The role of miRNAs in the regulation of EMT processes during fibrosis

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1 – Introduction

The human body is subject to magnificently intricate mechanisms of regulation, methods of control and recovery and repair systems which all contribute to the greater goal of optimal functioning and homeostasis of our delicate being. For centuries, scientific research has laid down the groundwork for understanding these systems, and our ability to work in cooperation with the understood mechanisms has provided us with the opportunity to prolong functioning and performance. From extreme sports and exposure to the elements to infection with diseases and illnesses, the body has found a way to respond to most all situations, be this with desirable outcomes or not.

Of late, the genetic code has been under close inspection in attempts to further understand the functioning of proteins and genes. In the 1970s and 1980s, technological advances including the sequencing of small RNAs such as 5s RNA, transfer RNA and small nuclear RNA, and through the use of RT-PCR the discovery of ribozymes and RNA-binding proteins (RBPs), understanding RNA and its effects on gene regulation and binding was made clearer (Burd, 1994; Keene, 2010; Kenan, 1994). The total RNA and all its subsequent complements such as these small RNAs give rise to the transcriptome, including all the accumulated levels of all expressed RNA. In 1993, Victor Ambros' Lab and Ruvkun's Lab discovered small regulatory RNAs in *Caenorhabditis elegans*, now known as microRNAs (miRNAs) (Keene, 2010; Lee, 1993; Wightman 1993). With better understanding of the regulatory functions of RNA also came the discovery that these so called small RNAs are the 'little regulators' of the 'big regulator' – miRNAs regulate RNA. The discovery of the miRNA was based on single antisense interactions between each of two small RNA transcripts (lin-4) and a single mRNA (lin-14) (Keene, 2009; Lee, 1993; Wightman 1993). Lin-14 mRNA is targeted by the RNA that is encoded by lin-4. The lin-4 encoded RNA targets a sequence in the 3' untranslated region (UTR) of lin-14 and developmentally represses LIN-14 protein production (Olson and Ambros, 1999). Later, the discovery of multitargeting of the miRNAs came into play. This entails that miRNAs that serve similar functions have been reported to be clustered on neighboring transcripts. MiRNAs can also be transcribed individually.

Today the role of miRNAs has become significantly clearer with over 700 human miRNA genes identified, amounting to about 3% of all human protein-coding genes (Siomi, 2010). MiRNAs are derived from intergenic, intronic and exonic regions of host gene sequences, and intronic and exonic miRNAs are usually cotranscribed with their host genes. Starting out as a double stranded (ds) miRNA complex, they are reduced to a single strand (ss) miRNA that targets mRNA with a partially complementary sequence. MiRNAs are therefore described as the fine-tuners of the genetic code, allowing for change within a cell ranging from the induction of cell proliferation to apoptosis all while working on one of the smallest scales known in cellular functioning. They mediate the gene expression profile of a cell, steering a cell toward further action. Their function is of extreme importance, because not only can they regulate gene expression, but also the degree in which a gene is expressed.

Starting out as primary-miRNAs (pri-miRNAs) when initially transcribed in the nucleus, they are processed by a RNA polymerase II complex known as the Microprocessor. The Microprocessor consists of two main molecules: Drosha and DiGeorge Critical Region 8 (DGCR8). In collaboration with several cofactors that differ depending on the microenvironment, the Microprocessor cleaves the pri-miRNA, resulting in a 60-100 nucleotide (nt) hairpin known as precursor miRNA (pre-miRNA). This is transported to the cytoplasm, where the pre-miRNA is further 'diced' by the endonuclease Dicer giving a miRNA duplex. One of the strands associates with the RNA induced silencing complex (RISC) and is chosen to bind to its target mRNA based on several factors. Binding of the 'mi-RISC' complex to the target mRNA results in gene silencing, often inhibiting transcription or degrading target mRNA. They reduce the output of mRNA, quickly leading to a new gene expression profile (Ulvila et al, 2009) (see Fig. 1). There are also miRNAs that are known to enhance mRNA transcription and translation (Bhattacharyya, S.N., 2006; Smalheiser, 2008; Vasudevan et al, 2007; Vasudevan and Steitz, 2007). The mechanisms by which miRNAs induce their effects on the expression profile of a cell include feedback and feedforward loops and default repression and activation. The regulation of the miRNA signaling pathway components is also under tight control, providing opportunities to regulate at several positions throughout the pathway. A-to-I editing at the pri-miRNA level, where a base-pair switch can lead to a

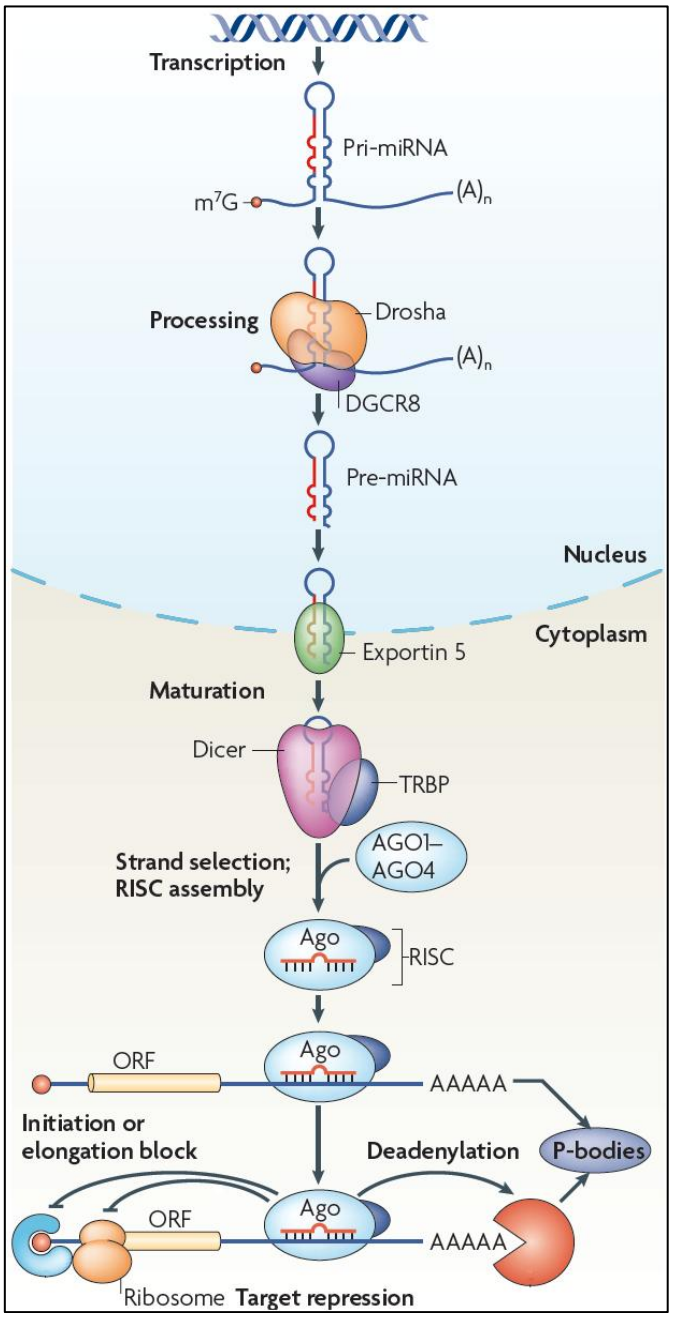

Fig. 1 – Biosynthesis of miRNA. Pri-miRNA is transcribed in the nucleus, and cropped by Drosha/DGCR8, the Microprocessor, into pre-miRNA. Exportin 5 transports pre-miRNA into the cytoplasm, where it is further diced by Dicer/TRBP. The resulting miRNA associates with AGO protein and becomes part of the RISC complex. Passenger strand miRNA* is degraded or stored in the P-Body, and mature miRNA directs the RISC complex toward target mRNA, where it represses or inhibits translation (Inui, 2008).

change in target mRNA and Microprocessor modulation, control of the Dicer at the pre-miRNA level, as well as posttranscriptional regulation can all be instructed to behave in a way which serves to maximize cell performance. All of these concepts will be explained in further detail in this thesis.

The constant uncovering of new phenomena such as the miRNAs also brings with it new questions. The age-old quest for longevity and the idea of 'healthy ageing' has become an everyday subject in today's society. An essential aspect of sustaining health is the body's ability to repair and regenerate after injury or damage. Medical assistance has come a long way in aiding repair and regeneration, but regaining full functioning after damage can still pose dilemmas.

