69	561	916	4.19
17546	hsa-miR-585	1.03	0.04	100	97	2.89
17572	hsa-miR-586	0.52	-0.95	61	119	2.34
17594	hsa-miR-587	0.96	-0.06	65	68	0.24
17630	hsa-miR-588	1.05	0.07	84	79	1.67
17570	hsa-miR-589	NA	NA	NA	NA	NA
17503	hsa-miR-590	0.78	-0.36	89	114	8.76
17404	hsa-miR-591	1.06	0.08	78	74	5.15
17312	hsa-miR-592	1.13	0.18	85	75	3.71
17564	hsa-miR-593	0.87	-0.20	72	84	10.06
17349	hsa-miR-595	1.06	0.09	84	79	3.92
17449	hsa-miR-596	0.81	-0.31	79	97	3.15
17424	hsa-miR-597	1.02	0.02	71	70	1.35
17637	hsa-miR-598	NA	NA	NA	NA	NA
17600	hsa-miR-599	1.06	0.08	83	79	1.44
17377	hsa-miR-600	0.95	-0.07	151	155	3.32
17498	hsa-miR-601	0.76	-0.40	84	110	1.09
17510	hsa-miR-602	1.09	0.13	1974	1808	1.17
17393	hsa-miR-603	1.11	0.15	86	78	1.68
17592	hsa-miR-604	0.95	-0.07	78	82	3.01
17374	hsa-miR-605	1.02	0.03	71	70	2.30
17387	hsa-miR-606	0.94	-0.09	64	68	0.17
17598	hsa-miR-607	0.97	-0.05	68	69	2.27
17443	hsa-miR-608	0.78	-0.36	82	105	5.02
17353	hsa-miR-609	1.12	0.17	76	68	2.35
17445	hsa-miR-610	1.04	0.06	70	68	3.20
17611	hsa-miR-611	0.79	-0.34	82	102	1.79
17346	hsa-miR-612	1.10	0.14	2937	2738	2.74
17577	hsa-miR-613	NA	NA	NA	NA	NA
17326	hsa-miR-614	1.02	0.02	89	89	1.85

17574	hsa-miR-615	0.45	-1.16	142	333	6.28
17289	hsa-miR-616	1.00	0.00	88	88	1.61
17552	hsa-miR-617	0.98	-0.03	193	200	6.25
17336	hsa-miR-618	1.16	0.22	93	80	1.57
17405	hsa-miR-619	1.04	0.06	78	76	5.20
15349	hsa-miR-620	1.12	0.16	82	73	2.26
17588	hsa-miR-621	0.95	-0.07	77	82	6.17
17493	hsa-miR-622	1.03	0.04	75	73	4.64
17309	hsa-miR-623	1.09	0.12	1816	1692	2.39
17635	hsa-miR-624	1.05	0.07	71	67	1.70
17573	hsa-miR-625	0.87	-0.20	336	384	1.63
17351	hsa-miR-626	1.02	0.03	67	67	4.35
17625	hsa-miR-627	0.85	-0.23	99	113	12.44
17471	hsa-miR-628	0.97	-0.04	220	228	3.86
17566	hsa-miR-629	0.72	-0.48	81	114	3.01
17327	hsa-miR-630	0.98	-0.03	196	200	3.72
17633	hsa-miR-631	1.08	0.11	100	93	6.27
17444	hsa-miR-632	1.00	0.00	81	81	3.70
15475	hsa-miR-633	1.05	0.08	77	72	2.87
17398	hsa-miR-634	1.26	0.33	133	107	2.09
17391	hsa-miR-635	0.92	-0.12	74	81	2.71
17479	hsa-miR-636	NA	NA	NA	NA	NA
17354	hsa-miR-637	0.86	-0.22	102	115	13.80
17550	hsa-miR-638	1.36	0.44	1228	905	8.04
17627	hsa-miR-639	NA	NA	NA	NA	NA
17579	hsa-miR-640	0.81	-0.31	71	88	7.25
17530	hsa-miR-641	0.94	-0.08	68	72	0.31
17305	hsa-miR-642	1.33	0.42	149	112	4.40
17325	hsa-miR-643	0.96	-0.05	85	89	3.09
17563	hsa-miR-644	1.10	0.14	73	66	3.41
17613	hsa-miR-645	1.04	0.06	93	89	2.09
17491	hsa-miR-646	0.94	-0.09	64	68	3.54
17516	hsa-miR-647	1.02	0.03	70	68	1.26
17441	hsa-miR-648	0.83	-0.27	106	130	2.54
15619	hsa-miR-649	1.04	0.06	71	69	3.10
17593	hsa-miR-650	NA	NA	NA	NA	NA
17394	hsa-miR-651	1.09	0.12	80	75	5.95
17281	hsa-miR-652	0.85	-0.24	122	143	2.94
15700	hsa-miR-653	1.13	0.18	90	81	3.18
17505	hsa-miR-654	0.92	-0.13	113	124	8.60
17286	hsa-miR-655	1.13	0.18	77	69	6.83
17356	hsa-miR-656	1.07	0.10	75	70	5.28
17460	hsa-miR-657	0.87	-0.20	105	121	3.14
17522	hsa-miR-658	0.89	-0.17	1643	1843	6.49
17322	hsa-miR-659	0.95	-0.08	751	781	2.51
17338	hsa-miR-660	0.88	-0.18	87	100	0.87
17582	hsa-miR-661	NA	NA	NA	NA	NA
17507	hsa-miR-662	0.89	-0.16	71	80	0.90
17558	hsa-miR-663	1.50	0.58	3400	2237	8.46
17939	hsa-miR-671	0.99	-0.02	2905	2984	5.69
17809	hsa-miR-769-3p	1.10	0.14	1898	1650	11.09

7190	hsa-miR-9	1.24	0.31	96	77	0.65
11185	hsa-miR-9*	1.11	0.16	76	68	4.37
11179	hsa-miR-92	0.79	-0.35	80	102	2.90
17718	hsa-miR-92b	0.69	-0.53	76	108	1.58
11180	hsa-miR-93	1.11	0.15	79	71	6.20
11181	hsa-miR-95	0.98	-0.02	120	123	3.87
13147	hsa-miR-96	1.19	0.25	110	93	4.57
11182	hsa-miR-98	1.12	0.16	284	254	4.13
11183	hsa-miR-99a	0.98	-0.03	71	73	0.35
11184	hsa-miR-99b	0.96	-0.05	70	73	3.48
17347	miRPlus_17347	1.05	0.08	81	78	6.06
17411	miRPlus_17411	1.10	0.14	90	82	1.13
17653	miRPlus_17653	NA	NA	NA	NA	NA
17808	miRPlus_17808	1.56	0.64	138	87	4.71
17810	miRPlus_17810	0.87	-0.21	117	135	5.75
17811	miRPlus_17811	1.00	0.01	72	72	3.34
17812	miRPlus_17812	0.84	-0.25	76	89	5.08
17813	miRPlus_17813	0.76	-0.40	74	99	1.59
17814	miRPlus_17814	NA	NA	NA	NA	NA
17815	miRPlus_17815	NA	NA	NA	NA	NA
17816	miRPlus_17816	NA	NA	NA	NA	NA
17817	miRPlus_17817	0.98	-0.02	74	76	4.69
17818	miRPlus_17818	0.94	-0.10	76	81	7.27
17819	miRPlus_17819	0.96	-0.06	69	74	2.76
17820	miRPlus_17820	0.86	-0.21	72	83	7.21
17821	miRPlus_17821	1.00	0.00	76	75	6.01
14261	spike_control_a	1.00	0.00	162	161	2.77
14263	spike_control_b	0.95	-0.07	211	219	3.62
14264	spike_control_c	0.88	-0.18	798	882	3.53
10904	spike_control_d	0.95	-0.08	5272	5472	5.30
10906	spike_control_e	0.93	-0.11	980	1018	5.54
14262	spike_control_f	0.89	-0.17	146	162	3.25
10905	spike_control_g	0.99	-0.02	660	673	5.10
10907	spike_control_h	0.90	-0.15	2888	3216	5.69
14257	spike_control_i	0.95	-0.07	13074	13625	4.50
10899	spike_control_j	1.00	0.00	17570	17232	6.24
17822	miRPlus_17822	0.95	-0.08	75	77	6.50
17823	miRPlus_17823	1.10	0.14	80	73	2.52
17824	miRPlus_17824	0.96	-0.06	74	76	4.21
17825	miRPlus_17825	0.96	-0.06	73	75	2.57
17826	miRPlus_17826	1.00	0.00	68	67	1.27
17827	miRPlus_17827	0.81	-0.30	75	91	3.23
17828	miRPlus_17828	0.75	-0.42	86	115	4.77
17829	miRPlus_17829	1.02	0.02	76	74	14.02
17830	miRPlus_17830	0.87	-0.21	288	331	1.00
17831	miRPlus_17831	1.03	0.04	74	71	1.62
17832	miRPlus_17832	1.05	0.06	1279	1244	5.29
17833	miRPlus_17833	0.86	-0.23	105	122	4.07
17834	miRPlus_17834	0.96	-0.07	159	166	4.92
17836	miRPlus_17836	0.94	-0.09	1151	1230	2.06
17837	miRPlus_17837	0.99	-0.01	69	71	2.27

17838	miRPlus_17838	1.00	0.00	74	73	2.69
17840	miRPlus_17840	0.87	-0.21	158	183	2.11
17841	miRPlus_17841	1.05	0.08	88	84	2.03
17842	miRPlus_17842	1.10	0.13	74	67	2.53
17843	miRPlus_17843	1.06	0.09	72	67	2.61
17844	miRPlus_17844	0.83	-0.27	76	94	3.76
17845	miRPlus_17845	0.83	-0.27	102	125	5.04
17847	miRPlus_17847	0.92	-0.11	82	89	2.91
17848	miRPlus_17848	0.96	-0.06	81	85	2.81
17849	miRPlus_17849	NA	NA	NA	NA	NA
17850	miRPlus_17850	1.05	0.07	75	73	5.19
17851	miRPlus_17851	0.87	-0.21	73	85	4.66
17852	miRPlus_17852	NA	NA	NA	NA	NA
17853	miRPlus_17853	0.99	-0.01	72	72	3.81
17854	miRPlus_17854	0.82	-0.29	97	119	6.89
17855	miRPlus_17855	1.10	0.14	103	94	3.67
17856	miRPlus_17856	1.35	0.43	5126	3737	5.54
17857	miRPlus_17857	1.15	0.20	104	89	4.23
17858	miRPlus_17858	1.32	0.40	133	102	4.67
17859	miRPlus_17859	0.95	-0.08	231	242	2.92
17860	miRPlus_17860	1.09	0.12	106	98	1.84
17861	miRPlus_17861	1.00	0.00	299	295	4.84
17862	miRPlus_17862	1.05	0.07	75	70	5.76
17863	miRPlus_17863	0.91	-0.14	125	135	2.39
17864	miRPlus_17864	1.36	0.45	4939	3577	6.08
17865	miRPlus_17865	1.21	0.27	1494	1205	4.83
17866	miRPlus_17866	NA	NA	NA	NA	NA
17867	miRPlus_17867	0.74	-0.44	76	106	5.94
17868	miRPlus_17868	0.77	-0.38	135	176	1.76
17869	miRPlus_17869	0.76	-0.39	8847	10655	9.06
17870	miRPlus_17870	0.71	-0.49	78	108	1.65
17871	miRPlus_17871	0.93	-0.10	2049	2192	4.63
17872	miRPlus_17872	0.61	-0.72	66	109	3.35
17873	miRPlus_17873	0.80	-0.32	73	91	8.92
17874	miRPlus_17874	0.68	-0.55	77	114	3.50
17875	miRPlus_17875	0.74	-0.43	77	107	5.12
17876	miRPlus_17876	0.84	-0.26	69	83	5.56
17877	miRPlus_17877	1.03	0.05	2507	2417	10.61
17878	miRPlus_17878	1.11	0.15	1522	1367	5.40
17879	miRPlus_17879	0.96	-0.06	77	80	3.91
17880	miRPlus_17880	1.00	-0.01	65	66	0.58
17881	miRPlus_17881	1.42	0.51	4484	3208	8.57
17882	miRPlus_17882	1.00	0.00	134	135	6.17
17883	miRPlus_17883	0.91	-0.14	73	80	1.41
17884	miRPlus_17884	0.71	-0.49	71	100	7.42
17885	miRPlus_17885	NA	NA	NA	NA	NA
17886	miRPlus_17886	NA	NA	NA	NA	NA
17887	miRPlus_17887	NA	NA	NA	NA	NA
17888	miRPlus_17888	NA	NA	NA	NA	NA
17889	miRPlus_17889	0.98	-0.02	66	67	4.31
17890	miRPlus_17890	1.19	0.25	2974	2493	1.96

