REGULATION OF ENDOTHELIAL–TO-MESENCHYMAL TRANSITION BY MICRORNAS IN CHRONIC ALLOGRAFT DYSFUNCTION

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ABBREVIATIONS PAGE

3’UTR, 3 prime untranslated region of mRNA

5’UTR, 5 prime untranslated region of mRNA

αSMA, α smooth muscle actin

CAD, chronic allograft dysfunction

CD31, platelet endothelial cell adhesion molecule (PECAM1)

CTGF, connective tissue growth factor

DPP-4, dipeptidyl peptidase 4

EndMT, endothelial-to-mesenchymal transition

eNOS, endothelial nitric oxide synthase

EPC, bone marrow derived endothelial progenitor cells

ER stress, endoplasmic reticular stress

ERK, extracellular signal regulated kinase

ET-1, Endothelin-1

FGF, fibroblast growth factor

FGFR, fibroblast growth factor receptor

FRS2α, fibroblast growth factor receptor subunit 2α

GFR, glomerular filtration rate

GRB2, growth factor receptor bound protein

GTP, guanosine triphosphate

HAEC, human aortic endothelial cells

HAVEC, human aortic valve endothelial cells

HCAEC, human coronary artery endothelial cells

HCV, hepatitis C virus

HIF-1α, hypoxia inducible factor-1α

HMVEC, human dermal microvascular endothelial cells

HUAEC, human umbilical artery endothelial cells

HUVEC, human umbilical vein endothelial cells

IL1β, interleukin 1β

INFY, interferon γ

KLF, krüppel-like factor

MALAT, metastasis associated lung adenocarcinoma transcript 1

MEEC, mouse embryonic endothelial cells

miRNA, mature microRNA

MMP, matrix metalloproteinase

mRNA, messenger RNA

NECD, notch extracellular domain

NICD, notch intracellular domain

NOX, NADPH oxidases

PAI-1, plasminogen activator inhibitor-1, also called CD31

p-Akt, phosphorylated Akt

PI3K, phosphoinositide 3-kinase

Pri-miRNA, primary transcript of microRNA

PTEN, phosphatase and tensin homolog

RNA, ribonucleic acid

HPEC, human pulmonary endothelial cells

ROCK, rho-associated protein kinase

ROS, reactive oxygen species

SARA, Smad anchor for receptor activation

TAC, transverse aortic constriction

TGFβ, transforming growth factor β

TGFβR, transforming growth factor β receptor

TNFα, tumour necrosis factor α

Abstract

Fibrosis is a universal finding in chronic allograft dysfunction and is characterized by an accumulation of extracellular matrix. The precise source of the myofibroblasts responsible for matrix deposition is not understood and pharmacological strategies for prevention or treatment of fibrosis remain limited. One source of myofibroblasts in fibrosis is EndMT, a process first described in heart development. Recently, lineage tracing of endothelial cells in mouse models allowed studies of EndMT *in vivo* and reported 27-35% of myofibroblasts involved in cardiac fibrosis and 16% of isolated fibroblasts in bleomycin-induced pulmonary fibrosis to be of endothelial origin.

Over the last decade, microRNAs have increasingly been described as key regulators of biological processes through repression or degradation of targeted mRNA. The stability and abundance of microRNAs in body fluids makes them attractive as potential biomarkers and progress is being made in developing microRNA targeted therapeutics.

In this review we will discuss evidence of microRNA regulation of EndMT from *in vitro* and *in vivo* studies and the potential relevance of this to heart, lung and kidney allograft dysfunction.

Introduction

Organ transplantation is the gold standard and in many cases the only treatment for patients with end-stage organ failure.[1] A limitation to successful transplantation is CAD where one of the processes leading failure of the transplanted organ is fibrosis.[2-4] Increasing graft survival remains a challenge; over the last two decades there have been substantial improvements in short-term outcomes after transplantation, but a reduction in late graft failure has been harder to achieve.[1,4,5]

Although fibrosis is common, fundamental questions about the mechanisms of fibrosis remain, including the origins of matrix producing myofibroblasts. EndMT is the process by which endothelial cells undergo a phenotypic change to become more like mesenchymal cells. The transition may be partial and reversible but in undergoing EndMT endothelial cells loose markers such as CD31 and VE-cadherin and gain mesenchymal ones including αSMA. Cell adhesion properties are lost and morphology changes from a closely abutting cobblestone pattern to elongated cells with increased potential for migration, therefore resembling myofibroblasts.[6-9]

MicroRNAs have been shown to be regulators of many cellular processes[10] and the purpose of this review is to examine the current evidence on their regulation of endothelial cell phenotype in fibrosis of heart, lung and kidney. Better understanding of this process could lead to new therapies to reduce fibrosis in allografts and therefore increase graft survival.