The process of fibrosis provides a very relevant example as to how miRNAs go about their work, and therefore serves as a perfect framework through which the regulative and regenerative capacities of miRNAs can be illustrated. Fibrosis is the persistent deposition of extracellular matrix (ECM) or the lack of breakdown of ECM – a result of an imbalance in ECM molecule turnover (Jiang, 2010). It limits function of the surrounding environment, or, when occurring in an organ such as the lungs, kidney, heart, liver or colon, imposes severely on the performance of said organ and can eventually lead to organ failure. Fibrosis is initiated by a process known as epithelial-to-mesenchymal transition (EMT). EMT refers to loss of the epithelial phenotype of cells and the taking on of a mesenchymal phenotype. Where the epithelium exists as a sheet of closely adhered cells by method of junctions and adhesion to the basement membrane, mesenchymal cells have free motility and do not possess the intracellular polarity found in epithelial cells. One of the consequences of EMT in fibrosis is that the mesenchymal cells that arise from EMT display a fibroblastic or myofibroblastic phenotype, depositing ECM molecules and encouraging the transition of more epithelial cells by secretion of EMT-driving factors such as TGF-β, cytokines and chemokines. TGF-β is the key factor in EMT, and exerts its effects both directly and indirectly. Smad-dependent pathways are directly activated by TGF-β, leading to the degradation and decreased expression of E-cadherin, which is an important adhesive molecule of epithelial cells. The loss of E-cadherin expression is replaced by N-cadherin, characteristic of mesenchymal cells. It is believed that the fibroblasts and myofibroblasts that result from EMT deliver a large fraction of fibrotic cells during fibrosis, making EMT an important contributor to fibrosis.

These factors that are involved in EMT provide numerous entry points for miRNAs to exert their regulatory function. A number of genes such as the zinc-finger E-Box Binding 1 and 2 (ZEB1/ZEB2) play an important role in initiating and stimulating fibrosis. The ZEB1/ZEB2 regulatory pathway works by repressing E-cadherin repression and forms a regulatory circuit with the miR-200 family. The miR-200 family in particular is known for its involvement in the regulation of EMT and forms a self-regulating circuit together with the ZEB genes.

The following thesis will therefore use fibrosis – and EMT in particular – as a framework through which the regulatory and regenerative capabilities of miRNAs will be portrayed. Focus on miRNA regulation of EMT, and not fibrosis as a whole, is deliberate, mainly because there are numerous EMT-miRNA pathways that have clearly been identified. The idea of 'let's start at the beginning' should also not be forgotten – it would be difficult to illustrate miRNA's effects on fibrosis if the upstream mechanisms and causes that lead up to fibrosis were unknown. In order to further specify the topic, this thesis will focus on the effect of miRNAs on EMT and fibrosis in the lungs where possible. A cell's behavior is highly susceptible to its surroundings and extracellular signals are in part responsible for shaping a cell's unique profile. This leads to the fact that the level of miRNA expression (and the exact miRNAs expressed) is not equal across all organs, so limiting the spectrum of this thesis by focusing on one organ will allow for dealing with the lungs in greater detail as well as for avoiding generalizations.

2 – MiRNAs and their role in RNA editing

Besides miRNAs, there are also other known regulators of RNA. Other small RNAs, such as small interfering RNA (siRNA), small nuclear RNA (snRNA) and small nucleolar RNA (snoRNA) are also known to have regulatory effects on gene expression (Dyce et al, 2010). The regulatory effects of miRNA cover a wide range of sites at which miRNAs can interfere. Of course this all starts with the biosynthesis of miRNA.

Biosynthesis of miRNA

The biosynthesis of miRNA is a highly controlled process, functions with extreme precision and can induce effects very rapidly. Synthesis of miRNA entails the conversion of double strand (ds) miRNA to single strand (ss) miRNA which binds to target mRNA. Intermediate steps are needed for cleavage and transport. MiRNA is initially transcribed by RNA polymerase II in the nucleus. This generates a primary miRNA, known as pri-miRNA (Siomi et al, 2010). Pri-miRNAs are endogenous hairpin transcripts, and a single pri-miRNA can give rise to several functional miRNAs (Siomi et al, 2010). Pri-miRNA is next processed by the RNase III enzyme Drosha. Drosha works in concert with its cofactor, DiGeorge Critical Region 8 (DGCR8)/Pasha. DGCR8/Pasha is a ds RNA binding domain (dsRBD) protein. These two components, in combination with a number of auxiliary cofactors such as the RNA helicases p68 and p72, form the core of the Microprocessor (Newman et al, 2010). These elements aid in promoting specificity and activity of Drosha. The Microprocessor converts pri-miRNA into a ~70 nucleotide (nt) long hairpin structured precursor-miRNA (pre-miRNA). The DGCR8/Pasha part of the Microprocessor helps by binding to the junction between the ss and ds region of the pri-miRNA stem, whereby it signals Drosha to cleave (Siomi et al, 2010). Cleaving of the pri-miRNA creates a short overhang on the 3’ end. This overhang is recognized by Exportin5, which exports the pre-miRNA to the cytoplasm. Once in the cytoplasm, the pre-miRNA is further cleaved by another RNase III enzyme; Dicer. Dicer is a dsRNA specific endonuclease, and works in combination with the dsRBD TRBP/PACT. It removes the loop from the pre-miRNA, after which the ~22 nt long miRNA strands are split. The two remaining strands are the mature (miRNA) and so-called ‘passenger’ (miRNA*) strands. Mature and passenger strands are chosen based on a number of factors. One important factor is the stability of the 5’ end of the miRNA. The strand with less stably base-paired sequence is most often chosen as the mature strand. The mature miRNA associates with an Argonaute (AGO) protein. The AGO is incorporated into the RNA induced silencing complex (RISC), which is directed by the miRNA toward miRNA-recognition elements (MRE) within the 3’-UTR of the target mRNA. Specificity of targeting is thought to be influence not only by MREs, but also by spacing between MREs, their proximity to the stop codon, the secondary structure of the mRNA and the target’s position within the 3’ untranslated region (UTR) (Dyce et al, 2010). The AGO protein is the effector component of all known RISCs, and of the four human argonautes known, only AGO2 has been shown to be able to cleave and degrade mRNA as opposed to inducing inhibition of transcription. It has been predicted that miRNA-mRNA complexes do not have to be perfectly complementary. On the contrary, most

miRNA-mRNA complexes only show high complementation within the seed region of the miRNA – a 7nt long region located 2-8 nt from the 5' end. Since binding of miRNA to its mRNA target does not have to be a perfect base pair match, cleavage and degradation of target mRNA can probably only occur when there is perfect base pair matching with AGO2 (Ulvila et al, 2009). In cases where complementation is of a low degree, translational repression results. The remaining passenger strand is thought to be degraded in the P-body, although its significance is still under discussion (see Fig.1, chapter 1). Studies have shown that due to its more complementary matching, miRNA* is not degraded, but associates with AGO1 or AGO2 (Czech et al., 2009; Ghildiyal et al., 2010; Okamura et al., 2008, Ulvila et al.,2009; Seitz et al., 2008).There are cases in which this leads to bifunctioning of the miRNA strands.

Although the same mechanism for biosynthesis is thought to be maintained throughout an organism, relative expression levels of miRNA-miRNA* strands differ among tissue types. This indicates that strand selection may not only rely on sequence features, but may be influenced by surrounding extracellular factors such as matrix signaling pathways or other stimulatory factors (Siomi et al, 2010).

Eventual target gene silencing is achieved through binding to the 3' UTR of the target mRNA, thereby repressing transcription of the accompanying protein. The target base pairing, as explained, partially influences the fate of the mRNA. While perfect seed region pairing to the target mRNA leads to degradation of mRNA through deadenylation, imperfect pairing leads to the inhibition of translation initiation or moves targets into cytoplasmic P-bodies (Liu et al, 2010).

Mechanisms of miRNA signaling

Although the exact mechanism by which miRNAs induce their effects on translation is still somewhat of an enigma, progress is being made by scientists daily. Through the use of knock-out mice, mimicking and inhibition of putative miRNA effects, phenotypic and physiological observations are made as to how miRNAs fine tune gene expression. Some aspects as to how they bring about their effects are known.

A cell is a constituent of tissue-specific microenvironment and miRNA expression profiles are unique for each of these environments. How a cell interprets extracellular signals is dependent on its history of received signals, as well as the intensity and duration of an incoming signal – they are context dependent. In addition, a cell must be able to distinguish between signals that provide or give rise to follow up a specific instruction, and a signal which does not surpass threshold levels and should thus not be processed. It does this through the use of signaling gradients and attenuation and amplification of signals. The results of these signaling filters are handed over to the default activation and repression mechanisms, and feedforward and feedbackward loops, introducing crucial signals to transcription regulators such as the miRNAs (Inui et al, 2010; Liu et al, 2010).