17891	miRPlus_17891	0.82	-0.28	76	93	1.20
17892	miRPlus_17892	NA	NA	NA	NA	NA
17893	miRPlus_17893	0.74	-0.43	71	95	14.50
17894	miRPlus_17894	1.16	0.21	425	351	6.40
17895	miRPlus_17895	0.92	-0.11	74	80	3.05
17896	miRPlus_17896	2.82	1.50	612	217	5.46
17897	miRPlus_17897	1.02	0.02	72	69	1.55
17898	miRPlus_17898	0.93	-0.10	73	78	3.46
17899	miRPlus_17899	0.84	-0.24	72	86	5.28
17900	miRPlus_17900	0.88	-0.19	166	189	3.63
17902	miRPlus_17902	NA	NA	NA	NA	NA
17903	miRPlus_17903	1.23	0.29	172	139	2.19
17904	miRPlus_17904	0.83	-0.27	220	262	3.67
17905	miRPlus_17905	0.95	-0.07	89	94	1.66
17906	miRPlus_17906	1.00	0.00	70	69	1.27
17907	miRPlus_17907	0.97	-0.05	68	70	2.29
17908	miRPlus_17908	NA	NA	NA	NA	NA
17909	miRPlus_17909	1.01	0.01	74	74	7.01
17910	miRPlus_17910	0.89	-0.17	71	79	0.28
17911	miRPlus_17911	0.98	-0.03	69	71	2.96
17912	miRPlus_17912	NA	NA	NA	NA	NA
17913	miRPlus_17913	NA	NA	NA	NA	NA
17914	miRPlus_17914	0.86	-0.22	71	83	2.99
17915	miRPlus_17915	0.87	-0.20	1928	2218	4.98
17916	miRPlus_17916	NA	NA	NA	NA	NA
17917	miRPlus_17917	NA	NA	NA	NA	NA
17918	miRPlus_17918	0.77	-0.38	96	126	4.92
17919	miRPlus_17919	0.95	-0.07	69	73	0.77
17920	miRPlus_17920	NA	NA	NA	NA	NA
17921	miRPlus_17921	0.96	-0.06	243	245	3.45
17922	miRPlus_17922	0.98	-0.04	70	72	0.89
17923	miRPlus_17923	NA	NA	NA	NA	NA
17924	miRPlus_17924	NA	NA	NA	NA	NA
17925	miRPlus_17925	0.76	-0.39	71	92	1.78
17926	miRPlus_17926	NA	NA	NA	NA	NA
17927	miRPlus_17927	0.71	-0.49	301	435	6.21
17928	miRPlus_17928	0.92	-0.12	74	80	3.43
17929	miRPlus_17929	NA	NA	NA	NA	NA
17930	miRPlus_17930	1.00	0.00	150	149	3.94
17931	miRPlus_17931	NA	NA	NA	NA	NA
17932	miRPlus_17932	1.04	0.06	67	64	2.06
17933	miRPlus_17933	0.74	-0.44	74	101	1.39
17934	miRPlus_17934	0.80	-0.33	71	89	7.01
17935	miRPlus_17935	NA	NA	NA	NA	NA
17936	miRPlus_17936	0.61	-0.72	73	120	1.82
17937	miRPlus_17937	NA	NA	NA	NA	NA
17938	miRPlus_17938	0.99	-0.02	68	69	1.97
17940	miRPlus_17940	0.98	-0.03	68	70	1.93
17941	miRPlus_17941	0.97	-0.04	69	71	2.21
17942	miRPlus_17942	0.98	-0.02	117	119	6.66
17943	miRPlus_17943	1.01	0.01	293	283	3.67

17944	miRPlus_17944	NA	NA	NA	NA	NA
17945	miRPlus_17945	0.97	-0.04	195	200	3.47
17946	miRPlus_17946	0.77	-0.38	94	123	5.04
17948	miRPlus_17948	NA	NA	NA	NA	NA
17949	miRPlus_17949	NA	NA	NA	NA	NA
17950	miRPlus_17950	1.17	0.22	669	582	4.74
17951	miRPlus_17951	NA	NA	NA	NA	NA
17952	miRPlus_17952	1.32	0.40	1783	1350	3.29
17953	miRPlus_17953	1.05	0.07	777	744	3.36
17954	miRPlus_17954	0.67	-0.57	74	112	5.31
17955	miRPlus_17955	0.72	-0.47	80	113	4.77
17956	miRPlus_17956	0.80	-0.32	71	90	9.42
17957	miRPlus_17957	1.02	0.03	79	76	2.01
17958	miRPlus_17958	NA	NA	NA	NA	NA
17959	miRPlus_17959	0.64	-0.63	78	121	1.67
17960	miRPlus_17960	1.06	0.09	2441	2289	2.73
17961	miRPlus_17961	0.80	-0.33	98	123	6.66

Table A.2 | LNA miRNA microarray data for LIM 1863 cells treated with TGF-β.

Gene Id	Annotation	Median Ratios	Log Median Ratios	Median Hy3	Median Hy5	CV
17748	hsa-let-7a	1.08	0.11	2838	2648	0.87
17749	hsa-let-7b	1.17	0.23	1168	1006	1.29
19004	hsa-let-7c	0.98	-0.02	2996	3042	3.49
17750	hsa-let-7d	0.93	-0.10	188	204	2.69
17751	hsa-let-7e	0.88	-0.19	114	131	2.74
17752	hsa-let-7f	0.79	-0.34	82	105	1.94
19602	hsa-let-7g	0.99	-0.01	155	154	1.89
19580	hsa-let-7i	1.06	0.09	386	363	0.29
10916	hsa-miR-1	1.38	0.46	105	76	2.77
19581	hsa-miR-100	NA	NA	NA	NA	NA
17615	hsa-miR-101	0.99	-0.02	86	87	2.59
10919	hsa-miR-103	1.02	0.03	650	628	1.84
10920	hsa-miR-105	NA	NA	NA	NA	NA
19582	hsa-miR-106b	0.85	-0.23	203	238	0.87
10923	hsa-miR-107	1.26	0.33	773	614	1.82
13485	hsa-miR-10a	1.07	0.10	1068	993	1.62
10925	hsa-miR-10b	0.86	-0.22	86	100	3.92
19583	hsa-miR-122a	1.09	0.13	67	61	0.22
14328	hsa-miR-124a	1.23	0.30	97	78	4.99
10928	hsa-miR-125a	0.93	-0.11	611	671	4.33
10929	hsa-miR-125b	0.92	-0.12	121	130	17.48
4610	hsa-miR-126	0.98	-0.03	69	71	2.11
10930	hsa-miR-126*	NA	NA	NA	NA	NA
10931	hsa-miR-127	0.80	-0.33	67	86	2.63
10932	hsa-miR-128a	1.06	0.08	87	83	3.19
19584	hsa-miR-128b	1.00	0.00	70	70	7.01
10934	hsa-miR-129	1.29	0.37	311	240	0.93
10935	hsa-miR-130a	NA	NA	NA	NA	NA
10936	hsa-miR-130b	1.05	0.07	166	158	0.75
10937	hsa-miR-132	NA	NA	NA	NA	NA
10938	hsa-miR-133a-133b	0.94	-0.09	88	94	3.80
10940	hsa-miR-134	0.88	-0.18	79	89	0.89
10941	hsa-miR-135a	1.63	0.70	124	76	2.28
10942	hsa-miR-135b	1.24	0.31	102	82	5.85
10943	hsa-miR-136	1.14	0.19	92	81	3.34
10944	hsa-miR-137	1.45	0.54	111	78	2.83
13140	hsa-miR-138	1.19	0.25	96	81	4.64
10945	hsa-miR-139	1.13	0.17	77	67	4.30
4700	hsa-miR-140	1.13	0.17	79	70	2.65
10946	hsa-miR-141	1.09	0.12	151	139	3.11
10947	hsa-miR-142-3p	0.95	-0.07	1181	1248	2.24
19015	hsa-miR-142-5p	0.96	-0.06	377	403	3.25
13177	hsa-miR-143	NA	NA	NA	NA	NA
10950	hsa-miR-144	1.09	0.13	75	69	6.44

10951	hsa-miR-145	1.07	0.10	79	74	2.31
10952	hsa-miR-146a	1.05	0.06	80	76	1.51
10306	hsa-miR-146b	0.98	-0.03	73	75	2.49
10954	hsa-miR-147	1.20	0.27	79	65	3.46
10955	hsa-miR-148a	1.22	0.29	129	104	3.54
19585	hsa-miR-148b	1.05	0.07	123	116	1.00
19586	hsa-miR-149	NA	NA	NA	NA	NA
19587	hsa-miR-150	0.91	-0.13	72	79	4.08
17463	hsa-miR-151	1.02	0.03	117	115	1.91
17676	hsa-miR-152	0.94	-0.09	75	79	3.35
10961	hsa-miR-153	NA	NA	NA	NA	NA
10962	hsa-miR-154	1.18	0.24	102	85	3.42
10963	hsa-miR-154*	0.99	-0.01	70	71	2.84
10964	hsa-miR-155	1.16	0.21	94	81	3.97
10965	hsa-miR-15a	0.96	-0.06	106	109	1.27
17280	hsa-miR-15b	0.88	-0.19	331	380	1.27
10967	hsa-miR-16	1.08	0.12	590	547	1.92
19588	hsa-miR-17-3p	1.03	0.04	215	205	2.45
17605	hsa-miR-17-5p-106a	0.91	-0.14	834	925	2.54
10971	hsa-miR-181a	1.05	0.07	72	68	3.34
11013	hsa-miR-181a*	NA	NA	NA	NA	NA
10972	hsa-miR-181b	1.14	0.19	83	73	5.41
10973	hsa-miR-181c	1.91	0.93	145	75	2.02
10974	hsa-miR-181d	1.16	0.21	78	67	2.71
10975	hsa-miR-182	1.14	0.19	169	150	1.39
10976	hsa-miR-182*	1.16	0.22	101	87	1.03
10977	hsa-miR-183	0.91	-0.14	182	204	2.45
10978	hsa-miR-184	1.19	0.26	257	213	3.09
5560	hsa-miR-185	0.93	-0.10	1828	1964	0.44
10979	hsa-miR-186	1.18	0.23	107	91	4.05
17643	hsa-miR-187	NA	NA	NA	NA	NA
19589	hsa-miR-188	1.09	0.12	69	63	1.58
10982	hsa-miR-189	0.95	-0.07	69	74	3.45
10983	hsa-miR-18a	0.99	-0.02	368	374	2.00
13178	hsa-miR-18a*	0.78	-0.35	72	91	0.49
13141	hsa-miR-18b	1.00	0.00	350	351	1.24
10984	hsa-miR-190	1.31	0.39	92	70	1.15
10985	hsa-miR-191	0.98	-0.03	532	550	2.53
13126	hsa-miR-191*	1.13	0.18	85	75	2.21
17732	hsa-miR-192	1.04	0.05	465	453	2.10
13172	hsa-miR-192	0.92	-0.12	69	74	4.51
10986	hsa-miR-193a	1.10	0.14	1618	1468	1.13
10987	hsa-miR-193b	0.88	-0.19	85	97	2.59
10988	hsa-miR-194	1.04	0.05	210	201	1.84
13148	hsa-miR-195	0.78	-0.36	70	89	1.18
10990	hsa-miR-196a	1.15	0.21	129	112	2.66
10991	hsa-miR-196b	1.35	0.44	116	85	2.69
10992	hsa-miR-197	1.11	0.15	140	125	3.73
10993	hsa-miR-198	0.96	-0.06	420	439	2.41
19590	hsa-miR-199a	0.83	-0.26	67	85	30.41