Origin of myofibroblasts

Myofibroblasts have been shown to be the key effector cells in fibrosis and whilst it is agreed they have multiple sources, their exact origins remain unclear and may vary with tissue type.[6,7,9,11-13] The current candidates are shown in Figure 1.

Using endothelial cell lineage tracing and double-labelling techniques in mouse models of cardiac and renal fibrosis, approximately 30% of all fibroblasts were found to be of endothelial origin.[14] Conversely, lineage tracing in an ischaemic model of cardiac fibrosis found <1% of myofibroblasts to be of endothelial origin.[15] This discrepancy may relate to the models of fibrosis and markers used.

Studies in pulmonary fibrosis models have provided further evidence of EndMT; co-localisation of αSMA with an endothelial marker has been demonstrated[16,17] and lineage tracing in bleomycin-induced pulmonary fibrosis identified 16% of isolated fibroblasts to be of endothelial origin.[18]

Staining on human allografts undergoing rejection have also provided evidence for the process of EndMT in these tissues. Compared to healthy controls, kidneys from patients diagnosed with CAD show increased expression of mesenchymal markers and a decreased expression of endothelial markers. Furthermore, double-staining found colocalisation of endothelial and mesenchymal markers in the CAD group providing further support for the role of EndMT in renal CAD.[19]

Similarly, in tissue from human cardiac allografts that had undergone rejection, the presence of EndMT has been suggested by a loss of FGFR1 staining and a gain in phosphorylated Smad2 when compared to controls.[20] Furthermore, in luminal endothelial cells colocalisation of CD31 with mesenchymal marker Notch3 was significantly greater in human coronary arteries from chronically rejecting heart allografts (evident in 80% of cases) when compared to normal human coronary arteries.[21]

Mechanism of EndMT in fibrosis

The cell signalling mechanisms that control EndMT are covered in detail elsewhere.[7,9] In brief, TGFβ signalling is the most clearly defined but several other pathways have been shown to be involved, a summary of which is provided in Figure 2.

Downstream of TGFβ signalling the Smad proteins are key effectors of changes in gene expression along with other transcriptional regulators such as Snail, Twist and Slug.[7] SARA gives Smads access to TGFβ to enable phosphorylation of Smad2 and Smad3 which then join Smad4 to alter gene expression.[22]

A parallel pathway downstream of TGFβ involves GRB2. GRB2 is bound to the TGFβ receptor and converts Ras to its active GTP-bound form that affects ROCK and RhoA-GTP activity downstream to modulate gene expression.[23]

Other pathways have also been found to interact with TGFβ signalling. ET-1 acts synergistically with TGFβ but cannot induce EndMT alone whereas PAI-1 inhibits TGFβ signalling.[24-26] There is a reciprocal inhibitory interaction between FGF signalling (via FGFR1 and its intracellular subunit FRS2α) and TGFβ signalling. Inflammatory cytokines IL1β, TNFα and INFϒ promote TGFβ indirectly through inhibition of FGF signalling.[9,20,21,27]

The Mitogen Activated Protein Kinase/ERK branch of the signalling cascade has been shown to be modulated during EndMT but may not be an essential element.[28] PI3K activity is affected by fibrotic stimuli but the response seen in EndMT is not consistent between studies and the impact of phosphorylated Akt levels on gene expression is not fully understood.[7,9,29-31]