Default repression

Default repression entails that gene expression is actively repressed, unless in the presence of activating signals. This mechanism is mostly used at the transcriptional level, whereby a repressor intentionally inhibits the activity of a pathway activator. Once in the presence of an activating signal, the repressor's inhibition is relieved, and transcription is activated. For example, miR-200 represses the gene expression of the transcription repressors of E-cadherin and this imposes a repressive signal. E-cadherin is characteristic of epithelial cells. Loss of E-cadherin expression causes the cell to take on a mesenchymal phenotype which contributes to fibrosis (see Chapter 3). ZEB1 and ZEB2 in turn repress the primary transcripts of miR-200 and results in repression of E-cadherin transcription factors (see Fig. 2) (Inui, 2010). Considering the potential impact of transcription, it is deemed unlikely that transcription relies on this activating signal alone. MiRNAs aid with signal interpretation by dampening positive mediators of signaling cascades. This raises the threshold needed to overcome in order to activate the pathway, creating a safety zone for appropriate signals and separating these from weak, inappropriate ones (Inui et al, 2010; Siomi et al, 2010).

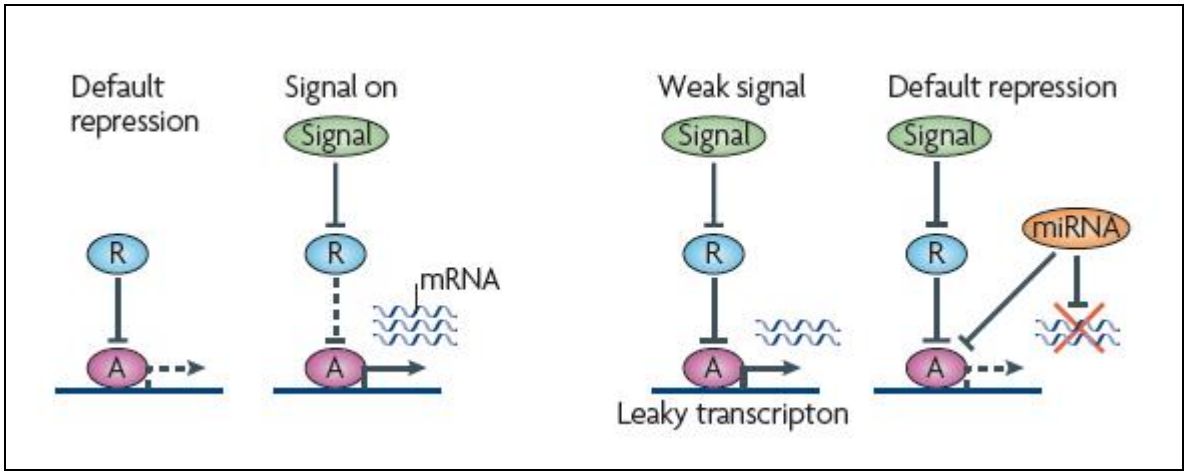

Fig. 2 – Default repression. Repressor (R) actively represses activator (A) of transcription. Only an incoming signal can inactivate R, letting A transcribe its target gene. MiR-200 represses transcription repressors of E-cadherin. ZEB1 and ZEB2 inhibit miR-200 transcripts, activating the transcription repressors. In the case of insufficient repression from R when challenged by a weak signal, further miRNA aids by suppressing A from transcribing its target gene. (Inui et al, 2010)

An example of a miRNA regulating a transcriptional repressor is the cardiac-specific miRNA miR-208. It orchestrates the repression of slow muscle fiber genes by silencing them. In the absence of miR-208, the repressor upregulates by roughly 2-fold, resulting in virtually complete inhibition of all its downstream target genes. (Liu et al, 2010)

Other examples include the Wnt, Notch and Hedgehog signaling pathways. They too are under the influence of strong default repression. Given their significance during organism development, miRNAs play a crucial role in maintaining their strictly regulated gene expression profile. Hedgehog, for instance, controls proliferation of cerebellar granule progenitor cells. These are essential for the generation of neurons in the mammalian brain. Abnormal Hedgehog pathway activity can lead to tumor formation in the brain, and

therefore control of Hedgehog transcription targets is of high importance. MiR324-5p has been identified as a suppressor of Hedgehog signaling by targeting transcription factor GL11. The loss of miR324-5p leads to upregulated pathway responsiveness, causing tumor formation (Inui et al, 2010).

MiRNAs can also use default repression to mediate their own functioning. For example, during DNA damage, a cascade of kinases activates the tumor suppressor gene p53. P53 activation in turn leads to cell cycle arrest, and apoptosis. Under normal circumstances, p53 is latent, and inhibited by ubiquitin-mediated degradation. Inhibition is completed by miR-125b targeting p53. However, loss of the repression signal from miR-125b activates p53-dependent apoptosis. While this may seem like the default repression seen earlier, miR-125b itself is part of the DNA damage network, being downregulated after genotoxic treatment. Therefore miR-125b ensures that a response by p53 to DNA damage is coordinated through a safe pathway (Inui et al, 2010).

Default activation

In much the same way as default repression, miRNAs can also maintain default activation. Here, miRNAs maintain an active signaling profile in order to keep downstream signals inhibited/activated depending on the most beneficial contribution to cell functioning. For instance, the hippo tumor suppressor pathway is involved in the coordination of cell proliferation and apoptosis by regulating proto-oncogenes. Yes-associated protein (YAP) and Taffazin (TAZ) are two such proto-oncogenes. In the absence of the Hippo signal, transcription of the YAP and TAZ target genes is free to occur (left panel). When the Hippo signal is turned on, the large tumor suppressor (LATS) phosphorylates YAP and TAZ, which inhibits their activity. Default active signaling from miR-372 and miR-373 causes activation of YAP and TAZ, because this causes inhibition of phosphorylation by the large tumor suppressor (LATS). Active signaling from the miRs prevents phosphorylation, and thereby causes nuclear retention of the transcription factors, allowing them to activate their target genes. (Hergovich, 2009; Inui, 2010) (see Fig. 3). Therefore, due to an upstream default activation of miRNA, downstream pathways are kept in tow to sustain maximal cell functioning. This example demonstrates the oncogenic potential of miRNAs that is kept controlled both through default activation and repression (Inui et al, 2010; Jiang et al, 2010).

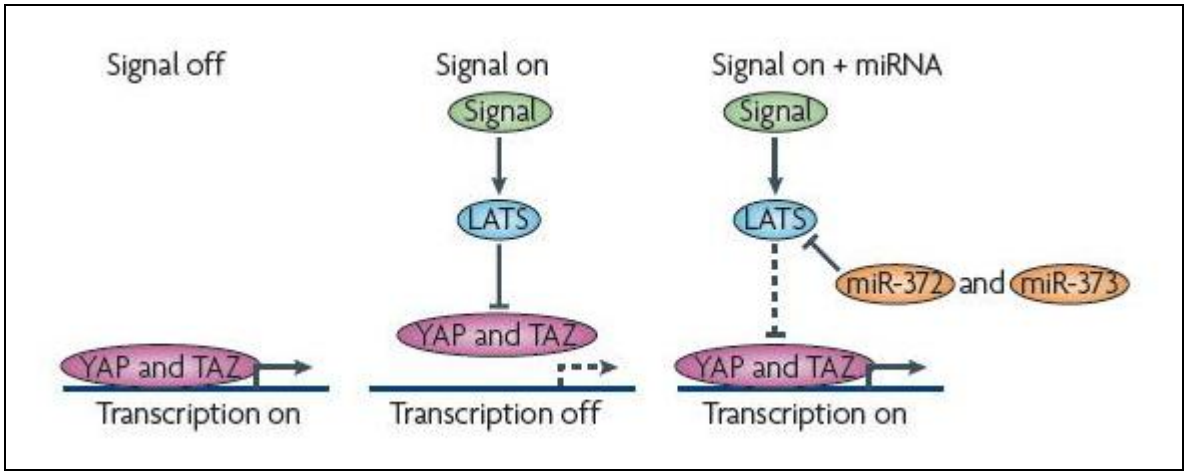

Fig. 3 – Default activation. An active miRNA signal induces phosphorylation of proto-oncogenes Yes-associated protein (YAP) and Taffazin (TAZ) need phosphorylation by large tumor suppressor (LATS). MiR-372 and miR-373 can inhibit LATS, inhibiting transcription of YAP and TAZ (Inui et al, 2010).