10995	hsa-miR-199a*	1.07	0.10	66	61	1.62
19591	hsa-miR-199b	NA	NA	NA	NA	NA
10997	hsa-miR-19a	1.09	0.13	187	170	2.22
10998	hsa-miR-19b	0.98	-0.04	130	133	0.55
11000	hsa-miR-200a	1.23	0.30	2277	1850	0.72
13127	hsa-miR-200a*	0.95	-0.08	68	73	13.09
9578	hsa-miR-200b	1.02	0.02	639	632	2.02
17427	hsa-miR-200c	1.15	0.20	643	560	2.42
11003	hsa-miR-202	0.94	-0.08	847	897	1.14
10314	hsa-miR-202*	1.92	0.94	133	72	8.23
11004	hsa-miR-203	1.27	0.34	215	169	2.16
11005	hsa-miR-204	1.14	0.19	88	78	2.89
11006	hsa-miR-205	0.91	-0.13	69	77	5.03
11007	hsa-miR-206	1.01	0.01	87	87	4.94
5730	hsa-miR-208	0.99	-0.02	110	117	6.28
11008	hsa-miR-20a	0.92	-0.11	932	1002	2.97
10999	hsa-miR-20a	0.97	-0.05	790	818	2.08
11009	hsa-miR-20b	0.92	-0.12	136	148	1.54
5740	hsa-miR-21	1.75	0.81	1082	627	1.88
13511	hsa-miR-210	1.09	0.12	944	869	5.04
11011	hsa-miR-211	1.16	0.22	89	78	2.47
19592	hsa-miR-212	0.76	-0.41	149	197	3.09
11014	hsa-miR-214	0.91	-0.13	1654	1811	2.16
11015	hsa-miR-215	1.02	0.02	323	310	2.48
11016	hsa-miR-216	1.03	0.05	70	68	4.73
19016	hsa-miR-217	1.03	0.04	67	65	2.34
11018	hsa-miR-218	1.05	0.08	75	72	1.85
11019	hsa-miR-219	NA	NA	NA	NA	NA
11020	hsa-miR-22	1.15	0.21	117	102	3.14
11021	hsa-miR-220	1.11	0.15	72	65	1.03
11022	hsa-miR-221	1.17	0.22	313	268	2.29
11023	hsa-miR-222	1.17	0.23	1897	1619	1.79
11024	hsa-miR-223	1.09	0.12	104	96	5.50
11025	hsa-miR-224	1.05	0.07	71	68	5.29
11026	hsa-miR-23a	1.37	0.46	639	467	0.54
11027	hsa-miR-23b	1.12	0.17	386	342	1.68
17506	hsa-miR-24	1.29	0.37	278	215	2.63
11029	hsa-miR-25	1.20	0.27	97	79	4.77
11030	hsa-miR-26a	1.33	0.41	319	240	0.93
11031	hsa-miR-26b	1.32	0.40	322	242	3.78
19593	hsa-miR-27a	1.17	0.23	135	115	2.15
13175	hsa-miR-27b	1.18	0.24	139	117	2.25
11034	hsa-miR-28	0.93	-0.11	71	78	3.76
19594	hsa-miR-296	1.23	0.30	212	173	1.38
11037	hsa-miR-299-3p	NA	NA	NA	NA	NA
11038	hsa-miR-299-5p	0.92	-0.11	75	81	4.27
11039	hsa-miR-29a	1.08	0.12	3376	3135	0.83
11040	hsa-miR-29b	1.21	0.27	268	223	0.97
11041	hsa-miR-29c	1.08	0.11	175	162	1.73
13143	hsa-miR-301	0.62	-0.68	64	103	3.20
11222	hsa-miR-302a	1.16	0.21	81	71	3.54

11042	hsa-miR-302a*	0.99	-0.01	81	82	1.15
11043	hsa-miR-302b	NA	NA	NA	NA	NA
5930	hsa-miR-302b*	1.03	0.04	76	74	18.72
11044	hsa-miR-302c	1.06	0.09	80	75	0.37
11045	hsa-miR-302c*	1.00	0.00	120	120	2.72
11046	hsa-miR-302d	1.11	0.15	78	70	3.75
19595	hsa-miR-30a-3p	1.04	0.06	64	61	1.30
11048	hsa-miR-30a-5p	0.99	-0.01	109	111	3.51
17565	hsa-miR-30b	1.11	0.15	373	342	4.78
17502	hsa-miR-30c	1.10	0.13	230	210	0.57
19596	hsa-miR-30d	0.97	-0.04	107	109	1.63
11224	hsa-miR-30e-3p	0.94	-0.09	72	77	3.05
13174	hsa-miR-30e-5p	1.03	0.05	82	79	2.54
11052	hsa-miR-31	1.08	0.10	136	127	0.88
11053	hsa-miR-32	0.87	-0.20	77	89	3.03
11054	hsa-miR-320	1.15	0.20	2858	2501	2.38
11055	hsa-miR-323	1.11	0.16	74	66	1.69
11056	hsa-miR-324-3p	0.98	-0.03	179	182	1.00
11057	hsa-miR-324-5p	0.96	-0.06	83	87	2.08
11058	hsa-miR-325	1.14	0.18	73	64	2.30
11059	hsa-miR-326	1.23	0.30	375	309	3.36
11060	hsa-miR-328	0.98	-0.03	69	70	0.83
11061	hsa-miR-329	NA	NA	NA	NA	NA
11062	hsa-miR-33	0.98	-0.03	76	84	7.64
11063	hsa-miR-330	0.90	-0.15	68	76	2.08
11064	hsa-miR-331	0.91	-0.14	90	99	2.24
11065	hsa-miR-335	0.94	-0.09	92	98	5.53
11066	hsa-miR-337	1.13	0.18	76	67	2.35
11067	hsa-miR-338	1.39	0.47	183	132	1.74
19597	hsa-miR-339	0.87	-0.21	72	82	2.24
17294	hsa-miR-33b	1.20	0.26	89	75	0.96
13144	hsa-miR-340	0.99	-0.02	84	85	2.36
11069	hsa-miR-342	NA	NA	NA	NA	NA
11070	hsa-miR-345	0.97	-0.05	102	106	2.70
19018	hsa-miR-346	0.92	-0.13	242	264	4.35
11072	hsa-miR-34a	1.27	0.34	97	77	1.02
11073	hsa-miR-34b	NA	NA	NA	NA	NA
11074	hsa-miR-34c	NA	NA	NA	NA	NA
14301	hsa-miR-361	0.80	-0.32	96	121	2.74
14279	hsa-miR-362	1.13	0.17	68	61	0.67
11077	hsa-miR-363	NA	NA	NA	NA	NA
11078	hsa-miR-365	0.99	-0.01	130	131	0.54
14280	hsa-miR-367	1.12	0.16	68	61	17.13
19598	hsa-miR-368	NA	NA	NA	NA	NA
11081	hsa-miR-369-3p	1.55	0.63	117	76	6.21
13145	hsa-miR-369-5p	1.09	0.12	70	65	2.51
11082	hsa-miR-370	1.08	0.11	250	236	2.20
11083	hsa-miR-371	1.11	0.15	76	69	3.34
11084	hsa-miR-372	1.12	0.16	71	64	5.37
11085	hsa-miR-373	1.18	0.24	71	60	4.21
11086	hsa-miR-373*	1.36	0.45	690	506	1.34

11087	hsa-miR-374	1.05	0.07	75	71	1.27
11088	hsa-miR-375	1.12	0.16	106	95	1.27
11089	hsa-miR-376a	1.04	0.06	65	62	3.17
14268	hsa-miR-376a*	0.97	-0.04	68	70	6.81
11090	hsa-miR-376b	1.11	0.15	76	68	6.34
11091	hsa-miR-377	1.11	0.15	81	73	5.72
11092	hsa-miR-378	1.00	-0.01	72	73	3.95
11093	hsa-miR-379	1.24	0.31	94	77	2.29
11094	hsa-miR-380-3p	1.11	0.15	67	60	2.15
13170	hsa-miR-380-5p	NA	NA	NA	NA	NA
14306	hsa-miR-381	0.96	-0.06	473	493	1.88
11097	hsa-miR-382	1.02	0.02	179	178	1.80
11098	hsa-miR-383	1.02	0.03	69	68	1.40
11099	hsa-miR-384	1.12	0.16	77	69	5.71
11240	hsa-miR-409-3p	0.96	-0.06	71	74	0.83
14310	hsa-miR-409-5p	0.86	-0.22	74	88	2.16
11102	hsa-miR-410	NA	NA	NA	NA	NA
17482	hsa-miR-411	NA	NA	NA	NA	NA
11103	hsa-miR-412	1.00	0.01	71	72	14.32
17474	hsa-miR-421	0.92	-0.12	98	107	2.76
11104	hsa-miR-422a	1.12	0.16	77	69	3.29
11105	hsa-miR-422b	0.91	-0.13	193	212	1.31
11106	hsa-miR-423	0.93	-0.11	151	162	2.76
11107	hsa-miR-424	0.88	-0.18	73	84	2.27
11108	hsa-miR-425-3p	NA	NA	NA	NA	NA
17608	hsa-miR-425-5p	1.12	0.17	123	110	3.65
13171	hsa-miR-429	0.97	-0.04	120	124	0.88
11110	hsa-miR-431	NA	NA	NA	NA	NA
11111	hsa-miR-432	1.22	0.29	81	66	1.45
13128	hsa-miR-432*	0.83	-0.26	85	104	3.38
11112	hsa-miR-433	1.01	0.02	73	72	2.87
11113	hsa-miR-448	1.11	0.15	72	65	2.27
11114	hsa-miR-449	1.08	0.11	86	79	6.10
17706	hsa-miR-449b	1.00	-0.01	69	69	2.03
17835	hsa-miR-450	0.98	-0.03	74	74	13.03
8538	hsa-miR-450	NA	NA	NA	NA	NA
11248	hsa-miR-451	0.83	-0.27	80	97	1.45
11116	hsa-miR-452	0.88	-0.19	350	404	1.73
13129	hsa-miR-452*	NA	NA	NA	NA	NA
11117	hsa-miR-453	1.11	0.15	77	69	12.91
17450	hsa-miR-454-3p	1.12	0.16	67	60	1.47
13179	hsa-miR-455	1.02	0.03	69	67	9.35
13180	hsa-miR-483	2.16	1.11	299	137	3.88
13181	hsa-miR-484	1.67	0.74	172	102	3.07
11118	hsa-miR-485-3p	1.03	0.04	88	86	2.80
11119	hsa-miR-485-5p	1.12	0.16	70	63	0.74
13182	hsa-miR-486	1.57	0.65	137	87	1.13
13183	hsa-miR-487a	0.95	-0.08	76	80	4.00
14285	hsa-miR-487b	0.57	-0.81	138	240	5.88
11120	hsa-miR-488	NA	NA	NA	NA	NA
11121	hsa-miR-489	0.84	-0.25	69	80	8.91

11122	hsa-miR-490	0.90	-0.15	120	135	2.25
11123	hsa-miR-491	1.00	0.00	78	78	2.96
11124	hsa-miR-492	1.33	0.41	2945	2257	2.61
14270	hsa-miR-493-3p	NA	NA	NA	NA	NA
11125	hsa-miR-493-5p	1.45	0.54	112	78	3.93
14287	hsa-miR-494	0.66	-0.60	1619	2457	2.30
17348	hsa-miR-495	0.97	-0.04	82	85	7.69
11128	hsa-miR-496	1.12	0.16	68	61	0.73
11129	hsa-miR-497	1.21	0.27	83	69	1.26
11130	hsa-miR-498	1.16	0.21	1926	1674	1.90
14313	hsa-miR-499	1.09	0.12	71	66	2.37
11132	hsa-miR-500	1.15	0.21	169	147	3.23
11133	hsa-miR-501	0.94	-0.10	69	74	4.58
11134	hsa-miR-502	1.11	0.15	82	76	2.99
11135	hsa-miR-503	1.00	-0.01	1108	1121	0.98
11136	hsa-miR-504	0.97	-0.04	72	75	8.45
14314	hsa-miR-505	NA	NA	NA	NA	NA
11138	hsa-miR-506	1.07	0.10	80	74	2.56
11139	hsa-miR-507	1.16	0.21	86	72	2.48
11140	hsa-miR-508	1.43	0.52	107	75	18.66
11141	hsa-miR-509	1.17	0.22	77	66	20.45
11142	hsa-miR-510	0.97	-0.05	164	169	1.28
11143	hsa-miR-511	1.04	0.05	71	68	3.37
11144	hsa-miR-512-3p	1.09	0.12	73	68	12.72
11145	hsa-miR-512-5p	0.94	-0.09	583	620	1.83
11146	hsa-miR-513	0.88	-0.19	1870	2135	2.64
11147	hsa-miR-514	1.11	0.15	75	66	2.56
11148	hsa-miR-515-3p	NA	NA	NA	NA	NA
11149	hsa-miR-515-5p	1.05	0.07	69	66	1.41
11150	hsa-miR-516-3p	1.07	0.10	74	70	4.07
11151	hsa-miR-516-5p	0.85	-0.24	98	115	3.52
13130	hsa-miR-517*	1.81	0.85	144	78	3.23
11153	hsa-miR-517a-517b	1.14	0.19	80	71	3.66
11154	hsa-miR-517c	1.16	0.21	84	73	2.59
11155	hsa-miR-518a	1.12	0.16	77	69	1.72
11156	hsa-miR-518b	1.14	0.20	106	93	4.03
11157	hsa-miR-518c	1.11	0.16	73	67	3.91
13131	hsa-miR-518c*	0.92	-0.12	1044	1140	2.54
11158	hsa-miR-518d	1.07	0.10	76	72	10.92
11159	hsa-miR-518e	NA	NA	NA	NA	NA
11160	hsa-miR-518f	1.02	0.03	72	71	2.39
10586	hsa-miR-518f*-526a	1.01	0.02	237	234	1.93
11161	hsa-miR-519a	NA	NA	NA	NA	NA
11162	hsa-miR-519b	1.12	0.16	70	62	3.88
10482	hsa-miR-519c	1.06	0.09	65	60	6.30
11163	hsa-miR-519d	0.80	-0.32	112	139	1.91
11164	hsa-miR-519e	0.79	-0.34	75	95	3.21
13132	hsa-miR-519e*	1.00	0.00	209	211	1.97
11165	hsa-miR-520a	NA	NA	NA	NA	NA