Notch signalling, ER stress, ROS and hypoxia have also been found to impact on EndMT . The Notch signalling cascade has increased activity in EndMT with NICD separating from NECD to affect gene expression directly or via the transcription factor Hey-1.[32] ER stress causes a reduction in hydrogen sulfide (H_2S) to affect gene expression.[33] ROS are generated from increased NOX and increase NFκB availability to alter gene expression.[34] Hypoxia activates HIF-1α which in turn affects gene expression either directly or via the transcription factor Snail1 whose levels also increase downstream of TGFβ through inhibition of its proteasomal degradation.[35]

How microRNAs work

MicroRNAs are short single-stranded non-coding RNA sequences around 22 nucleotides in length that regulate protein expression at the post-transcriptional level.[10,36] Given >60% of protein coding genes are thought to be under microRNA control,[10] these RNA sequences likely play a role in regulating almost every cellular process and there is increasing evidence for their involvement in fibrosis and EndMT.[7,37]

Mature, single-stranded microRNAs are produced by processing of their double-stranded precursors (Figure 3). Nucleotides 2-8 of the miRNA are referred to as the seed region and bind the target sequence on mRNA through Watson-Crick pairing to direct the miRNA-induced silencing complex. Predominantly, it is the 3'UTR of target mRNA to which the seed region binds but less frequently target sequences have also been identified in the open reading frame and the 5'UTR of mRNA.[38,39] Infrequently miRNAs have been associated with increased protein expression; this may be through inhibition of repressors or direct stimulation of mRNA translation.[39]

Evidence of microRNA involvement in EndMT in heart, lung and kidney fibrosis

In vitro studies

EndMT has been demonstrated *in vitro* on exposure of endothelial cells to fibrotic stimuli such as TGFβ [28,40,41] high glucose concentrations,[42,43] radiation[44] or hypoxia.[35,45] MicroRNA arrays have been used to screen for differentially expressed miRNAs in the presence or absence of fibrotic stimuli.[28,42,45] A summary of miRNAs whose levels have been found to be affected in EndMT is shown in Table 1.

A role in activating EndMT has been suggested for certain miRNAs as blocking them is effective in preventing the process. MiR-21 is expressed at higher levels in human cardiac fibroblasts than in endothelial cells and is upregulated in response to TGFβ stimulation in HUVEC.[34,46] MiR-21 blockade partly rescues endothelial marker expression that was suppressed by TGFβ in these cells and prevents changes in gene expression associated with EndMT.[46] Furthermore, exposure to miR-21 directly stimulated EndMT in the absence of TGFβ. In HPECs however, although miR-21 was upregulated it was found to be dispensable in EndMT occurring in response to radiation as its inhibition did not affect this process.[44] In human lung fibroblasts miR-21 expression increased and a miR-21 antagomir inhibited expression of collagen type IIIA supporting involvement in fibrosis although not necessarily in EndMT.[44]

Upregulating other miRNAs can prevent EndMT indicating that they function as inhibitors of this process with their downregulation being required to allow EndMT. For instance, in high glucose conditions, miR-18a-5p[47], miR-200b[43] and miR-320[42] are downregulated and their upregulation appears to protect against EndMT and maintain normal endothelial function

In the case of miR-200a, which has been shown to oppose EndMT, overexpression can increase endothelial marker expression above baseline in the absence of TGFβ.[23] Other miRNAs appear to have a more purely regulatory role rather than being capable of stimulating a change.[22]

Serum from patients with fibrotic disease has been shown to modulate miRNA levels. Incubating HUVEC with serum from patients with Kawasaki disease was associated with lower expression of miR-483 when compared with cells incubated with healthy sera. Overexpression of miR-483 attenuated the EndMT response to diseased sera.[48]

Certain upregulated miRNAs may play a role in negative feedback within the signalling pathway for EndMT. Although miR-155 is upregulated under fibrotic conditions, TGFβ stimulation and hypoxia, overexpression with pre-miR-155 blocks the ability of TGFβ to induce EndMT in mouse embryonic endothelial cells. In support of an inhibitory role, antagomirs to miR-155 were able to induce αSMA expression in the absence of TGFβ.[45]

Pre-clinical models

As with *in vitro* studies, microRNA array data on animal tissue has also demonstrated differential expression of certain miRNAs between organs that have undergone fibrosis in response to transplantation, radiation,[44] chronic hypoxia[31] or diabetes[27] and control uninjured tissues. Although this does not necessarily indicate involvement in the process of EndMT, it can help to focus the direction of further studies.[27,31,44] Table 2 shows changes in miRNA expression identified in different models of fibrosis.