Feedforward and feedbackward loops

MiRNAs use feedback and feedforward loops to control expression of their targets. Factors within a signaling pathway report back to a previous regulatory factor (feedback) in order to achieve a desired effect, or induce a reaction of a downstream factor (feedforward). As miRNAs can also regulate their own expression, these loops can also be used to control their own expression. Through the use of a feedforward loop, miRNAs can repress a negative regulator of a pathway, enhancing its target gene expression. For example, in muscle cells, miR-1 represses histone deacetylase 4 (HDAC4), thereby stimulating the myocyte enhancer factor 2 (MEF2) activity. MEF2 is an important transcription factor involved in cellular differentiation and thus embryonic development. By stimulating MEF2, miR-1 expression is enhanced (see Fig. 4A) (Liu et al, 2010).

A feedback loop is established through miRNA repression of an activator of miRNA or mRNA expression. Such a loop is also found in the muscle cell, where serum response factor (SRF) regulates the expression of miR-133. SRF is a highly conserved DNA binding protein that is required for specification of smooth, cardiac and skeletal muscle (Majesky, 2003). MiR-133, in turn, represses expression of SRF. This way, a self regulatory pathway is created, providing feedback control (see Fig. 4B).

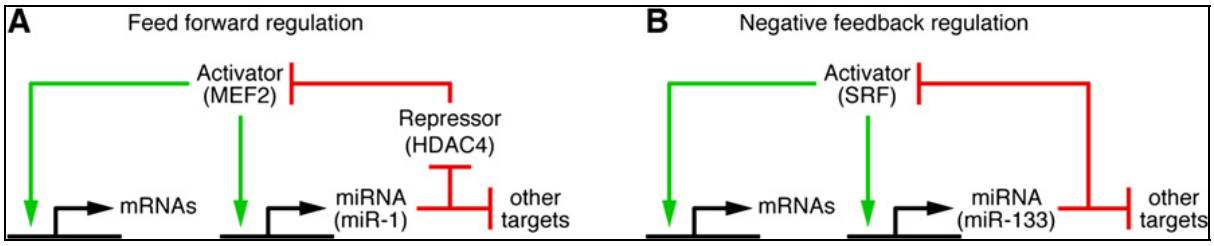

Fig. 4. Feedforward and feedbackward loops. A – A feedforward is created by MEF2 activation of miR-1, which in turn regulates expression of MEF2 repressor HDAC4, as well as other targets. B – activator SRF controls mRNA and miRNA miR-133 expression. miR-133 can repress SRF expression, as well as other targets. Both loops are self regulatory circuits. (Liu et al, 2010)

A common result of the above mentioned mechanisms of miRNA signaling is their eventual silencing effect on mRNA, or, in some cases, themselves. It has been predicted that binding of the miRNA-RISC complex to mRNA prevents the cap-dependent initiation of mRNA translation (Ulvila et al, 2009). This directs mRNA toward degradation in the p-bodies of the cell (Kiriakidou et al, 2007; Ulvila et al, 2009).

Although the above mentioned mechanisms are not representative of miRNA's full repertoire of pathway regulation methods – as their exact systems of operation are still largely unknown – they do illustrate some of the great capabilities of miRNAs.

Regulation of miRNA pathways and their constituents

As with any biological pathway, the miRNA biosynthesis pathway and its constituents require constant regulation and guidance in order to maintain maximum cell functioning and efficiency. This regulation starts as early as modifying the seed region to optimally target its accompanying mRNA, all the way down to posttranscriptional regulation of miRNA and mRNA. Since the processes of transcription and translation are physically separated by the nuclear membrane, they cannot be directly coupled (Keene, 2010). Although this seems to implicate that the final decision as to which genes are expressed lies within the cytoplasm, regulation within the nucleus also occurs and can be of great influence as to which gene are expressed.

A-to-I editing

Adenosine to inosine (or A-to-I) editing refers to the replacement of an adenosine by an inosine by method of deamination. Base pair modification is carried out by adenosine deaminases acting on RNA (ADAR). Because inosine closely resembles guanine in its properties, inosine is interpreted as guanine in effect making it an 'A-to-G' edit. A-to-I editing can therefore change the meaning of codons, and because it does not require breaking of the RNA backbone, modification happens with relatively little intrusion. This type of editing primarily occurs in protein-coding exons in pre-mRNAs, as well as in pri-miRNA, and can lead to the generation or destruction of splice sites, regulate alternative splicing and influence the structure of existing splice sites (Farajollahi et al, 2010). In some cases, A-to-I editing can prevent pri-miRNA processing by Drosha, or even degradation by directing the pri-miRNA complex to Tudor SN (Tudor staphylococcal nuclease homolog) (Siomi et al, 2010) (see Fig. 5). When base pair modification gives rise to a different amino acid, this can influence the effect of the resulting protein. When base pair editing causes a change such that the effects of the resulting protein are different from that of the original protein, this is known as a non-synonymous change (Farajollahi et al, 2010; Siomi et al, 2010). For example, the codon CUA codes for leucine. A-to-I editing would give CU'I' where the I is interpreted as a G, giving CUG. CUG also codes for leucine. Here the base pair switching does not influence the resulting amino acid and therefore will not affect the effects of the final protein. However, ACU codes for threonine. A-to-I editing would give 'I'CU, which is interpreted as GCU. GCU codes for the alanine amino acid. This is known as a

non-synonymous change because alanine may induce a different effect on the functions of the resulting protein than threonine may have.

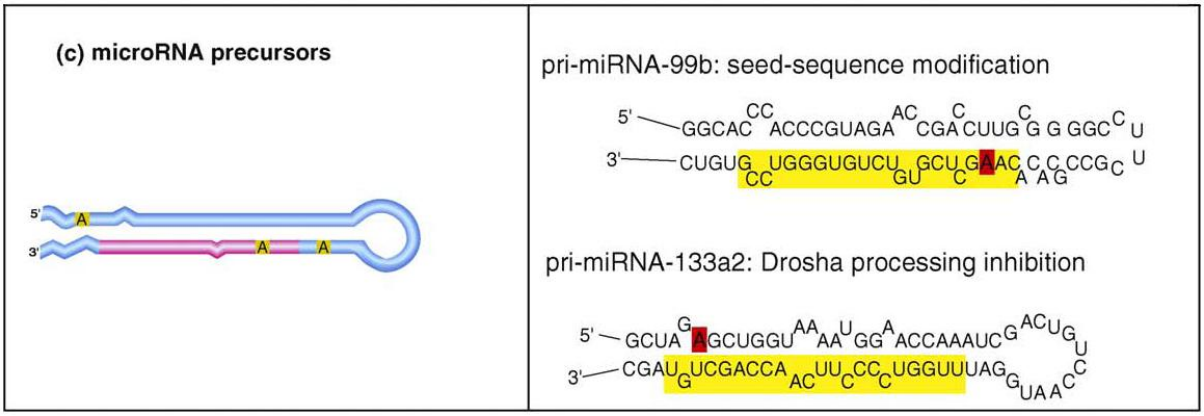

Fig. 5 – A-to-I editing in miRNA precursors. Editing can lead to a recoding event, as is the case in pri-miRNA-99b seed sequence modification. Seed sequence is marked in yellow, the modification in red. Mature miRNA-99b can now potentially alter target mRNA interaction. In pri-miRNA133a2, a modification outside the seed region (yellow) causes a change in processing rate by Drosha, but does not change the target mRNA interaction (Farajollahi et al, 2010).

The Microprocessor

The Microprocessor's main constituents – Drosha and DGCR8 – are under constant observation by their RNA helicase cofactors p68 and p72. These cofactors promote Drosha cleavage of miRNAs, and their depletion can lead to a reduction in the level of miRNAs as they can no longer be cleaved (Siomi et al, 2010). P68 and p72 are the core cofactors of the Microprocessor but, depending on the situation, often work in combination with other cofactors. For instance, tumor suppressor protein p53 also acts as a cofactor of the Microprocessor during DNA damage, where p53 associates with Drosha and p58, enhancing Drosha processing of miRNAs. Another example is the interaction of estradiol receptor α (ERα) with Drosha in a p68-/p72-dependent manner. ERα is bound to estradiol (E2), and its association with the Microprocessor inhibits the production of a subset of miRNAs in the uterus after ovariectomization in mice (Siomi et al, 2010).

Where the site-specific factors may instruct the Microprocessor to induce a precise effect, the presence of the core cofactors p68 and p72 seem to be of persistent importance, and the loss of function of either cofactor is lethal (Newman et al, 2010).

Drosha and DGCR8 themselves are also under the watchful eye of a feedback circuit. DGCR8 directly stabilizes Drosha, giving Drosha the opportunity to cleave two hairpin structures of the 5'UTR and coding sequences of the DGCR8 mRNA. This leads to a degradation of mRNA. As both aspects of the circuit induce negative effects, this is known as a double negative feedback loop. This way, the RBPs become 'regulators of regulators'. The up- and downstream regulation of transcription, translation and posttranscriptional processes is by this method given an extra element of security and stabilization, as they create interdependency. This also makes them more resilient against incoming signals for global

RNA networks, allowing them to maintain their delicate fine tuning of these processes (Farajollahi et al, 2010; Keene, 2010; Siomi et al, 2010).

