6.1. SUMMARY

TGF-β controls a diverse set of cellular processes including cell proliferation, differentiation, apoptosis and several pathological conditions, thereby a deeper understanding of the transcriptional and posttranscriptional regulation of TGF-β signalling cascade is of paramount clinical importance. In this study, we have explored the physiological post-transcriptional regulation of TGF-β1 by miRNAs with a perspective towards identifying a novel mechanism for inhibiting TGF-β1 induced fibrosis in the lung. Anti-TGF-β antibodies and small molecule inhibitors of TGF-β receptors have been previously explored in a number of diseases, including lung fibrosis, but showed only moderate success in pre-clinical models and human trials till date. In this study, we employed a combination of in silico and in vitro approaches to identify microRNAs which could be involved in the post-transcription regulation of TGF-β1.

In this study, we report for the first time that miR-326 and miR-609 are involved in the post-transcriptional regulation of TGF-β1. Ectopic expression of miRNA mimics could downregulate TGF-β1 expression at the protein and mRNA levels in epithelial cells. In contrast, antagonir approach to knockdown basal miRNA expression in epithelial cells could upregulate TGF-β1 expression. An inverse correlation was observed between miR-326 and miR-609 with TGF-β1 in cell-lines of multiple origins, thus indicating a physiological role of these microRNAs in regulating TGF-β expression. A downregulation of miR-326 levels were also observed during Epithelial-mesenchymal transition in cultured human lung cells, and this model was further utilized to test the efficacy of miR-326. Here, we observed that while miR-326...
mimics reduced TGF-β1 and prevented EMT, ectopic expression of miR-326 antagonir significantly upregulated TGF-β1 expression and enhanced EMT. This is a novel finding and was confirmed further by staining for EMT markers.

We further investigated the biological importance of miR-326 using a well-established model of bleomycin-induced pulmonary fibrosis. Although miR-609 showed in vitro efficacy in regulating TGF-β1 levels, we could not perform any in vivo studies due to the absence of a mouse ortholog. We found that the increased expression of TGF-β1 in bleomycin induced lung fibrosis model was associated with loss of miR-326. Further, inhaled delivery of hairpin nucleotides mimicking miR-326 was sufficient to inhibit TGF-β1 expression in this model and attenuate the fibrotic process.

A limitation of this approach is that the bleomycin model does not fully replicate all the clinical features of human IPF. Since bleomycin is known to initiate a TGF-β-dependent induction of pulmonary fibrosis, success in this model could only reassure that our findings regarding miR-326 mediated repression of TGF-β1 are correct and could not be extrapolated directly towards potential benefit in IPF. Hence, we further extended our study with human IPF subjects and observed that the lungs from IPF patients have lower levels of miR-326, which was inversely correlated with TGF-β. Given that IPF is a progressively lethal disease without any current effective treatment, it is likely that our findings may have future clinical applications. Moreover, TGF-β1 induced fibrosis is important in many diseases and miR-326 may have implications for the applicability as a treatment. Consistently, databases also
suggest that besides human lungs, miR-326 is highly expressed in other cell-types such as blood cells, brain, etc and may be investigated in future for therapeutic applications (Figure 61). In particular, direct inhibition of TGF-β1 synthesis via a mechanism suitable for local administration, for example by using inhaled modified nucleotides, could be advantageous in IPF as compared to antibody based neutralization strategies. Systemic administration of antibodies may not sufficiently inhibit the local TGF-β1 autocrine loop in lungs, whereby TGF-β1 secreted by epithelial cells acts upon the cells to further increase TGF-β1 expression, and may have unintended consequences due to the potent immunomodulatory roles of TGF-β1.

One advantage of using physiological miRNA target-site based nucleotide therapy as compared to siRNAs, which target specific regions of the gene, is that related genes within a functional network share regulatory motifs. Leads from real-time based analysis of fibrosis-related genes indicate a functional role of miR-326 in fibrosis by affecting multiple genes. Molecular modeling with Ingenuity Pathway Analysis was confirmatory, as it suggested both direct and indirect interactions of miR-326 with genes involved in TGF-β signalling and fibrosis-related pathways. Consistent with these predictions, we demonstrated a functional role of miR-326 in modulating the key genes of TGF-β signalling pathway, including Smad3 and Smad7 at the protein level. While western blot and immunohistochemical analyses showed a significant downregulation in Smad3 levels with miR-326 expression, an opposite effect was seen on Smad7 protein levels. This is particularly interesting, as Smad7 is a known negative inhibitor of TGF-β signalling pathway. The precise molecular mechanism for this upregulation is intriguing and would be an interesting question for future studies.
Such a miRNA-mediated upregulation of the target gene is supported by recent reports, which suggest that microRNAs can posttranscriptionally stimulate gene expression through direct and indirect mechanisms (Shobha, 103; Nicole, 104; Yin, 105). These studies indicate that microRNAs can associate with protein complexes (microribonucleoproteins or microRNPs) to participate in translation upregulation and significant alterations in gene expression.

Our molecular modeling result also indicates Ets-1 as a central mediator of the interactions. Moreover, as Ets-1 is a known target of miR-326, we also demonstrate that miR-326 targets Ets-1 in transfected cells as well as miRNA treated lung sections. Further, to dissect the interplay between TGF-β1, miR-326 and Ets-1, we performed a sequential knockdown of Ets-1 protein using miR-326, Ets-1 siRNA or both. This suggested that while miR-326 can downregulate TGF-β1 and Ets-1 individually, siRNA-mediated knockdown of Ets-1 showed a synergistic effect with miR-326 in downregulating TGF-β1 expression. This was an interesting finding and further confirmed that an overexpression of miR-326 alleviates the features of fibrosis by simultaneous effects on both Ets-1 and TGF-β1 expression.

In a nutshell, our results suggest for the first time that TGF-β1 expression is regulated by miR-326 and an increased expression of this microRNA in mouse lungs targets several fibrosis-specific genes, thus resulting in the attenuation of bleomycin-induced lung fibrosis. Overall, our data suggest that miR-326 is a central mediator in the pathogenesis of lung fibrosis and a potential target for developing novel therapeutics in treating fibrotic diseases, including human IPF.
6.2. CONCLUSIONS

- TGF-β is regulated at the post-transcriptional level by hsa-miR-326 and hsa-miR-609.
- miR-326 and miR-609 are inversely correlated with TGF-β in multiple human cell lines. miR-326 physiologically represses TGF-β1 expression in epithelial cells, and this effect is reversible under stimulatory conditions.
- Increase in TGF-β1 expression during the progression of bleomycin induced pulmonary fibrosis is associated with loss of miR-326.
- Intranasal administration of miR-326 mimics was sufficient to inhibit TGF-β1 expression and attenuate the fibrotic process.
- Human IPF lung specimens had markedly diminished miR-326 expression compared to non-fibrotic control lungs.
- Ectopic expression of miR-326 mimics inhibits TGF-β production and epithelial-mesenchymal transition (EMT) in cultured human lung cells.
- Molecular modelling identified additional miR-326 target genes involved in TGF-β signalling and fibrosis-related pathways.
- miR-326 downregulates pro-fibrotic genes, such as Ets1, Smad3 and MMP-9, while upregulating anti-fibrotic genes, such as Smad7.

In summary, our results suggest for the first time that miR-326 plays a key role in regulating TGF-β1 expression as well as other pro-fibrotic genes and could be useful in developing better therapeutic strategies for alleviating pulmonary fibrosis.