13133	hsa-miR-520a*	0.85	-0.23	76	89	1.59
11166	hsa-miR-520c-520b	1.07	0.10	66	62	3.18
11168	hsa-miR-520d	1.16	0.21	72	62	1.70
13134	hsa-miR-520d*	0.80	-0.32	86	108	3.84
11169	hsa-miR-520e	1.56	0.64	117	75	4.90
11167	hsa-miR-520f-520c	1.18	0.24	73	62	2.85
13146	hsa-miR-520g-520h	NA	NA	NA	NA	NA
11171	hsa-miR-521	1.07	0.10	75	70	3.94
11172	hsa-miR-522	1.21	0.28	78	64	2.92
11173	hsa-miR-523	1.07	0.10	74	68	3.49
10618	hsa-miR-524*	0.79	-0.33	71	89	0.96
11175	hsa-miR-525	0.80	-0.33	169	214	3.55
11174	hsa-miR-525*-524	1.03	0.05	74	71	5.65
11176	hsa-miR-526b	0.86	-0.22	86	100	10.52
13136	hsa-miR-526b*	1.54	0.62	116	76	2.97
13137	hsa-miR-526c	1.16	0.22	132	113	1.31
11177	hsa-miR-527	0.92	-0.12	205	224	1.11
17624	hsa-miR-532	0.92	-0.12	85	91	2.09
14271	hsa-miR-539	1.18	0.24	68	59	4.82
14315	hsa-miR-542-3p	1.10	0.13	72	65	8.50
14273	hsa-miR-542-5p	1.02	0.03	70	70	1.71
13712	hsa-miR-544	1.08	0.11	65	60	1.31
17846	hsa-miR-545	1.07	0.09	80	75	0.99
13721	hsa-miR-545	0.99	-0.01	68	69	11.82
17535	hsa-miR-548a	1.06	0.09	74	69	3.50
17298	hsa-miR-548b	1.18	0.24	84	71	2.12
15313	hsa-miR-548c	1.08	0.11	72	67	6.68
17533	hsa-miR-548d	0.90	-0.16	70	78	8.60
17370	hsa-miR-549	0.98	-0.03	70	73	2.84
17660	hsa-miR-550	0.87	-0.20	89	102	2.19
17272	hsa-miR-551a	1.04	0.06	303	296	2.76
17500	hsa-miR-551b	1.00	0.00	71	70	5.39
17668	hsa-miR-552	0.89	-0.16	79	88	1.18
17271	hsa-miR-553	1.09	0.13	72	66	3.41
17640	hsa-miR-554	1.01	0.01	78	77	3.20
17612	hsa-miR-555	0.98	-0.02	70	71	2.74
17426	hsa-miR-556	1.08	0.10	75	69	2.38
17376	hsa-miR-557	1.13	0.18	805	717	0.73
17652	hsa-miR-558	NA	NA	NA	NA	NA
14755	hsa-miR-559	0.99	-0.02	69	68	4.07
17456	hsa-miR-560	NA	NA	NA	NA	NA
14773	hsa-miR-561	1.16	0.21	83	71	3.20
17536	hsa-miR-562	NA	NA	NA	NA	NA
17569	hsa-miR-563	NA	NA	NA	NA	NA
17645	hsa-miR-564	1.23	0.29	99	80	2.21
17413	hsa-miR-565	0.94	-0.09	122	129	1.96
17634	hsa-miR-567	0.95	-0.08	64	69	13.27

17661	hsa-miR-568	0.97	-0.04	73	76	2.92
14854	hsa-miR-569	1.06	0.09	72	68	3.77
14863	hsa-miR-570	NA	NA	NA	NA	NA
17490	hsa-miR-571	NA	NA	NA	NA	NA
17551	hsa-miR-572	1.44	0.52	2285	1596	1.24
17641	hsa-miR-573	1.06	0.09	84	79	1.61
17662	hsa-miR-574	0.79	-0.34	71	91	14.68
17626	hsa-miR-575	1.40	0.49	118	83	6.98
17396	hsa-miR-576	0.90	-0.15	77	84	5.80
17420	hsa-miR-577	NA	NA	NA	NA	NA
17302	hsa-miR-578	1.05	0.08	72	69	2.08
17628	hsa-miR-579	1.04	0.06	67	65	2.27
17459	hsa-miR-580	1.07	0.10	71	65	3.67
14962	hsa-miR-581	1.24	0.31	92	74	1.96
17380	hsa-miR-582	1.11	0.15	70	63	1.76
17295	hsa-miR-583	0.91	-0.13	1125	1216	2.79
17423	hsa-miR-584	0.58	-0.78	522	878	4.12
17546	hsa-miR-585	1.11	0.15	95	85	8.05
17572	hsa-miR-586	0.56	-0.85	60	111	6.62
17594	hsa-miR-587	NA	NA	NA	NA	NA
17630	hsa-miR-588	1.05	0.07	77	74	4.31
17570	hsa-miR-589	NA	NA	NA	NA	NA
17503	hsa-miR-590	0.80	-0.33	78	97	4.51
17404	hsa-miR-591	0.96	-0.06	72	76	14.88
17312	hsa-miR-592	1.15	0.20	80	71	4.52
17564	hsa-miR-593	NA	NA	NA	NA	NA
17349	hsa-miR-595	1.07	0.10	77	72	2.79
17449	hsa-miR-596	0.83	-0.26	73	87	0.93
17424	hsa-miR-597	NA	NA	NA	NA	NA
17637	hsa-miR-598	NA	NA	NA	NA	NA
17600	hsa-miR-599	1.09	0.13	79	72	4.07
17377	hsa-miR-600	1.01	0.02	138	136	2.41
17498	hsa-miR-601	0.78	-0.36	72	93	3.32
17510	hsa-miR-602	1.04	0.05	2236	2137	3.83
17393	hsa-miR-603	1.06	0.09	76	73	6.65
17592	hsa-miR-604	1.01	0.02	72	70	3.85
17374	hsa-miR-605	1.03	0.05	68	66	2.92
17387	hsa-miR-606	NA	NA	NA	NA	NA
17598	hsa-miR-607	NA	NA	NA	NA	NA
17443	hsa-miR-608	0.82	-0.28	73	90	3.28
17353	hsa-miR-609	1.11	0.15	71	64	1.91
17445	hsa-miR-610	1.07	0.10	67	62	2.41
17611	hsa-miR-611	0.86	-0.22	78	91	1.27
17346	hsa-miR-612	1.19	0.25	3329	2767	1.35
17577	hsa-miR-613	NA	NA	NA	NA	NA
17326	hsa-miR-614	1.03	0.04	83	81	2.12
17574	hsa-miR-615	0.54	-0.89	118	220	15.50
17289	hsa-miR-616	0.98	-0.02	80	81	4.15
17552	hsa-miR-617	0.96	-0.06	560	588	3.42
17336	hsa-miR-618	1.15	0.21	86	73	1.71
17405	hsa-miR-619	1.07	0.10	72	68	2.14

15349	hsa-miR-620	1.12	0.16	75	67	2.81
17588	hsa-miR-621	0.99	-0.02	72	75	3.78
17493	hsa-miR-622	1.02	0.02	69	68	1.22
17309	hsa-miR-623	1.13	0.18	2292	2053	3.21
17635	hsa-miR-624	0.87	-0.21	67	81	30.57
17573	hsa-miR-625	0.89	-0.17	350	405	4.31
17351	hsa-miR-626	NA	NA	NA	NA	NA
17625	hsa-miR-627	1.04	0.06	90	91	5.58
17471	hsa-miR-628	0.93	-0.11	544	580	3.93
17566	hsa-miR-629	0.81	-0.31	89	112	6.51
17327	hsa-miR-630	0.99	-0.02	405	416	1.55
17633	hsa-miR-631	1.11	0.16	92	81	5.04
17444	hsa-miR-632	1.03	0.04	77	75	4.92
15475	hsa-miR-633	0.98	-0.04	72	75	5.34
17398	hsa-miR-634	1.19	0.25	118	99	2.73
17391	hsa-miR-635	0.92	-0.12	67	74	23.57
17479	hsa-miR-636	NA	NA	NA	NA	NA
17354	hsa-miR-637	0.88	-0.19	91	103	2.92
17550	hsa-miR-638	1.21	0.28	1743	1438	3.37
17627	hsa-miR-639	NA	NA	NA	NA	NA
17579	hsa-miR-640	0.80	-0.32	68	83	6.59
17530	hsa-miR-641	NA	NA	NA	NA	NA
17305	hsa-miR-642	1.28	0.36	135	104	44.46
17325	hsa-miR-643	0.97	-0.05	79	82	3.39
17563	hsa-miR-644	1.10	0.13	70	63	3.81
17613	hsa-miR-645	1.05	0.06	84	80	1.78
17491	hsa-miR-646	NA	NA	NA	NA	NA
17516	hsa-miR-647	1.07	0.09	66	62	1.44
17441	hsa-miR-648	0.84	-0.25	99	118	2.69
15619	hsa-miR-649	1.04	0.06	66	64	0.49
17593	hsa-miR-650	NA	NA	NA	NA	NA
17394	hsa-miR-651	1.13	0.17	73	65	22.80
17281	hsa-miR-652	0.86	-0.22	111	129	2.62
15700	hsa-miR-653	1.11	0.15	83	76	4.00
17505	hsa-miR-654	0.94	-0.08	109	116	5.36
17286	hsa-miR-655	1.09	0.13	69	63	2.66
17356	hsa-miR-656	NA	NA	NA	NA	NA
17460	hsa-miR-657	0.83	-0.28	86	104	2.04
17522	hsa-miR-658	0.92	-0.12	2413	2614	0.60
17322	hsa-miR-659	0.92	-0.12	860	934	2.08
17338	hsa-miR-660	0.92	-0.11	83	90	1.56
17582	hsa-miR-661	NA	NA	NA	NA	NA
17507	hsa-miR-662	NA	NA	NA	NA	NA
17558	hsa-miR-663	1.67	0.74	4227	2561	2.41
17939	hsa-miR-671	1.06	0.08	3488	3230	3.19
17809	hsa-miR-769-3p	1.07	0.10	2415	2233	2.66
7190	hsa-miR-9	1.20	0.27	89	76	2.59
11185	hsa-miR-9*	1.04	0.05	72	69	4.33
11179	hsa-miR-92	0.79	-0.34	75	93	2.46
17718	hsa-miR-92b	0.73	-0.46	71	99	2.58
11180	hsa-miR-93	0.95	-0.08	73	77	12.70

11181	hsa-miR-95	1.05	0.07	113	107	1.91
13147	hsa-miR-96	1.13	0.18	98	86	3.07
11182	hsa-miR-98	1.06	0.08	238	226	2.01
11183	hsa-miR-99a	NA	NA	NA	NA	NA
11184	hsa-miR-99b	NA	NA	NA	NA	NA
17347	miRPlus_17347	1.05	0.07	76	72	2.62
17411	miRPlus_17411	1.03	0.04	74	72	2.59
17653	miRPlus_17653	NA	NA	NA	NA	NA
17808	miRPlus_17808	1.60	0.67	124	78	2.31
17810	miRPlus_17810	0.88	-0.18	116	131	2.45
17811	miRPlus_17811	0.92	-0.12	68	74	15.12
17812	miRPlus_17812	0.91	-0.13	72	79	4.38
17813	miRPlus_17813	0.82	-0.29	70	86	1.36
17814	miRPlus_17814	NA	NA	NA	NA	NA
17815	miRPlus_17815	NA	NA	NA	NA	NA
17816	miRPlus_17816	NA	NA	NA	NA	NA
17817	miRPlus_17817	NA	NA	NA	NA	NA
17818	miRPlus_17818	0.92	-0.12	69	75	4.19
17819	miRPlus_17819	NA	NA	NA	NA	NA
17820	miRPlus_17820	0.90	-0.15	67	74	2.75
17821	miRPlus_17821	0.97	-0.05	72	73	6.52
14261	spike_control_a	0.98	-0.04	152	155	1.75
14263	spike_control_b	0.90	-0.15	182	204	2.66
14264	spike_control_c	0.85	-0.24	708	827	2.03
10904	spike_control_d	0.99	-0.02	5826	5829	3.05
10906	spike_control_e	0.93	-0.11	908	972	2.28
14262	spike_control_f	0.87	-0.20	124	142	1.61
10905	spike_control_g	0.97	-0.04	559	577	4.26
10907	spike_control_h	0.89	-0.17	2506	2753	3.42
14257	spike_control_i	0.93	-0.11	11857	12861	2.61
10899	spike_control_j	0.99	-0.02	17164	16971	4.72
17822	miRPlus_17822	0.99	-0.01	70	70	3.81
17823	miRPlus_17823	1.06	0.09	73	68	7.05
17824	miRPlus_17824	0.97	-0.04	69	70	5.59
17825	miRPlus_17825	NA	NA	NA	NA	NA
17826	miRPlus_17826	1.04	0.05	66	63	0.35
17827	miRPlus_17827	0.80	-0.32	77	97	4.10
17828	miRPlus_17828	0.99	-0.02	99	101	1.78
17829	miRPlus_17829	1.01	0.02	72	71	7.88
17830	miRPlus_17830	0.88	-0.18	321	364	1.72
17831	miRPlus_17831	NA	NA	NA	NA	NA
17832	miRPlus_17832	1.00	0.00	1397	1362	3.49
17833	miRPlus_17833	0.87	-0.21	97	112	4.22
17834	miRPlus_17834	0.88	-0.18	113	128	5.38
17836	miRPlus_17836	0.93	-0.11	1555	1669	3.08
17837	miRPlus_17837	NA	NA	NA	NA	NA
17838	miRPlus_17838	NA	NA	NA	NA	NA
17840	miRPlus_17840	0.87	-0.19	137	159	1.71
17841	miRPlus_17841	1.05	0.07	79	76	1.76
17842	miRPlus_17842	1.12	0.17	71	64	1.20
17843	miRPlus_17843	NA	NA	NA	NA	NA