Dicer regulation

Dicer processing of pre-miRNA seems to happen quite sufficiently, as pre-miRNA levels are quite low in relation to pri- and mature miRNA. This could indicate that substrate turnover is run by a rapid mechanism, requiring little regulation. However, Dicer itself may act as an important point for miRNA biogenesis regulation (Siomi et al, 2010). Dicer activity is regulated indirectly by withholding nuclear miRNA, denying it access due to Dicer's location in the cytoplasm. In a more direct manner, Dicer is regulated by its products. Dicer contains let-7 target sites, suggesting that let-7 miRNA directly targets Dicer. Let-7 is a miRNA superfamily, composed of 12 precursor transcripts. LIN28, an RBP, represses both Drosha and Dicer activity by directing terminal uridylyl transferase 4 (TUT4) to add a uridine tail to let-7. Such a long hairpin extension cannot be processed by Dicer, inhibiting dicing of pre-miRNA, and inducing degradation. This creates a sense of competition between Dicer and LIN28 for pre-let7 (Newman et al, 2010; Siomi et al, 2010) (see Fig. 6).

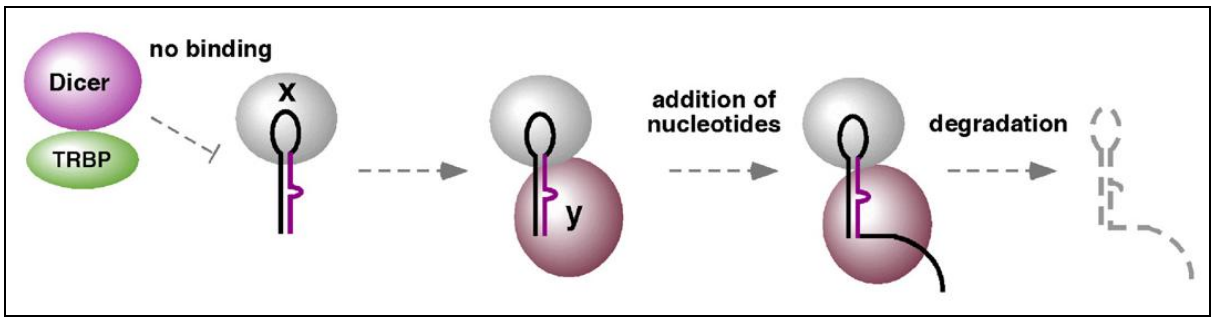

Fig.6 – Dicer regulation by method of competition. RBP LIN28 (marked X) competes with Dicer for pre-let-7. LIN28 recruits additional factors such as TUT4 to add a uridine tail (marked Y), whereby recognition and processing by Dicer is inhibited. This induces degradation of the pre-miRNA (Siomi et al, 2010).

Homeostasis of miRNA expression

Many miRNAs are encoded as clusters in the genome, meaning that transcription of miRNA often results in transcription of a polycistronic primary transcript, known as global miRNA. However, processing of an individual miRNA can also be independently regulated where the desired miRNA is singled out from its cluster. As the primary transcript is processed in the context of the initial cluster, this indicates that posttranscriptional regulation of miRNA is possible. This is an important contributor to the homeostasis of miRNA expression, because it functions as a filter, causing degradation of unnecessary miRNAs and performance of beneficial miRNA to cell functioning only. Argonaute (AGO) proteins also play an important role in maintaining homeostasis by binding to both the 5' and 3' ends of miRNA. This protects miRNA from exonucleases and thus degradation. However, cells seem to have developed a method whereby they can dislodge AGO from miRNA, exposing miRNA to the exonuclease XRN-2 for degradation. While cells have found a way to circumvent the protection provided by AGO, miRNA association with target RNA nullifies this effect, creating several positions throughout the miRNA biosynthesis pathway by which

homeostasis can be maintained (Liu et al, 2010; Siomi et al, 2010).

3 – Regulation of EMT by miRNAs

Besides the importance of EMT during embryonic formation, implantation and organ development, EMT has taken an increasingly center-stage position in wound healing, tissue regeneration and organ fibrosis (Kalluri, 2009). Its role in fibrosis has long created a paradigm in pathological research, but convincing empirical evidence has shown that EMT does in fact induce the generation of repair-associated mesenchymal cells in the form of fibroblasts and myofibroblasts (Kalluri, 2009). Myofibroblasts and fibroblasts have shown to be the key mediators of fibrosis. Over the years, miRNA families and individual miRNAs have been identified as key mediators of EMT in fibrosis, such as the miR-200 family and the miR-205 family. They work in a regulatory circuit with the ZEB1 and ZEB2 genes, which stimulate EMT in part by inhibiting E-cadherin expression of the epithelial cells in exchange for the elevated expression of N-cadherin. E-cadherin is essential for the maintenance of an epithelial phenotype, as it ensures cell-to-cell adhesion. Loss of E-cadherin expression leads to free mobility of the cells as they are no longer anchored to the basement membrane. Precise workings of the ZEB genes and their connection with miR-200 and miR-205 will be explained in detail later on in this chapter.

General mechanism of EMT

In the event of an injury, or where wound healing is necessary, epithelial and/or endothelial cells release inflammatory signals, triggering blood clotting and the deposition of a temporary extracellular matrix (ECM). Blood clots attract platelets and other immunological components such as macrophages and neutrophils. Myofibroblasts already at the site of injury produce metalloproteinases (MMPs – notably MMP2, MMP3 and MMP9) which can be seen as an initiating step of EMT. Epithelial cells in the lungs are called alveolar epithelial cells, or AECs, and come in two subtypes: alveolar epithelial type II cells (AT2), which are believed to serve as progenitor cells for repair after injury, capable of self-renewal and able to give rise to the second type of epithelial cell: alveolar epithelial type I (AT1) (Willis and Borok, 2007). AECs work in concert with macrophages, neutrophils, myofibroblasts and use MMPs to degrade the basement membrane to which they are attached, collagen IV and laminin, and encourage the recruitment of inflammatory cells. Growth factors such as fibroblast growth factor 2 (FGF-2), epidermal growth factor (EGF) and transforming growth factor β (TGF-β) are also employed and aid further ECM formation. TGF-β, in combination with cytokine interleukin-13 (IL-13) and platelet-derived growth factor (PDGF) activate fibroblasts, which can differentiate into myofibroblasts. Myofibroblasts are crucial contributors to ECM formation, being key depositors of collagen (Wynn, 2008).
This generalized process of wound healing can develop into fibrosis when the balance between collagen deposition and its subsequent degradation is deregulated and leads to a net collagen increase. The fibroblasts and myofibroblasts that orchestrate fibrosis in the lungs are in large part derived from the AECs that have undergone EMT. Fibroblasts are not only derived from EMT, but also appear to be recruited from the bloodstream after being released by bone marrow (Kalluri and Neilson, 2003). The alveolar epithelium is an

important source of TGF-β and profibrotic cytokines, creating a perfect niche for fibrosis development. In lung diseases such as idiopathic pulmonary fibrosis (IPF) and usual interstitial pneumonia (UIP), it has long been suspected that the pathology found in the lungs was due to persistent inflammation but research has shown that AECs express fibroblast markers and display fibroblast-like phenotypes (Willis et al, 2006; Iyonaga et al, 1997; Selman and Pardo, 2003). The exposure of AECs to TGF-β causes down-regulation of surfactant protein C production, which is important for effective gaseous exchange across the alveolar membrane. Research has also shown that cells undergoing EMT express both mesenchymal markers such as fibroblast-specific protein 1 (FSP1) and α-smooth muscle actin (α-SMA) as well as epithelial markers like E-cadherin, but under the influence of TGF-β, PDGF, EGF and FGF-2 as well as through myofibroblasts signaling, AECs dismember themselves from the basement membrane, and migrate along chemokine and cytokine gradients into the interstitium, taking on a complete fibroblastic phenotype (Strutz et al, 1995; Okada et al, 1997; Zeisberg et al, 2003) (see Fig. 7).

EMT readies AECs for reshaping and migration by stripping them of their characteristics such as cell polarity, use of adherens and tight junctions, desmosomes and hemidesmosomes and its mechanical strength derived from the cytoskeleton. Once in the interstitium, these newly acquired fibroblasts and myofibroblasts in the form of mesenchymal cells play a crucial role in the contribution to organ fibrosis by method of excessive ECM deposition. While the degradation of the basement membrane has previously been described as the completing step of EMT, the impact EMT eventually has on organism functioning is not encountered until later.