Changes in expression observed *in vitro* do not consistently translate to *in vivo* models (Table 1). Although TGFβ treatment was associated with downregulation of miR-29b and upregulation of miR-216a in HUVEC, these changes were not seen in endothelial cells sorted from hearts of TAC operated mice when compared to sham-operated controls. There was, however, evidence of EndMT occurring in these hearts.[46]

Involvement of miRNA in the process of EndMT *in vivo* has been suggested through correlation between expression of specific miRNAs, extent of EndMT and amount of fibrosis in the studied tissues. For instance, the CD1 strain of mice experience more renal fibrosis in response to diabetes than 129Sv mice. The mechanism of fibrosis in CD1 mice is at least partially driven by EndMT as demonstrated by increased colocalisation of endothelial and mesenchymal markers in diabetic kidneys vs controls. This pattern was seen to a much lesser extent in 129Sv mice. Interestingly, in CD1 mice but not in 129Sv mice, diabetes was associated with suppression of let-7 and miR-29 in the kidney.[27]

Consistent with the finding that miR-21 promotes EndMT *in vitro*[46], miR-21 levels were found to be increased compared to controls in lung tissue from a mouse model of radiation-induced pulmonary fibrosis. In situ hybridisation showed this increase to be specific to radiation-damaged areas of lung. As the presence of EndMT *in vivo* was not investigated, a direct effect of miRNA on EndMT could not be demonstrated.[44]

The impact of specific miRNAs on the process of EndMT has been investigated *in vivo* through manipulation of their levels. An inhibitory effect of let-7 on EndMT was supported by the increased expression of mesenchymal markers in endothelial cells,

associated with administering let-7 antagomirs in adult mice.[21] This role was further examined using a transplant model of aorta allograft in transgenic mice that allow fate mapping of endothelial cells by labelling with green fluorescent protein. Tissue was examined 2 weeks after transplantation, when rejection was occurring.I In mice given let-7 antagomirs the proportion of luminal endothelial cells undergoing EndMT had increased from 61% to 80-90%. Conversely, let-7 mimic reduced collagen deposition and the proportion of endothelial cells undergoing EndMT to 33.7%. Together these results support a role for miRNA in modulating EndMT.[21]

miR-133a is another inhibitor of EndMT *in vivo* as transgenic mice that upregulate miR-133a specifically in heart were shown to be partially protected from EndMT seen in diabetic cardiac fibrosis.[49] Similarly, overexpression of miR-200b in endothelial cells reduced cardiac fibrosis in diabetic mice and prevented EndMT. Interestingly, the downregulation of miR-200b seen in diabetic hearts compared to controls occurred only in endothelial cells, not cardiomyocytes, further supporting involvement in the process of EndMT.[43]

Consistent with findings in lung that miR-21 levels increase in pulmonary fibrosis,[44] Kumarswamy et al.[46] saw the same trend in hearts that had undergone TAC. Furthermore, administration of miR-21 inhibitors was shown to reduce the proportion of cells undergoing EndMT supporting a function of miR-21 in activating EndMT.[46]

Human studies

Human studies on the involvement of microRNA in EndMT during fibrosis of heart, lung and kidney remain sparse. EndMT has been shown to occur in heart and kidney CAD, as discussed earlier, but these studies did not investigate changes in

miRNA.[20,21] Others have found differential expression of miRNAs in human fibrotic disease but not identified a direct link with EndMT.[31,48]

MiRNA levels have been shown to correlate with disease states. A strong correlation was shown between miR-126a-5p serum levels and severity of disease in neonates with pulmonary hypertension.[31] Likewise, miR-21[50,51] have been demonstrated to increase with decreasing GFR in renal transplantation whereas miR-200b[51] falls. In Kawasaki disease there was an inverse correlation between serum levels of miR-483 and severity of disease. Interestingly, it seems that miR-483 comes from endothelial cells as there was also an inverse correlation between CD31-positive microparticles containing miR-483 and degree of coronary artery damage.[48] Certain miRNAs, previously shown to have a role in EndMT through *in vitro* or animal studies, have also been implicated in the process of graft rejection in humans and so it could be hypothesized that these they miRNA changes affect endothelial function.