17844	miRPlus_17844	0.92	-0.12	72	79	1.52
17845	miRPlus_17845	0.79	-0.35	78	102	5.06
17847	miRPlus_17847	0.94	-0.10	76	82	1.76
17848	miRPlus_17848	0.96	-0.06	76	79	2.06
17849	miRPlus_17849	NA	NA	NA	NA	NA
17850	miRPlus_17850	1.03	0.04	71	70	5.15
17851	miRPlus_17851	0.76	-0.40	73	98	11.80
17852	miRPlus_17852	0.94	-0.08	69	73	1.10
17853	miRPlus_17853	NA	NA	NA	NA	NA
17854	miRPlus_17854	0.80	-0.32	86	107	1.43
17855	miRPlus_17855	1.10	0.14	95	85	2.99
17856	miRPlus_17856	1.47	0.56	6031	4093	1.55
17857	miRPlus_17857	1.17	0.23	95	81	5.22
17858	miRPlus_17858	1.29	0.37	123	95	3.95
17859	miRPlus_17859	0.96	-0.05	305	323	2.79
17860	miRPlus_17860	1.16	0.21	98	85	3.62
17861	miRPlus_17861	0.89	-0.16	239	261	2.89
17862	miRPlus_17862	1.04	0.06	69	66	1.97
17863	miRPlus_17863	0.82	-0.28	94	113	0.97
17864	miRPlus_17864	1.57	0.65	5832	3728	1.23
17865	miRPlus_17865	1.24	0.31	1346	1087	0.37
17866	miRPlus_17866	NA	NA	NA	NA	NA
17867	miRPlus_17867	NA	NA	NA	NA	NA
17868	miRPlus_17868	0.80	-0.32	119	150	1.40
17869	miRPlus_17869	0.96	-0.06	10006	10308	2.42
17870	miRPlus_17870	0.77	-0.38	75	98	2.45
17871	miRPlus_17871	0.93	-0.10	2395	2586	1.53
17872	miRPlus_17872	0.72	-0.47	66	92	3.21
17873	miRPlus_17873	0.85	-0.24	70	83	3.44
17874	miRPlus_17874	0.72	-0.48	72	100	1.41
17875	miRPlus_17875	0.79	-0.35	76	98	2.21
17876	miRPlus_17876	0.80	-0.32	66	82	5.27
17877	miRPlus_17877	0.96	-0.06	2679	2789	1.22
17878	miRPlus_17878	1.15	0.20	2118	1850	1.89
17879	miRPlus_17879	0.99	-0.01	72	73	4.34
17880	miRPlus_17880	NA	NA	NA	NA	NA
17881	miRPlus_17881	1.49	0.58	5448	3626	1.48
17882	miRPlus_17882	1.10	0.13	138	126	3.89
17883	miRPlus_17883	0.94	-0.09	70	75	7.73
17884	miRPlus_17884	0.80	-0.32	66	82	4.76
17885	miRPlus_17885	NA	NA	NA	NA	NA
17886	miRPlus_17886	NA	NA	NA	NA	NA
17887	miRPlus_17887	NA	NA	NA	NA	NA
17888	miRPlus_17888	0.89	-0.17	67	76	2.05
17889	miRPlus_17889	NA	NA	NA	NA	NA
17890	miRPlus_17890	1.11	0.15	3001	2702	1.52
17891	miRPlus_17891	0.81	-0.30	72	87	3.48
17892	miRPlus_17892	0.91	-0.13	66	72	4.64
17893	miRPlus_17893	0.81	-0.30	68	84	3.20
17894	miRPlus_17894	0.97	-0.04	693	708	1.96
17895	miRPlus_17895	0.89	-0.16	69	77	3.82

17896	miRPlus_17896	1.56	0.64	333	211	2.77
17897	miRPlus_17897	NA	NA	NA	NA	NA
17898	miRPlus_17898	NA	NA	NA	NA	NA
17899	miRPlus_17899	0.84	-0.26	67	80	4.47
17900	miRPlus_17900	0.91	-0.14	160	176	1.19
17902	miRPlus_17902	NA	NA	NA	NA	NA
17903	miRPlus_17903	1.20	0.27	169	142	1.96
17904	miRPlus_17904	0.86	-0.22	185	217	1.58
17905	miRPlus_17905	1.01	0.01	91	92	3.78
17906	miRPlus_17906	NA	NA	NA	NA	NA
17907	miRPlus_17907	NA	NA	NA	NA	NA
17908	miRPlus_17908	NA	NA	NA	NA	NA
17909	miRPlus_17909	0.99	-0.02	68	69	3.35
17910	miRPlus_17910	NA	NA	NA	NA	NA
17911	miRPlus_17911	0.88	-0.19	66	77	22.88
17912	miRPlus_17912	NA	NA	NA	NA	NA
17913	miRPlus_17913	NA	NA	NA	NA	NA
17914	miRPlus_17914	0.90	-0.15	68	75	7.30
17915	miRPlus_17915	1.03	0.05	2303	2204	2.09
17916	miRPlus_17916	NA	NA	NA	NA	NA
17917	miRPlus_17917	NA	NA	NA	NA	NA
17918	miRPlus_17918	0.85	-0.23	98	115	1.85
17919	miRPlus_17919	NA	NA	NA	NA	NA
17920	miRPlus_17920	NA	NA	NA	NA	NA
17921	miRPlus_17921	0.93	-0.10	860	906	1.98
17922	miRPlus_17922	NA	NA	NA	NA	NA
17923	miRPlus_17923	NA	NA	NA	NA	NA
17924	miRPlus_17924	NA	NA	NA	NA	NA
17925	miRPlus_17925	NA	NA	NA	NA	NA
17926	miRPlus_17926	NA	NA	NA	NA	NA
17927	miRPlus_17927	0.77	-0.38	309	399	1.15
17928	miRPlus_17928	NA	NA	NA	NA	NA
17929	miRPlus_17929	NA	NA	NA	NA	NA
17930	miRPlus_17930	0.99	-0.01	188	190	2.86
17931	miRPlus_17931	NA	NA	NA	NA	NA
17932	miRPlus_17932	1.05	0.07	64	61	1.54
17933	miRPlus_17933	0.75	-0.41	70	94	1.60
17934	miRPlus_17934	0.81	-0.30	66	83	6.06
17935	miRPlus_17935	NA	NA	NA	NA	NA
17936	miRPlus_17936	0.70	-0.51	75	109	4.24
17937	miRPlus_17937	NA	NA	NA	NA	NA
17938	miRPlus_17938	NA	NA	NA	NA	NA
17940	miRPlus_17940	NA	NA	NA	NA	NA
17941	miRPlus_17941	0.71	-0.49	66	94	15.78
17942	miRPlus_17942	1.04	0.05	112	108	2.72
17943	miRPlus_17943	0.94	-0.09	358	375	2.88
17944	miRPlus_17944	NA	NA	NA	NA	NA
17945	miRPlus_17945	0.99	-0.01	180	184	2.41
17946	miRPlus_17946	0.85	-0.24	90	106	4.53
17948	miRPlus_17948	NA	NA	NA	NA	NA
17949	miRPlus_17949	NA	NA	NA	NA	NA

17950	miRPlus_17950	1.08	0.11	893	828	2.09
17951	miRPlus_17951	NA	NA	NA	NA	NA
17952	miRPlus_17952	1.55	0.63	2610	1674	1.74
17953	miRPlus_17953	0.93	-0.10	1350	1449	1.64
17954	miRPlus_17954	0.69	-0.53	71	101	1.48
17955	miRPlus_17955	0.76	-0.40	69	91	1.41
17956	miRPlus_17956	0.85	-0.23	72	83	0.97
17957	miRPlus_17957	NA	NA	NA	NA	NA
17958	miRPlus_17958	NA	NA	NA	NA	NA
17959	miRPlus_17959	0.82	-0.29	90	111	3.18
17960	miRPlus_17960	0.98	-0.03	2479	2558	1.26
17961	miRPlus_17961	0.87	-0.20	103	117	3.42

Table A.3 | LNA miRNA microarray data for LIM 1863 cells treated with TNF-α.

Gene Id	Annotation	Median Ratios	Log Median Ratios	Median Hy3	Median Hy5	CV
17748	hsa-let-7a	0.92	-0.12	2215	2407	4.20
17749	hsa-let-7b	0.97	-0.04	953	984	2.51
19004	hsa-let-7c	0.92	-0.12	2091	2262	1.01
17750	hsa-let-7d	0.83	-0.27	172	211	2.93
17751	hsa-let-7e	0.92	-0.12	130	138	4.18
17752	hsa-let-7f	0.77	-0.37	83	107	3.43
19602	hsa-let-7g	0.90	-0.15	140	156	0.45
19580	hsa-let-7i	1.00	0.01	365	367	2.52
10916	hsa-miR-1	1.40	0.48	107	76	6.05
19581	hsa-miR-100	NA	NA	NA	NA	NA
17615	hsa-miR-101	0.99	-0.02	88	89	3.58
10919	hsa-miR-103	0.99	-0.01	586	587	1.01
10920	hsa-miR-105	NA	NA	NA	NA	NA
19582	hsa-miR-106b	0.94	-0.10	221	235	1.44
10923	hsa-miR-107	1.07	0.09	646	600	1.41
13485	hsa-miR-10a	0.78	-0.35	782	996	1.29
10925	hsa-miR-10b	0.80	-0.32	80	101	2.00
19583	hsa-miR-122a	1.11	0.15	68	62	6.50
14328	hsa-miR-124a	1.26	0.33	96	77	3.85
10928	hsa-miR-125a	0.91	-0.14	304	333	4.24
10929	hsa-miR-125b	1.02	0.03	136	133	4.15
4610	hsa-miR-126	0.99	-0.02	70	71	2.08
10930	hsa-miR-126*	NA	NA	NA	NA	NA
10931	hsa-miR-127	0.83	-0.27	70	84	5.04
10932	hsa-miR-128a	1.07	0.09	88	82	2.59
19584	hsa-miR-128b	1.07	0.09	70	65	17.25
10934	hsa-miR-129	1.19	0.25	254	219	2.67
10935	hsa-miR-130a	1.03	0.05	68	66	2.09
10936	hsa-miR-130b	1.01	0.01	161	162	1.13
10937	hsa-miR-132	NA	NA	NA	NA	NA
10938	hsa-miR-133a-133b	0.93	-0.10	88	95	3.87
10940	hsa-miR-134	0.91	-0.14	78	86	1.89
10941	hsa-miR-135a	1.70	0.77	130	77	2.91
10942	hsa-miR-135b	1.32	0.40	112	82	6.38
10943	hsa-miR-136	1.20	0.27	98	81	2.42
10944	hsa-miR-137	1.50	0.58	116	77	3.29
13140	hsa-miR-138	1.24	0.31	102	83	5.17
10945	hsa-miR-139	1.02	0.03	78	75	6.36
4700	hsa-miR-140	1.13	0.17	82	73	2.25
10946	hsa-miR-141	1.04	0.06	148	140	2.58
10947	hsa-miR-142-3p	1.01	0.01	1209	1205	1.99
19015	hsa-miR-142-5p	1.10	0.13	446	413	2.73
13177	hsa-miR-143	NA	NA	NA	NA	NA
10950	hsa-miR-144	1.18	0.23	77	66	11.97
10951	hsa-miR-145	1.11	0.15	79	71	3.20