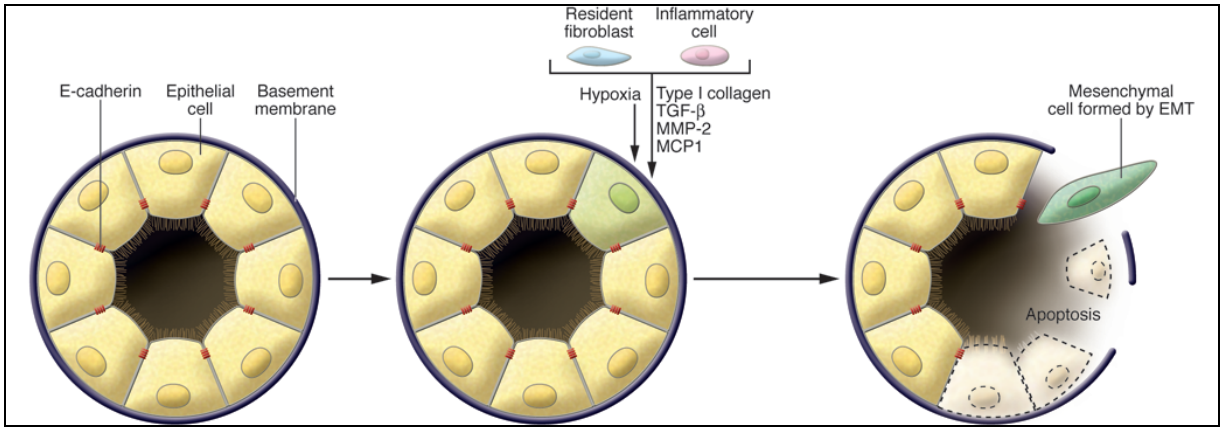

Fig. 7 – Epithelial-to-mesenchymal transition (EMT). Epithelial cells are bound to the basement membrane and each other with junctions. Under the influence of signals from fibroblasts and inflammatory cells, dismemberment from the basement membrane and each other causes them to lose their epithelial phenotype and take on a mesenchymal phenotype, migrating to the interstitium (Kalluri and Weinberg, 2009).

Key fibrotic regulatory factors come from TGF-β

Of the many factors and regulators involved in EMT and lung fibrosis, TGF-β is without doubt the most important one, as it is able to initiate and maintain EMT by activating signaling pathways and transcriptional regulators involved in tissue fibrosis (Bedi et al, 2006;

Zavadil et al, 2005). It exists in three isoforms, TGF-β1, -2, and -3. Tissue fibrosis is primarily attributed to TGF-β1, its main cellular sources being macrophages and monocytes (Wynn, 2008). TGF-β1 is upregulated in fibrotic lungs. It can use both direct and indirect pathways to steer EMT and fibrosis. The most significant pathways are the Smad-dependent and Smad-independent pathways (Bedi et al, 2008; Hills and Squires, 2010; Willis and Borok, 2007; Willis et al, 2006) (see Fig. 8).

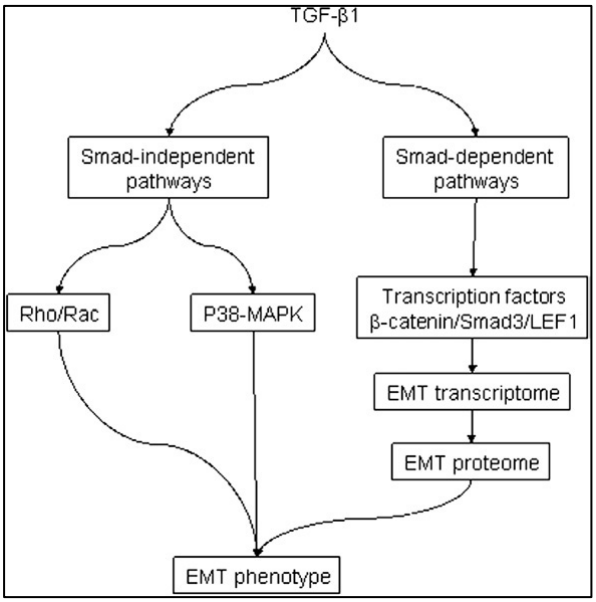

Fig. 8 – Smad-independent and –dependent pathways of TGF-β profibrotic effect. Dependent pathways lead to EMT proteome production, the independent pathway uses Rho/Ras and p38/MAPK pathways to lead to an EMT phenotype. (Bedi et al, 2008)

The Smad-dependent pathway uses Smad proteins Smad2, Smad3, Smad4 and Smad7, although Smad3 is known as the main mediator. TGF-β1 signals are received by serine/threonine kinase type I and type II receptors. A received signal from TGF-β1 causes internalization into endosomes of the receptors where Smad anchor for receptor activation (SARA) causes a formational change in Smad2 or Smad3. Smad2 and Smad3 are then phosphorylated by the type I receptor and they associate with Smad4 after which they translocate to the nucleus. Here they interact with transcription factors, which cause the expression of connective tissue growth factor (CTGF), α-smooth muscle actin (α-SMA), collagen type I and III, Slug, Snail, Scatter, lymphoid enhancing factor-1 (lef-1) and β-catenin (Willis and Borok, 2007; Willis et al, 2006; Wynn, 2008). As is evident from this long list of factors that Smad is responsible for, research done with a TGF-β1 receptor mutation showed that Smad signaling is a prerequisite for TGF-β-induced responses (Itoh et al, 2003; Willis and Borok, 2007). The activated transcription factors all form part of the EMT transcriptome – the total transcription-related factors for EMT. The translation of these transcription factors leads to the production of the EMT proteome – all proteins associated with EMT. They are also responsible for the cellular machinery needed for junction disassembly, cytoskeletal rearrangement and cellular motility leading to the presentation of a full EMT phenotype (Willis et al, 2006).

TGF-β has also been found to direct EMT through a Smad-independent pathway, although evidence to support this is mainly in vitro. Using RhoA, Ras and MAPK signaling, the Smad-independent pathway is triggered by TGF-β to induce EMT. In mink lung epithelial cells, RhoA has been linked to α-SMA activation in EMT intermediate cells, as well as to the regulation of cytoskeletal and adherens junction rearrangement. Tight junction disassembly

may also be under the influence of TGF-β, where TGF-β receptors are recruited to tight junctions after which it interacts with polarity protein Par6. Phosphorylation of Par6 leads to an accumulation of Smurf1 which degrades RhoA and results in dissolution of tight junctions, and disassembly of the actin cytoskeleton (Willis and Borok, 2007). Distinction between Smad-dependent and independent pathways can be difficult, as there seems to be a lot of crosstalk between the two pathways (Inui, 2010). Smad3 has, for instance, been sighted in the independent pathway, while its main contribution is believed to be in the Smad-dependent pathway and MAPK interacts with Smad dependent pathways to inhibit EMT (Davies et al, 2005; Willis and Borok, 2007).

While TGF-β is considered a key regulator and inducer of fibrosis in lung, there are important contributors that come in the form of neither TGF-β nor Smad. Cytokines such as IL-4, IL-5, IL-13 and IL-21 are known to act separately from the TGF-β/Smad pathway to stimulate collagen deposition (Wynn, 2008). The effects of IL-13 have been investigated in detail, and reveal that IL-13 induces production of latent TGF-β1 in macrophages, thereby indirectly activating TGF-β. It does this by cleaving latency-associated protein (LAP) which is responsible for keeping TGF-β latent (Wynn, 2008).

The Zinc-finger E Box Binding 1 and 2 (ZEB1/ZEB2)–miR-200 double negative feedback loop and the miR-200 family

As has been demonstrated, EMT is under the influence of a large spectrum of factors, including both genetic as well as cellular contributors. TGF-β remains to play an essential role in the coordination of EMT induction and it is therefore not unthinkable that TGF-β is also involved in some of the known miRNA pathways that lead to fibrosis through EMT encouragement. Over the years, one particular miRNA family has gained increasing credits for its involvement in EMT. This is the miR-200 family, which works in close combination with the miR-205 family. While evidence concerning other miRNAs and miRNA's general role in EMT and fibrosis development is constantly being acquired, the exact interactions and underlying mechanisms between the supporting factors is still being investigated. Recently, the miR-200 and miR-205 families have been reported to work in close association with ZEB1 and ZEB2 (where ZEB2 is also known as Smad interacting protein (SIP1)). ZEB1 and ZEB2 are transcription factors that repress the expression of E-cadherin by binding to the promoter of E-cadherin using their zinc-finger motifs. Zinc finger motifs are domains that are stabilized by a zinc ion and besides having protein folding properties, can also recognize DNA (Klug, 2010). The zinc-finger motifs are located at the ZEB1/2 N-and C-terminal regions and bind to the E-boxes of the E-cadherin promoter whereby E-cadherin expression is repressed (Cano et al, 2008; Gregory et al, 2008a). The loss of E-cadherin expression contributes to the loss of the epithelial profile, and its expression is replaced by the increased expression of N-cadherin, characteristic of mesenchymal cells (Gregory et al, 2008a; Katoh and Katoh, 2008).

The miR-200 and miR-205 families have been reported to work in close association with the ZEB transcription factors, with the miR-200 family having a particularly direct influence. The miR-200 family can be grouped into two sub-families according to their seed sequence. MiR-200a and miR-141 share identical seed sequences, as do miR-200b, miR-200c and miR-429 (see Fig. 9) (Cano et al, 2008). The ZEB mRNAs have several binding sites which the miR-200 family can target. The ZEB2 3' UTR contains six target sites for miR-200b (and therefore also for miR-200c and miR-429, as they share the same seed sequence), three target sites for miR-200a (and therefore also for miR-141) and two target sites for miR-205. The ZEB1 3'UTR was also found to contain multiple target sites including five miR-200b sites (and seed sequence sharers), three miR-200a sites (and miR-141) and one miR-205 site (Gregory et al, 2008b). MiR-200b has the most binding sites of all the miR-200 family members which is in concurrence with the finding that it is most effective at repressing ZEB1 and ZEB2 (Gregory et al, 2008b).

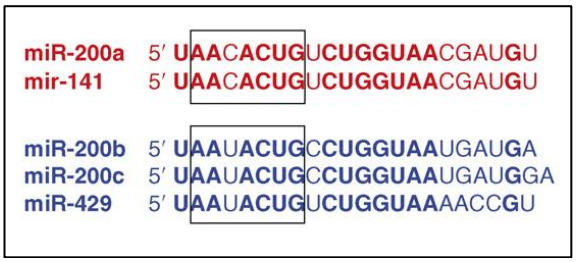

Fig. 9 – Seed sequence similarity amongst the miR-200 family (seed sequences boxed). MiR-200a and miR-141 (in red) share an identical seed sequence, as do miR-200b, miR-200c and miR-429 (in blue) (Cano et al, 2008).

The relationship between the miR-200 family and the ZEB transcription factors has been closely studied and has led to the discovery of a double negative feedback loop, where miR-200 family members are regulated by and regulate the expression and functioning constituents of the ZEB transcription factors. With the onset of EMT, TGF-β contributes to the activation of ZEB1/ZEB2. ZEB1/ZEB2 transcriptionally repress the miR-200 family by binding paired E-boxes close to the miR-200b, miR200a and miR-429 transcription start site (TSS). (see Fig. 10) The identified transcripts also contain all the elements needed for epithelial-specific expression. For both of the transcripts, ZEB1 and ZEB2 negatively regulate the transcription of the enclosed genes by directing E-box binding upstream of the assumed TSS (Bracken et al, 2008b).

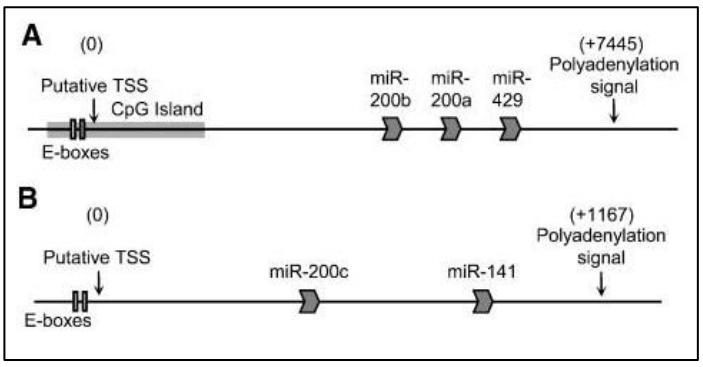

Fig. 10 – E-boxes bind to the polycistronic transcript containing (A) miR-200b, miR-200a, miR-429 and (B) miR-200c and miR-141 genes. E-box binding is close to the assumed transcription start site (TSS), effectively repressing transcription of the genes (Bracken et al, 2008b).

While the ZEB factors have primarily been portrayed as promoters of EMT, their regulation has also been noted. They are regulated by the precise factors that they themselves suppress – by the miR-200 family (Bracken et al, 2008b). Studies where miR200c and miR0299b were overexpressed showed

that ZEB1 and ZEB2 were regulated, respectively. In addition, the repression of ZEB1/ZEB2 gave way to an upregulation of E-cadherin – an indication that the restoration of an epithelial phenotype might be possible (Christoffersen et al, 2007; Hurteau et al 2007).

This regulation pathway between ZEB1/ZEB2 and miR-200/miR-205 creates a negative feedback loop (see Fig. 11). Feedback loops are not an uncommon method of regulation, as was seen earlier in the regulatory mechanisms implemented by miRNA. The use of such loops has multiple advantages as it creates the option of reversibility, robustness by limiting the option of interference from outside networks and switchability between epithelial and mesenchymal phenotypes (Bracken et al, 2008a; Hornstein, 2006; Tsang et al, 2007).

TGF-β is certainly also a contributor to EMT induction through meddling with the ZEB1/ZEB2 pathways. It reduces miR-200 transcription and increases ZEB1 expression indirectly by binding to Smad-coactivators, which in turn have been activated by TGF-β (Bracken et al, 2008a; Gregory et al, 2008a).

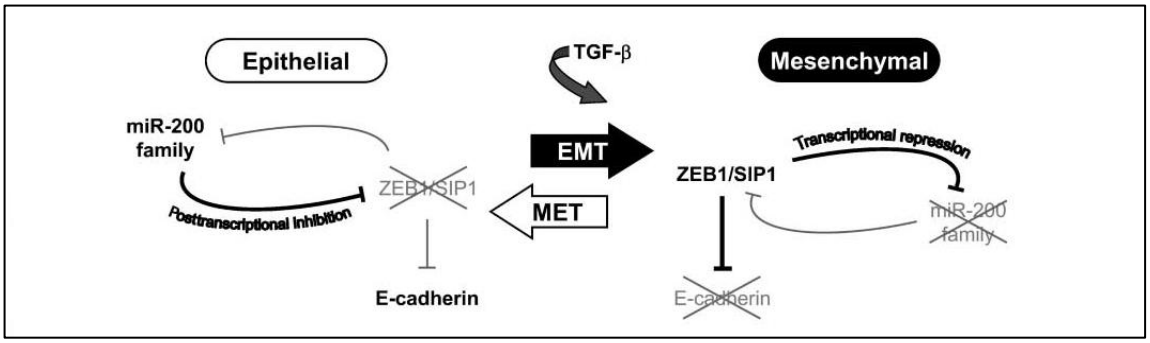

Fig. 11 – the double negative feedback loop regulating ZEB1/ZEB2 (ZEB2=SIP1) and miR-200 family expression through MET (mesenchymal-epithelial transition) and EMT. miR-200 can posttranscriptionally inhibit ZEB1/ZEB2 expression, activating E-cadherin expression and sustaining an epithelial-like phenotype. Onset of EMT encourages ZEB1/ZEB2 to repress transcription of the miR-200 family, and inhibit E-cadherin expression, resulting in a mesenchymal phenotype (Bracken et al, 2008b).

Snail, Twist, Goosecoid and other genetic regulators of EMT

The ZEB pathways alone are not the only way in which miRNAs exert their influence on EMT and fibrosis. The expression of Twist, Goosecoid and other transcription factors downregulate E-cadherin, which is key for maintaining cytoskeletal strength and the epithelial phenotype (Thiery et al, 2009). Indirect factors assist in EMT regulation and promotion, one such factor being Snail1. Snail1 directly represses the transcription of E-cadherin by binding E-boxes to the E-cadherin promoter in much the same way as ZEB does. It also makes use of corepressors including histone deacetylase 1 and 3 (HDAC1/HDAC3). As a result, the cell polarity characteristic of epithelial cells is lost, a crucial step in EMT (Gregory et al, 2008a). In addition, Snail1 induces the expression of metalloproteinases, causing delamination of the basement membrane and thereby progressing EMT. This mechanisms works with a feedback loop too, for expression of MMP3 triggers EMT by

increasing the level of reactive oxygen species giving way to further activation of Snail1 expression (Thiery et al, 2009).