10952	hsa-miR-146a	1.14	0.19	88	79	3.29
10306	hsa-miR-146b	0.99	-0.01	74	75	6.21
10954	hsa-miR-147	1.19	0.25	81	69	5.21
10955	hsa-miR-148a	1.16	0.22	121	103	3.35
19585	hsa-miR-148b	0.97	-0.04	115	118	2.54
19586	hsa-miR-149	NA	NA	NA	NA	NA
19587	hsa-miR-150	0.92	-0.12	74	80	2.81
17463	hsa-miR-151	0.98	-0.03	113	115	3.01
17676	hsa-miR-152	0.95	-0.07	77	81	5.78
10961	hsa-miR-153	1.00	0.00	72	73	15.69
10962	hsa-miR-154	1.19	0.25	102	85	3.22
10963	hsa-miR-154*	0.90	-0.14	70	79	10.28
10964	hsa-miR-155	1.18	0.24	95	81	3.98
10965	hsa-miR-15a	0.93	-0.10	101	109	0.26
17280	hsa-miR-15b	0.93	-0.11	324	353	4.77
10967	hsa-miR-16	1.02	0.02	563	551	1.48
19588	hsa-miR-17-3p	1.12	0.16	247	214	3.47
17605	hsa-miR-17-5p-106a	0.92	-0.13	826	894	2.03
10971	hsa-miR-181a	1.09	0.12	74	68	11.08
11013	hsa-miR-181a*	NA	NA	NA	NA	NA
10972	hsa-miR-181b	1.19	0.25	84	70	3.23
10973	hsa-miR-181c	1.99	1.00	146	74	5.78
10974	hsa-miR-181d	1.20	0.26	80	66	5.11
10975	hsa-miR-182	1.10	0.14	158	144	1.07
10976	hsa-miR-182*	1.17	0.22	104	89	3.54
10977	hsa-miR-183	1.08	0.11	209	194	1.11
10978	hsa-miR-184	1.24	0.32	210	169	5.15
5560	hsa-miR-185	1.02	0.03	1164	1138	2.54
10979	hsa-miR-186	1.19	0.25	108	92	3.58
17643	hsa-miR-187	NA	NA	NA	NA	NA
19589	hsa-miR-188	1.10	0.14	68	63	0.61
10982	hsa-miR-189	0.97	-0.04	69	71	5.82
10983	hsa-miR-18a	0.97	-0.05	365	375	1.72
13178	hsa-miR-18a*	0.81	-0.31	75	92	1.56
13141	hsa-miR-18b	0.97	-0.04	338	344	3.19
10984	hsa-miR-190	1.29	0.37	93	72	2.47
10985	hsa-miR-191	0.97	-0.05	494	508	2.87
13126	hsa-miR-191*	1.16	0.21	88	76	2.55
17732	hsa-miR-192	0.92	-0.12	407	445	2.22
13172	hsa-miR-192	0.91	-0.14	69	78	5.60
10986	hsa-miR-193a	1.28	0.36	1891	1473	1.00
10987	hsa-miR-193b	0.82	-0.29	80	100	5.62
10988	hsa-miR-194	1.01	0.02	198	195	1.69
13148	hsa-miR-195	0.80	-0.32	70	88	3.16
10990	hsa-miR-196a	1.15	0.20	128	112	2.30
10991	hsa-miR-196b	1.17	0.23	103	88	2.75
10992	hsa-miR-197	1.07	0.10	132	123	7.80
10993	hsa-miR-198	0.98	-0.03	336	340	2.96
19590	hsa-miR-199a	0.96	-0.06	68	71	6.55
10995	hsa-miR-199a*	1.05	0.07	65	62	0.84

19591	hsa-miR-199b	NA	NA	NA	NA	NA
10997	hsa-miR-19a	1.13	0.17	193	171	1.00
10998	hsa-miR-19b	1.01	0.01	137	136	1.59
11000	hsa-miR-200a	1.14	0.19	2159	1876	0.80
13127	hsa-miR-200a*	1.03	0.05	72	69	0.29
9578	hsa-miR-200b	0.99	-0.01	627	629	0.38
17427	hsa-miR-200c	1.05	0.07	548	520	0.97
11003	hsa-miR-202	1.00	0.00	535	535	1.16
10314	hsa-miR-202*	1.97	0.98	142	72	2.52
11004	hsa-miR-203	1.12	0.16	179	161	1.45
11005	hsa-miR-204	1.21	0.27	92	76	2.24
11006	hsa-miR-205	0.96	-0.06	70	73	0.32
11007	hsa-miR-206	1.01	0.02	89	85	3.74
5730	hsa-miR-208	0.99	-0.02	94	95	2.34
11008	hsa-miR-20a	0.93	-0.10	905	970	2.88
10999	hsa-miR-20a	0.91	-0.13	761	831	0.71
11009	hsa-miR-20b	0.89	-0.16	137	154	1.03
5740	hsa-miR-21	1.58	0.66	970	625	2.31
13511	hsa-miR-210	0.99	-0.01	580	594	2.81
11011	hsa-miR-211	1.20	0.26	91	76	8.66
19592	hsa-miR-212	0.78	-0.36	150	194	3.97
11014	hsa-miR-214	0.95	-0.08	917	959	3.52
11015	hsa-miR-215	0.92	-0.12	294	318	2.92
11016	hsa-miR-216	1.07	0.09	71	67	9.50
19016	hsa-miR-217	1.04	0.05	68	66	2.08
11018	hsa-miR-218	1.06	0.08	76	72	6.43
11019	hsa-miR-219	1.04	0.05	68	65	2.32
11020	hsa-miR-22	1.17	0.22	118	100	4.37
11021	hsa-miR-220	1.14	0.18	73	64	4.07
11022	hsa-miR-221	1.03	0.05	306	297	2.51
11023	hsa-miR-222	1.04	0.05	1661	1610	1.27
11024	hsa-miR-223	1.18	0.24	109	93	7.24
11025	hsa-miR-224	0.97	-0.04	70	73	8.60
11026	hsa-miR-23a	1.19	0.26	535	448	1.73
11027	hsa-miR-23b	0.93	-0.10	315	338	1.97
17506	hsa-miR-24	1.14	0.19	248	218	0.75
11029	hsa-miR-25	1.25	0.33	98	79	5.24
11030	hsa-miR-26a	1.20	0.27	274	232	3.84
11031	hsa-miR-26b	1.27	0.35	294	233	2.87
19593	hsa-miR-27a	1.09	0.12	128	118	1.66
13175	hsa-miR-27b	1.11	0.15	129	118	3.06
11034	hsa-miR-28	0.95	-0.07	73	77	4.19
19594	hsa-miR-296	1.08	0.11	187	175	0.59
11037	hsa-miR-299-3p	NA	NA	NA	NA	NA
11038	hsa-miR-299-5p	0.96	-0.05	75	78	1.78
11039	hsa-miR-29a	1.07	0.09	3237	3067	3.36
11040	hsa-miR-29b	1.22	0.28	265	217	2.85
11041	hsa-miR-29c	1.02	0.03	162	161	1.16
13143	hsa-miR-301	0.64	-0.64	66	105	4.28
11222	hsa-miR-302a	1.15	0.20	82	72	8.65
11042	hsa-miR-302a*	1.06	0.08	83	80	2.41

11043	hsa-miR-302b	0.99	-0.01	72	74	20.27
5930	hsa-miR-302b*	1.09	0.12	79	73	6.72
11044	hsa-miR-302c	1.18	0.24	83	71	2.60
11045	hsa-miR-302c*	1.05	0.06	122	116	2.09
11046	hsa-miR-302d	1.07	0.10	78	73	6.97
19595	hsa-miR-30a-3p	1.04	0.06	65	62	0.77
11048	hsa-miR-30a-5p	0.90	-0.15	101	111	0.92
17565	hsa-miR-30b	0.93	-0.10	315	336	0.98
17502	hsa-miR-30c	0.96	-0.05	198	207	3.08
19596	hsa-miR-30d	0.90	-0.15	97	108	1.50
11224	hsa-miR-30e-3p	0.97	-0.04	73	75	5.43
13174	hsa-miR-30e-5p	1.04	0.05	82	81	2.36
11052	hsa-miR-31	1.12	0.17	139	123	1.84
11053	hsa-miR-32	0.88	-0.18	79	91	3.39
11054	hsa-miR-320	1.02	0.03	2161	2138	1.64
11055	hsa-miR-323	1.09	0.13	74	67	2.08
11056	hsa-miR-324-3p	1.00	0.00	149	148	2.42
11057	hsa-miR-324-5p	0.99	-0.02	86	86	4.53
11058	hsa-miR-325	1.19	0.25	73	63	2.90
11059	hsa-miR-326	1.15	0.20	343	300	1.77
11060	hsa-miR-328	1.00	-0.01	70	72	3.43
11061	hsa-miR-329	NA	NA	NA	NA	NA
11062	hsa-miR-33	0.97	-0.05	77	80	5.73
11063	hsa-miR-330	0.89	-0.16	69	77	2.65
11064	hsa-miR-331	0.89	-0.16	88	99	2.34
11065	hsa-miR-335	0.93	-0.11	92	97	5.47
11066	hsa-miR-337	1.15	0.21	79	68	5.14
11067	hsa-miR-338	1.26	0.33	165	131	0.84
19597	hsa-miR-339	0.88	-0.18	71	81	2.58
17294	hsa-miR-33b	1.20	0.27	89	74	1.65
13144	hsa-miR-340	0.99	-0.01	84	84	1.06
11069	hsa-miR-342	NA	NA	NA	NA	NA
11070	hsa-miR-345	1.01	0.01	106	106	4.21
19018	hsa-miR-346	0.89	-0.17	158	180	1.55
11072	hsa-miR-34a	1.27	0.35	97	76	3.77
11073	hsa-miR-34b	1.04	0.06	65	62	0.68
11074	hsa-miR-34c	1.10	0.14	73	67	4.48
14301	hsa-miR-361	0.80	-0.32	101	122	3.76
14279	hsa-miR-362	1.11	0.15	68	61	3.20
11077	hsa-miR-363	1.05	0.07	73	70	7.08
11078	hsa-miR-365	0.99	-0.01	132	132	0.72
14280	hsa-miR-367	1.12	0.16	68	61	1.18
19598	hsa-miR-368	NA	NA	NA	NA	NA
11081	hsa-miR-369-3p	1.63	0.70	128	77	10.14
13145	hsa-miR-369-5p	1.06	0.08	73	69	3.54
11082	hsa-miR-370	1.17	0.23	249	211	3.32
11083	hsa-miR-371	1.13	0.17	78	68	3.43
11084	hsa-miR-372	1.03	0.04	73	72	11.67
11085	hsa-miR-373	1.17	0.23	71	60	1.28
11086	hsa-miR-373*	1.06	0.09	485	463	3.59
11087	hsa-miR-374	1.07	0.10	76	70	2.38

11088	hsa-miR-375	1.13	0.17	106	93	2.19
11089	hsa-miR-376a	NA	NA	NA	NA	NA
14268	hsa-miR-376a*	0.93	-0.11	71	77	10.42
11090	hsa-miR-376b	1.16	0.22	78	67	6.98
11091	hsa-miR-377	1.17	0.23	82	70	0.71
11092	hsa-miR-378	0.99	-0.01	74	75	2.69
11093	hsa-miR-379	1.25	0.32	97	78	5.79
11094	hsa-miR-380-3p	1.12	0.17	70	62	18.10
13170	hsa-miR-380-5p	1.02	0.03	66	65	1.31
14306	hsa-miR-381	0.94	-0.09	323	348	2.04
11097	hsa-miR-382	1.04	0.05	167	161	1.58
11098	hsa-miR-383	1.09	0.12	70	64	6.70
11099	hsa-miR-384	1.13	0.18	80	69	2.33
11240	hsa-miR-409-3p	1.02	0.02	75	75	2.24
14310	hsa-miR-409-5p	0.89	-0.17	74	83	2.73
11102	hsa-miR-410	1.01	0.02	69	68	3.91
17482	hsa-miR-411	NA	NA	NA	NA	NA
11103	hsa-miR-412	0.95	-0.08	72	77	8.35
17474	hsa-miR-421	0.87	-0.20	96	110	3.35
11104	hsa-miR-422a	1.09	0.12	78	73	5.92
11105	hsa-miR-422b	0.86	-0.22	195	225	1.58
11106	hsa-miR-423	0.87	-0.21	113	130	1.94
11107	hsa-miR-424	0.94	-0.08	77	80	2.10
11108	hsa-miR-425-3p	0.80	-0.33	67	85	1.05
17608	hsa-miR-425-5p	1.13	0.17	124	110	2.99
13171	hsa-miR-429	1.02	0.02	126	123	1.97
11110	hsa-miR-431	NA	NA	NA	NA	NA
11111	hsa-miR-432	1.26	0.34	84	67	4.16
13128	hsa-miR-432*	0.86	-0.22	87	102	3.33
11112	hsa-miR-433	0.92	-0.12	73	80	7.99
11113	hsa-miR-448	1.06	0.08	71	67	6.18
11114	hsa-miR-449	1.17	0.23	87	74	4.72
17706	hsa-miR-449b	1.03	0.04	72	70	1.13
17835	hsa-miR-450	0.97	-0.05	73	76	4.64
8538	hsa-miR-450	1.07	0.10	72	66	7.45
11248	hsa-miR-451	0.79	-0.33	78	99	3.97
11116	hsa-miR-452	0.96	-0.06	277	284	2.83
13129	hsa-miR-452*	0.87	-0.20	72	86	25.36
11117	hsa-miR-453	1.20	0.27	80	66	8.09
17450	hsa-miR-454-3p	1.11	0.15	67	61	1.67
13179	hsa-miR-455	1.03	0.05	69	66	1.48
13180	hsa-miR-483	2.17	1.11	279	129	3.74
13181	hsa-miR-484	1.68	0.75	170	99	6.86
11118	hsa-miR-485-3p	1.05	0.06	86	83	3.97
11119	hsa-miR-485-5p	NA	NA	NA	NA	NA
13182	hsa-miR-486	1.60	0.68	140	87	3.47
13183	hsa-miR-487a	0.97	-0.05	78	80	3.71
14285	hsa-miR-487b	0.58	-0.78	139	233	7.28
11120	hsa-miR-488	NA	NA	NA	NA	NA
11121	hsa-miR-489	0.84	-0.25	72	89	9.37
11122	hsa-miR-490	0.89	-0.18	112	128	2.27