Another way in which Snail1 promotes EMT is through indirect promotion of ZEB2. It does this by regulating a natural antisense transcript (NAT), which prevents splicing in the 5' UTR of the ZEB2 gene thereby increasing ZEB2 production. In addition, Snail factors can bind to Smad proteins, promoting TGF-β–induced EMT (Thiery et al, 2009; Vincent et al., 2009). Snail also comes in another variant known as Snail2 or Slug and although Snail2 seems play a more prominent role in neural crest development (where EMT is also of great importance), Snail2 has also been linked to the degradation of Snail1, once again pointing to a regulatory feedback loop (Thiery et al, 2009; Wang et al., 2009). Nonetheless, as has been proven in the past with feedback loops, Snail2 assists Snail1 by activating ZEB1 (Thiery et al, 2009).

The dismemberment of E-cadherin in epithelial cells is also indirectly promoted by other transcriptional factors such as Twist and Goosecoid. Twist is a basic helix-loop-helix (two α-helices connected by a loop) transcription factor, and like Snail and ZEB binds E-box elements to the E-cadherin promoter. This causes inhibition of E-cadherin expression and thereby indirectly supports class switching to N-cadherin (Gregory et al, 2008a). It also promotes cell invasion and motility by activating miR-10b (Bracken et al, 2008a). The mechanism by which Goosecoid operates is still under investigation but for now has been assumed to indirectly promote EMT by aiding the repression of E-cadherin expression (Bracken et al, 2008a; Thiery et al, 2009) (see Fig. 12).

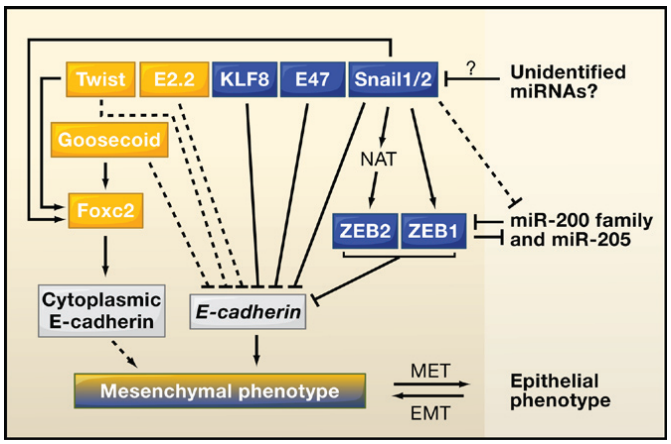

Fig. 12 – schematic presentation of direct (blue) and indirect (yellow) pathways through which the mesenchymal phenotype is expressed (Thiery et al, 2009)

4 – Future clinical prospects for the use of miRNA in the treatment of EMT

The discovery of miRNAs and their function has without doubt made a large impact on the way research is conducted. The majority of laboratories now use different RNA interference methods to get a better and more complete understanding of the biological systems and disease models they investigate (Keene, 2009). MiRNAs propose very attractive opportunities for new therapeutic treatment. Their effect on unstressed adult tissue seems to be minimal, while their contribution to remodeling of diseased tissue is promising. Studies done on cardiovascular disorder models using antagonistic miRNAs (antagomiRs) and miR mimics have given confident results that suggest that disease phenotypes are subject to change through the application of miRNAs (Bonauer et al., 2009; Caré et al, 2007 (on behalf of Lui and Olson); Liu and Olson, 2010; Thum et al, 2008). These methods using a mimic and an antagonist of the miRNA under investigation have shown to be a very effective method of discovering miRNA function and implications on up- and downstream pathways (Liu and Olson, 2010). The clustering of miRNAs on proximate transcripts that serve a similar function allows for easier localization of unknown miRNAs when looking for miRNAs with a particular function or, in reverse, the role of a miRNA with an unknown function can be predicted based on its position and proximity to other miRNAs of which the functions are known. In addition, the fact that miRNAs are often grouped according to function means that potential therapeutic use of miRNAs can target an array of genes as opposed to a single gene (Abdellatif, 2010). This is beneficial because it may reduce an unwanted effect such as EMT on several levels as opposed to one level. Obviously a lot amount of research still remains to be done before treatment using miRNAs can be applied clinically. However, miRNA does hold a lot of potential and could provide a very precise and effective new method of treatment. Where drugs that single out a sole target may only address a small portion of the problem, miRNA treatment could for instance target the ZEB transcription factors which, as has been demonstrated, influence EMT through several pathways. This may also avoid redundancy to a certain degree, as multiple mRNAs are targeted instead of having to implicate several mechanisms to inhibit a single target – ‘killing two birds with one stone’, so to speak (Liu and Olson, 2010). Another advantage presents itself here, because a more complete treatment through miRNA targeting may also mean that fewer treatments are needed, as EMT is targeted in multiple ways in a single treatment. Keeping in mind the attempt to sustain a maximal quality of life, fewer treatments would mean less imposition and intrusion on lifestyle and thus a relatively better quality of life.

Nonetheless, the high level of regulation and the fact that some globally expressed miRNAs may later be transcribed as individual miRNAs makes it difficult to confirm target mRNAs. The conditions under which miRNAs are identified can also be of influence on their behavior, as this pulls other factors such as experimental controls into play which may manipulate targeting. It would (and has proven to) be difficult to distill the natural conditions of miRNA behavior, because investigative controls influence this behavior

(Abdellatif, 2010). On the other hand, miRNA regulation is very tightly controlled, as was demonstrated with the feedback mechanisms and default repression and activation methods. This makes miRNA highly resistant to interfering genetic networks, and this has been proven by the fact that some miRNA sequences have been highly conserved over the course of evolution (Maurer et al, 2010; Lui and Paroo, 2010). Although this may eliminate some of the uncertainty around target determination, the field of miRNA research is nowhere near complete. A microarray reflecting expression at a certain point in time is a snap-shot of a situation which is actually highly dynamic and versatile. The transcriptome is a balance between on-going transcription and on-going changes in RNA stability and the transcriptome constantly changes to maintain this balance. It is the combination of the changes in translation and posttranscription that determines the gene expression profile (Keene, 2009).

MiRNAs therefore hold a lot of potential, and their regulatory capacities need to be harnessed and channeled toward therapeutic treatment of EMT. This will require many more years of research but will undoubtedly bear fruitful results in the end.

5 – Conclusion

In this thesis, EMT in the lungs has served as a frame through which the regulatory capacities of miRNA have been demonstrated. MiRNAs work as the fine tuners of the gene expression profile and are of great essence for the maximal performance of an organism. MiRNA families miR-200 and miR-205 have shown to be part of a meticulously regulated circuit with the ZEB1 and ZEB2 transcription factors. ZEB1 and ZEB2 activation contributes to the process of EMT, initiating and sustaining fibrosis.

The world of miRNAs present regulatory mechanisms that work with extreme precision. The biosynthesis of miRNA is regulated on a number of levels, adding to the precision with which miRNAs carry out their function. Through feedback and feedforward loops, default repression and activation, regulation of biosynthesis components such as the Microprocessor and Dicer as well as global and individual miRNA transcription, miRNAs are able to induce their regulatory function with extreme precision. It is therefore that miRNAs are known as the fine-tuners of the gene expression profile and can aid in determining the degree in which genes – and thus their subsequent proteins – are expressed. MiRNAs play a large role in determining the fate a cell, ranging from cell proliferation to apoptosis. In the case of EMT, activation of the miR-200 and miR-205 family has proven to be capable of changing the entire phenotype of a cell! The consequences of such a change have proven to contribute to the detrimental condition of fibrosis. Not only through the direct pathway of ZEB1 and ZEB2 interaction have miRNAs shown to exert their influence. Indirect pathways of miRNA activation can lead to increased levels of TGF-β – the key protein that orchestrates EMT and the furthering of fibrosis. TGF-β sets a number of new pathways in motion (such as the Smad dependent and Smad independent pathways) whereby EMT continues to contribute to fibrosis. Therefore, the impact that miRNAs have on the cell propagates and echoes down numerous other processes. Their impact on cell and organ functioning are of great magnitude and miRNAs provide opportunities through which the effects of EMT may potentially be prevented. This is because the uncovering of miRNAs and their extensive influence on cell behavior has brought with it a new field for therapeutics. The possible use or targeting of miRNAs to treat conditions such as the demonstrated EMT proposes exciting new opportunities with a lot of potential. The fact that miRNAs can target several mRNAs at a time eliminates a factor of redundancy because a miRNA could be able to influence several aspects of a condition whereas other drug treatments may need individual drugs to target the individual aspects. Nonetheless, there still remains a lot to be refined and determined. Although other forms of gene therapy have become increasingly acceptable and more frequently used, the genetic code is the building blocks of life, and intervention should proceed with extreme caution. The regulatory capacities of the miRNA should be harnessed and directed toward therapeutic treatment of EMT and fibrosis. Although this still requires a lot of research the potential miRNA holds is most definitely worth exploring and developing.

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