11123	hsa-miR-491	1.02	0.03	81	80	1.38
11124	hsa-miR-492	1.14	0.19	1759	1541	2.77
14270	hsa-miR-493-3p	NA	NA	NA	NA	NA
11125	hsa-miR-493-5p	1.46	0.55	113	77	2.04
14287	hsa-miR-494	0.65	-0.62	1432	2162	5.89
17348	hsa-miR-495	1.02	0.03	78	76	12.96
11128	hsa-miR-496	NA	NA	NA	NA	NA
11129	hsa-miR-497	1.22	0.29	86	71	4.04
11130	hsa-miR-498	0.99	-0.01	1469	1497	4.76
14313	hsa-miR-499	1.08	0.11	73	70	5.14
11132	hsa-miR-500	1.14	0.18	159	140	4.26
11133	hsa-miR-501	0.98	-0.03	70	71	0.71
11134	hsa-miR-502	1.15	0.21	85	74	0.63
11135	hsa-miR-503	0.96	-0.05	901	936	1.64
11136	hsa-miR-504	1.07	0.10	72	68	4.58
14314	hsa-miR-505	NA	NA	NA	NA	NA
11138	hsa-miR-506	1.09	0.12	81	73	3.75
11139	hsa-miR-507	1.18	0.24	89	76	3.21
11140	hsa-miR-508	1.48	0.57	111	75	5.36
11141	hsa-miR-509	1.10	0.14	76	68	15.69
11142	hsa-miR-510	1.00	0.00	137	136	1.55
11143	hsa-miR-511	1.05	0.07	73	69	11.14
11144	hsa-miR-512-3p	1.09	0.13	76	70	3.55
11145	hsa-miR-512-5p	0.98	-0.02	437	444	4.72
11146	hsa-miR-513	0.89	-0.16	1781	1965	2.32
11147	hsa-miR-514	1.10	0.14	76	69	4.56
11148	hsa-miR-515-3p	NA	NA	NA	NA	NA
11149	hsa-miR-515-5p	1.05	0.07	72	68	10.25
11150	hsa-miR-516-3p	1.05	0.06	76	70	4.78
11151	hsa-miR-516-5p	0.96	-0.05	113	121	7.00
13130	hsa-miR-517*	1.92	0.94	147	77	4.44
11153	hsa-miR-517a-517b	1.17	0.23	83	71	4.17
11154	hsa-miR-517c	1.23	0.30	85	69	1.60
11155	hsa-miR-518a	1.14	0.19	78	68	7.87
11156	hsa-miR-518b	1.17	0.23	111	93	4.41
11157	hsa-miR-518c	1.10	0.14	75	67	3.87
13131	hsa-miR-518c*	1.05	0.06	865	828	1.98
11158	hsa-miR-518d	1.06	0.09	77	73	7.61
11159	hsa-miR-518e	1.12	0.17	71	63	0.74
11160	hsa-miR-518f	0.97	-0.05	74	75	1.81
10586	hsa-miR-518f*-526a	1.06	0.09	251	233	2.10
11161	hsa-miR-519a	1.05	0.08	67	64	1.18
11162	hsa-miR-519b	1.14	0.19	71	62	1.17
10482	hsa-miR-519c	1.06	0.09	64	61	2.77
11163	hsa-miR-519d	0.81	-0.31	111	139	2.37
11164	hsa-miR-519e	0.78	-0.37	80	103	6.87
13132	hsa-miR-519e*	1.00	0.01	204	209	4.89
11165	hsa-miR-520a	1.03	0.04	71	69	4.64
13133	hsa-miR-520a*	0.88	-0.19	81	91	3.30

11166	hsa-miR-520c-520b	1.07	0.10	67	63	0.71
11168	hsa-miR-520d	1.16	0.21	74	64	4.10
13134	hsa-miR-520d*	0.95	-0.08	94	99	3.84
11169	hsa-miR-520e	1.63	0.70	119	73	5.69
11167	hsa-miR-520f-520c	1.18	0.24	74	61	6.51
13146	hsa-miR-520g-520h	1.10	0.14	67	61	2.09
11171	hsa-miR-521	1.04	0.06	76	71	12.70
11172	hsa-miR-522	1.15	0.20	79	67	5.48
11173	hsa-miR-523	0.98	-0.03	74	76	4.45
10618	hsa-miR-524*	0.80	-0.33	75	92	6.88
11175	hsa-miR-525	0.81	-0.30	165	203	4.25
11174	hsa-miR-525*-524	1.04	0.05	77	74	12.33
11176	hsa-miR-526b	0.89	-0.17	89	99	1.88
13136	hsa-miR-526b*	1.52	0.60	112	75	3.20
13137	hsa-miR-526c	1.13	0.18	125	109	2.72
11177	hsa-miR-527	0.98	-0.03	302	309	1.76
17624	hsa-miR-532	0.91	-0.14	82	90	3.28
14271	hsa-miR-539	1.16	0.21	69	59	5.31
14315	hsa-miR-542-3p	1.12	0.16	72	64	5.80
14273	hsa-miR-542-5p	1.07	0.10	70	66	0.87
13712	hsa-miR-544	1.08	0.11	65	60	2.84
17846	hsa-miR-545	1.12	0.16	82	73	2.16
13721	hsa-miR-545	1.03	0.05	69	66	2.33
17535	hsa-miR-548a	1.06	0.09	76	71	3.26
17298	hsa-miR-548b	1.19	0.25	84	71	1.84
15313	hsa-miR-548c	1.01	0.02	75	74	10.23
17533	hsa-miR-548d	0.95	-0.07	71	74	8.09
17370	hsa-miR-549	1.01	0.02	71	70	10.59
17660	hsa-miR-550	0.85	-0.24	90	104	5.12
17272	hsa-miR-551a	1.12	0.16	193	172	5.76
17500	hsa-miR-551b	1.11	0.15	74	66	2.80
17668	hsa-miR-552	0.78	-0.35	69	88	2.82
17271	hsa-miR-553	1.00	0.00	73	73	6.88
17640	hsa-miR-554	1.02	0.03	79	77	0.82
17612	hsa-miR-555	0.97	-0.05	77	81	10.40
17426	hsa-miR-556	1.06	0.09	77	73	7.48
17376	hsa-miR-557	1.09	0.12	440	405	3.75
17652	hsa-miR-558	1.00	0.00	71	71	4.84
14755	hsa-miR-559	1.06	0.08	69	66	2.26
17456	hsa-miR-560	NA	NA	NA	NA	NA
14773	hsa-miR-561	1.16	0.22	84	72	3.88
17536	hsa-miR-562	0.98	-0.03	70	71	6.06
17569	hsa-miR-563	1.05	0.07	67	64	6.15
17645	hsa-miR-564	1.25	0.32	101	80	3.06
17413	hsa-miR-565	1.04	0.06	138	130	2.20
17634	hsa-miR-567	NA	NA	NA	NA	NA
17661	hsa-miR-568	1.04	0.05	75	73	3.72

14854	hsa-miR-569	1.10	0.13	73	67	12.39
14863	hsa-miR-570	NA	NA	NA	NA	NA
17490	hsa-miR-571	NA	NA	NA	NA	NA
17551	hsa-miR-572	1.12	0.17	1533	1365	0.29
17641	hsa-miR-573	1.08	0.11	87	82	1.96
17662	hsa-miR-574	NA	NA	NA	NA	NA
17626	hsa-miR-575	1.50	0.59	125	84	5.99
17396	hsa-miR-576	0.93	-0.10	78	83	1.81
17420	hsa-miR-577	NA	NA	NA	NA	NA
17302	hsa-miR-578	1.06	0.09	74	68	5.44
17628	hsa-miR-579	1.03	0.04	67	66	0.34
17459	hsa-miR-580	1.06	0.09	72	69	6.17
14962	hsa-miR-581	1.28	0.36	94	74	3.18
17380	hsa-miR-582	1.08	0.11	70	64	17.59
17295	hsa-miR-583	1.04	0.06	1109	1061	1.68
17423	hsa-miR-584	0.51	-0.97	409	806	1.01
17546	hsa-miR-585	1.08	0.11	94	87	6.05
17572	hsa-miR-586	0.55	-0.86	62	112	4.90
17594	hsa-miR-587	NA	NA	NA	NA	NA
17630	hsa-miR-588	1.08	0.11	80	75	3.54
17570	hsa-miR-589	NA	NA	NA	NA	NA
17503	hsa-miR-590	0.81	-0.30	78	97	5.89
17404	hsa-miR-591	1.01	0.01	73	72	6.66
17312	hsa-miR-592	1.12	0.16	81	71	3.58
17564	hsa-miR-593	NA	NA	NA	NA	NA
17349	hsa-miR-595	1.08	0.10	80	74	2.36
17449	hsa-miR-596	0.85	-0.23	74	86	3.30
17424	hsa-miR-597	NA	NA	NA	NA	NA
17637	hsa-miR-598	NA	NA	NA	NA	NA
17600	hsa-miR-599	1.09	0.12	80	74	1.35
17377	hsa-miR-600	0.90	-0.14	119	132	1.65
17498	hsa-miR-601	0.82	-0.29	78	95	0.96
17510	hsa-miR-602	0.94	-0.08	1366	1466	1.60
17393	hsa-miR-603	1.05	0.07	80	77	18.42
17592	hsa-miR-604	0.99	-0.01	71	73	7.12
17374	hsa-miR-605	1.02	0.04	69	67	4.67
17387	hsa-miR-606	NA	NA	NA	NA	NA
17598	hsa-miR-607	NA	NA	NA	NA	NA
17443	hsa-miR-608	0.88	-0.19	82	93	5.39
17353	hsa-miR-609	1.12	0.16	72	64	0.40
17445	hsa-miR-610	1.03	0.04	66	63	5.86
17611	hsa-miR-611	0.89	-0.17	90	109	21.84
17346	hsa-miR-612	1.03	0.04	2973	2877	1.70
17577	hsa-miR-613	NA	NA	NA	NA	NA
17326	hsa-miR-614	1.06	0.09	87	82	1.09
17574	hsa-miR-615	0.62	-0.68	178	302	5.57
17289	hsa-miR-616	1.01	0.02	81	79	0.68
17552	hsa-miR-617	0.96	-0.06	332	357	3.14
17336	hsa-miR-618	1.19	0.25	86	72	1.85
17405	hsa-miR-619	1.02	0.03	75	73	3.88
15349	hsa-miR-620	1.11	0.15	76	68	2.02

17588	hsa-miR-621	0.98	-0.02	71	73	5.97
17493	hsa-miR-622	0.96	-0.06	72	75	7.89
17309	hsa-miR-623	1.01	0.01	1702	1686	1.08
17635	hsa-miR-624	NA	NA	NA	NA	NA
17573	hsa-miR-625	0.89	-0.17	316	354	1.37
17351	hsa-miR-626	NA	NA	NA	NA	NA
17625	hsa-miR-627	1.03	0.05	105	96	6.84
17471	hsa-miR-628	0.92	-0.12	319	346	2.67
17566	hsa-miR-629	0.87	-0.20	106	121	5.23
17327	hsa-miR-630	1.02	0.03	365	353	2.03
17633	hsa-miR-631	1.12	0.16	93	83	5.55
17444	hsa-miR-632	1.07	0.09	79	74	2.55
15475	hsa-miR-633	1.10	0.13	72	68	8.26
17398	hsa-miR-634	1.20	0.27	121	99	3.64
17391	hsa-miR-635	0.81	-0.31	71	90	11.53
17479	hsa-miR-636	NA	NA	NA	NA	NA
17354	hsa-miR-637	0.83	-0.26	88	106	4.01
17550	hsa-miR-638	1.12	0.16	1210	1074	3.14
17627	hsa-miR-639	NA	NA	NA	NA	NA
17579	hsa-miR-640	0.81	-0.30	67	83	10.81
17530	hsa-miR-641	NA	NA	NA	NA	NA
17305	hsa-miR-642	1.31	0.39	133	100	1.58
17325	hsa-miR-643	0.96	-0.06	79	81	2.42
17563	hsa-miR-644	1.11	0.15	68	61	1.49
17613	hsa-miR-645	1.06	0.09	88	83	3.86
17491	hsa-miR-646	NA	NA	NA	NA	NA
17516	hsa-miR-647	1.04	0.06	65	63	1.64
17441	hsa-miR-648	0.88	-0.19	101	116	1.52
15619	hsa-miR-649	NA	NA	NA	NA	NA
17593	hsa-miR-650	NA	NA	NA	NA	NA
17394	hsa-miR-651	0.86	-0.21	83	97	6.22
17281	hsa-miR-652	0.89	-0.17	109	123	5.83
15700	hsa-miR-653	1.15	0.20	84	73	0.88
17505	hsa-miR-654	0.82	-0.28	91	111	2.02
17286	hsa-miR-655	1.11	0.16	68	61	2.14
17356	hsa-miR-656	NA	NA	NA	NA	NA
17460	hsa-miR-657	0.85	-0.23	85	100	1.66
17522	hsa-miR-658	0.94	-0.09	1621	1706	1.35
17322	hsa-miR-659	1.00	0.01	598	590	1.14
17338	hsa-miR-660	0.90	-0.15	81	90	3.25
17582	hsa-miR-661	NA	NA	NA	NA	NA
17507	hsa-miR-662	NA	NA	NA	NA	NA
17558	hsa-miR-663	1.19	0.25	3040	2599	3.04
17939	hsa-miR-671	0.97	-0.04	2670	2765	2.30
17809	hsa-miR-769-3p	0.99	-0.02	1642	1674	1.76
7190	hsa-miR-9	1.24	0.31	93	74	2.42
11185	hsa-miR-9*	1.09	0.12	73	67	10.05
11179	hsa-miR-92	0.82	-0.29	78	95	1.60
17718	hsa-miR-92b	0.75	-0.42	72	98	4.71
11180	hsa-miR-93	1.07	0.09	74	69	8.67
11181	hsa-miR-95	0.88	-0.18	97	111	2.76

13147	hsa-miR-96	1.21	0.28	104	86	5.21
11182	hsa-miR-98	1.05	0.07	253	243	1.19
11183	hsa-miR-99a	NA	NA	NA	NA	NA
11184	hsa-miR-99b	NA	NA	NA	NA	NA
17347	miRPlus_17347	1.07	0.10	78	73	2.06
17411	miRPlus_17411	0.93	-0.10	73	78	3.74
17653	miRPlus_17653	NA	NA	NA	NA	NA
17808	miRPlus_17808	1.64	0.72	127	78	5.55
17810	miRPlus_17810	0.91	-0.14	116	127	3.20
17811	miRPlus_17811	NA	NA	NA	NA	NA
17812	miRPlus_17812	0.86	-0.22	72	84	4.30
17813	miRPlus_17813	0.82	-0.28	72	88	4.08
17814	miRPlus_17814	NA	NA	NA	NA	NA
17815	miRPlus_17815	NA	NA	NA	NA	NA
17816	miRPlus_17816	NA	NA	NA	NA	NA
17817	miRPlus_17817	NA	NA	NA	NA	NA
17818	miRPlus_17818	0.91	-0.13	69	77	10.88
17819	miRPlus_17819	0.89	-0.16	68	77	15.46
17820	miRPlus_17820	0.93	-0.10	69	74	4.99
17821	miRPlus_17821	0.96	-0.06	73	77	2.84
14261	spike_control_a	1.00	-0.01	151	153	1.93
14263	spike_control_b	0.92	-0.12	189	203	1.50
14264	spike_control_c	0.89	-0.17	691	770	2.13
10904	spike_control_d	1.04	0.05	5465	5311	2.32
10906	spike_control_e	0.94	-0.09	866	924	3.56
14262	spike_control_f	0.91	-0.14	128	140	2.16
10905	spike_control_g	1.04	0.06	564	551	2.89
10907	spike_control_h	0.95	-0.08	2448	2687	3.19
14257	spike_control_i	0.98	-0.02	12353	12502	2.31
10899	spike_control_j	1.01	0.02	16600	16551	2.99
17822	miRPlus_17822	NA	NA	NA	NA	NA
17823	miRPlus_17823	1.14	0.19	74	64	2.82
17824	miRPlus_17824	0.99	-0.01	70	71	5.75
17825	miRPlus_17825	0.95	-0.07	69	72	0.67
17826	miRPlus_17826	1.03	0.05	66	63	1.77
17827	miRPlus_17827	0.88	-0.18	73	85	6.35
17828	miRPlus_17828	0.85	-0.23	87	103	2.78
17829	miRPlus_17829	1.01	0.01	73	71	5.40
17830	miRPlus_17830	1.00	0.00	239	234	2.79
17831	miRPlus_17831	0.87	-0.20	69	80	23.91
17832	miRPlus_17832	1.01	0.02	1008	997	2.41
17833	miRPlus_17833	0.96	-0.06	113	117	6.82
17834	miRPlus_17834	0.89	-0.16	104	116	2.75
17836	miRPlus_17836	0.99	-0.01	1024	1026	1.71
17837	miRPlus_17837	0.98	-0.02	69	70	7.81
17838	miRPlus_17838	0.92	-0.12	73	80	9.35
17840	miRPlus_17840	0.92	-0.12	141	153	1.45
17841	miRPlus_17841	1.06	0.09	80	76	3.87
17842	miRPlus_17842	1.08	0.11	72	66	4.24
17843	miRPlus_17843	1.06	0.09	69	65	4.61
17844	miRPlus_17844	0.92	-0.12	73	80	1.36

17845	miRPlus_17845	0.89	-0.17	92	104	4.67
17847	miRPlus_17847	0.97	-0.05	78	80	1.42
17848	miRPlus_17848	0.98	-0.02	78	79	4.37
17849	miRPlus_17849	NA	NA	NA	NA	NA
17850	miRPlus_17850	1.01	0.02	72	70	3.34
17851	miRPlus_17851	0.92	-0.12	70	77	0.75
17852	miRPlus_17852	0.93	-0.10	70	75	3.62
17853	miRPlus_17853	0.99	-0.02	70	74	19.29
17854	miRPlus_17854	0.83	-0.26	85	104	1.53
17855	miRPlus_17855	1.08	0.12	91	84	5.00
17856	miRPlus_17856	1.12	0.16	3981	3655	3.32
17857	miRPlus_17857	1.20	0.27	97	81	6.98
17858	miRPlus_17858	1.33	0.41	125	94	4.17
17859	miRPlus_17859	1.01	0.01	254	257	2.20
17860	miRPlus_17860	1.13	0.17	95	84	4.56
17861	miRPlus_17861	1.06	0.09	272	262	2.29
17862	miRPlus_17862	1.08	0.11	71	65	1.64
17863	miRPlus_17863	0.90	-0.15	98	109	1.51
17864	miRPlus_17864	1.14	0.19	4006	3516	1.36
17865	miRPlus_17865	1.06	0.08	1195	1144	1.97
17866	miRPlus_17866	NA	NA	NA	NA	NA
17867	miRPlus_17867	NA	NA	NA	NA	NA
17868	miRPlus_17868	0.84	-0.24	125	147	0.35
17869	miRPlus_17869	0.90	-0.15	10412	11420	2.30
17870	miRPlus_17870	0.72	-0.48	85	120	9.27
17871	miRPlus_17871	0.94	-0.09	1964	2075	2.17
17872	miRPlus_17872	0.73	-0.46	66	91	3.96
17873	miRPlus_17873	0.89	-0.17	72	81	7.62
17874	miRPlus_17874	0.75	-0.42	75	100	10.15
17875	miRPlus_17875	0.77	-0.38	74	97	2.73
17876	miRPlus_17876	NA	NA	NA	NA	NA
17877	miRPlus_17877	1.01	0.01	2496	2465	2.37
17878	miRPlus_17878	1.03	0.04	1332	1300	1.13
17879	miRPlus_17879	0.99	-0.02	74	76	3.64
17880	miRPlus_17880	NA	NA	NA	NA	NA
17881	miRPlus_17881	1.15	0.20	4619	4021	4.61
17882	miRPlus_17882	0.94	-0.09	131	136	3.62
17883	miRPlus_17883	0.86	-0.22	69	81	10.89
17884	miRPlus_17884	0.82	-0.29	67	82	0.88
17885	miRPlus_17885	NA	NA	NA	NA	NA
17886	miRPlus_17886	NA	NA	NA	NA	NA
17887	miRPlus_17887	NA	NA	NA	NA	NA
17888	miRPlus_17888	NA	NA	NA	NA	NA
17889	miRPlus_17889	NA	NA	NA	NA	NA
17890	miRPlus_17890	0.96	-0.06	2284	2404	1.73
17891	miRPlus_17891	0.82	-0.29	71	90	9.12
17892	miRPlus_17892	NA	NA	NA	NA	NA
17893	miRPlus_17893	0.78	-0.37	68	88	4.28
17894	miRPlus_17894	0.96	-0.07	355	371	2.61
17895	miRPlus_17895	0.92	-0.12	69	75	6.62
17896	miRPlus_17896	1.54	0.62	303	197	1.15

17897	miRPlus_17897	NA	NA	NA	NA	NA
17898	miRPlus_17898	NA	NA	NA	NA	NA
17899	miRPlus_17899	NA	NA	NA	NA	NA
17900	miRPlus_17900	0.92	-0.13	156	172	2.23
17902	miRPlus_17902	NA	NA	NA	NA	NA
17903	miRPlus_17903	1.05	0.08	150	142	4.36
17904	miRPlus_17904	0.87	-0.19	181	207	1.95
17905	miRPlus_17905	1.02	0.02	92	90	4.69
17906	miRPlus_17906	1.01	0.02	68	67	1.18
17907	miRPlus_17907	NA	NA	NA	NA	NA
17908	miRPlus_17908	NA	NA	NA	NA	NA
17909	miRPlus_17909	1.07	0.10	71	66	2.21
17910	miRPlus_17910	NA	NA	NA	NA	NA
17911	miRPlus_17911	1.00	0.00	66	65	2.61
17912	miRPlus_17912	NA	NA	NA	NA	NA
17913	miRPlus_17913	NA	NA	NA	NA	NA
17914	miRPlus_17914	0.81	-0.30	67	83	11.33
17915	miRPlus_17915	0.97	-0.04	1791	1845	2.33
17916	miRPlus_17916	NA	NA	NA	NA	NA
17917	miRPlus_17917	NA	NA	NA	NA	NA
17918	miRPlus_17918	0.89	-0.17	101	113	1.37
17919	miRPlus_17919	NA	NA	NA	NA	NA
17920	miRPlus_17920	NA	NA	NA	NA	NA
17921	miRPlus_17921	0.94	-0.09	633	669	1.29
17922	miRPlus_17922	NA	NA	NA	NA	NA
17923	miRPlus_17923	NA	NA	NA	NA	NA
17924	miRPlus_17924	NA	NA	NA	NA	NA
17925	miRPlus_17925	NA	NA	NA	NA	NA
17926	miRPlus_17926	NA	NA	NA	NA	NA
17927	miRPlus_17927	0.75	-0.42	273	369	1.03
17928	miRPlus_17928	NA	NA	NA	NA	NA
17929	miRPlus_17929	NA	NA	NA	NA	NA
17930	miRPlus_17930	0.88	-0.18	150	170	2.12
17931	miRPlus_17931	NA	NA	NA	NA	NA
17932	miRPlus_17932	1.05	0.07	64	61	0.66
17933	miRPlus_17933	0.77	-0.39	71	92	4.90
17934	miRPlus_17934	NA	NA	NA	NA	NA
17935	miRPlus_17935	NA	NA	NA	NA	NA
17936	miRPlus_17936	0.77	-0.38	82	107	5.75
17937	miRPlus_17937	NA	NA	NA	NA	NA
17938	miRPlus_17938	NA	NA	NA	NA	NA
17940	miRPlus_17940	0.73	-0.46	73	101	7.24
17941	miRPlus_17941	0.71	-0.50	72	102	11.14
17942	miRPlus_17942	0.93	-0.10	98	105	2.75
17943	miRPlus_17943	0.94	-0.09	348	370	1.72
17944	miRPlus_17944	NA	NA	NA	NA	NA
17945	miRPlus_17945	0.89	-0.18	149	169	1.29
17946	miRPlus_17946	0.84	-0.25	89	107	1.35
17948	miRPlus_17948	NA	NA	NA	NA	NA
17949	miRPlus_17949	NA	NA	NA	NA	NA
17950	miRPlus_17950	0.95	-0.08	575	598	3.52

17951	miRPlus_17951	0.83	-0.28	68	83	1.24
17952	miRPlus_17952	1.23	0.30	1594	1306	0.80
17953	miRPlus_17953	0.91	-0.14	749	820	0.93
17954	miRPlus_17954	0.72	-0.46	70	95	2.23
17955	miRPlus_17955	0.72	-0.47	73	101	2.25
17956	miRPlus_17956	NA	NA	NA	NA	NA
17957	miRPlus_17957	NA	NA	NA	NA	NA
17958	miRPlus_17958	NA	NA	NA	NA	NA
17959	miRPlus_17959	0.73	-0.45	83	113	2.37
17960	miRPlus_17960	0.99	-0.02	1959	1995	2.51
17961	miRPlus_17961	0.83	-0.28	95	115	2.40