For instance, miR-21 and miR-155 have been found to be upregulated in human cardiac allograft rejection. Admittedly, in this study the focus was on acute rejection with biopsies taken within a year of transplantation. Also in the case of miR-21 the upregulation was only significant when comparing tissue showing severe rejection with control biopsies taken from native heart to investigate unexplained ventricular tachyarrhythmias rather than non-rejecting transplants. However, miR-155 was significantly upregulated in rejecting vs non-rejecting cardiac transplant biopsies and levels were found to parallel rejection severity.[52]

miR-21 has also been implicated in lung allograft rejection as although it was not detected in normal human lung it was shown to be strongly expressed in chronic rejection, particularly in fibroblasts and epithelial cells in affected areas.[53]

In kidney transplantation, of the miRNAs already discussed as playing a role in EndMT, it is miR-21, miR-200b and miR-155 for which there is most evidence to support a function in kidney CAD. Analysis of urine samples from living donor renal transplant recipients with proven rejection on biopsy showed increased levels of miR-21 and decreased levels of miR-200b when compared with healthy controls. However, in the case of miR-21 this difference did not maintain significance when those with rejection were compared to those with stable graft function,[50,51] which is similar to the picture seen in heart.[52]

In keeping with previous findings of miR-155 being upregulated in EndMT,[41,45] levels in blood samples were significantly higher in patients with renal allograft rejection compared to those with stable graft function or healthy controls.[50]

How MicroRNAs regulate EndMT in fibrosis

Modulating expression of specific miRNAs can impact on EndMT and has been used to investigate their function. Current knowledge on the ways in which miRNAs interact with the pathways regulating EndMT in fibrosis are summarized in Figure 2 with more detail provided in Table 3. Various mechanisms of action have been implicated and miRNAs may be effective at modulating EndMT only within a precise time window. For instance, miR-20a mimics reduced the number of cells that entered EndMT *in vitro* but had no effect on cells that had already started the program.[22]

A positive feedback loop has been demonstrated between miR-29, let-7 and FGFR1 which functions to inhibit TGFβ signaling.[21] Another example of a positive feedback loop is found in the interaction between miR-130a and NFκB. miR-130a is upregulated in a NFκB dependent fashion in both fibrotic mouse lungs and cells treated with TGFβ1 in vitro. MiR-130a mimics were found to increase NFκB-linked

luciferase activity whereas antagomirs decreased it indicating a function in promoting NFκB expression. As miRNAs primarily inhibit gene expression it is possible that there are other steps downstream of miR-130a leading to promotion of NFκB expression.[54]

In the case of miR-145, a reciprocal inhibitory interaction exists with the long non-coding RNA MALAT1 which is upregulated downstream of TGFβ and acts to reduce production of mature miR-145.[40]

For other miRNAs there is more conflicting data on their modes of action. miR-155 appears to function as a negative regulator of EndMT as it is upregulated by fibrotic stimuli but inhibits EndMT when overexpressed and prevents the activation of RhoA downstream of TGFβ when studied in mouse embryonic endothelial cells.[41,45] Furthermore, miR-155 has been found to directly target TGFβ inhibitor c-Ski mRNA to inhibit its expression and so disinhibit TGFβ signaling, consistent with inhibiting EndMT.[41] However, this may be a tissue specific role as it is not in keeping with findings in human and mouse heart transplantation where, although miR-155 was similarly upregulated with fibrosis, graft survival was increased in miR-155 knockout mice so supporting a function of miR-155 to promote fibrosis.[52] *In vitro* work on human coronary artery endothelial cells found miR-155 to promote EndMT as the addition of its inhibitor hindered TGF-β induced changes in markers.[41]

Studies differ in their findings of whether PI3K activity is increased or decreased in EndMT but miRNAs have been shown to interact with this level of the pathway nonetheless.[29,31,46] Kumarswamy et al.,[46] suggest that the proportion of p-Akt is increased on activation of TGFβ signaling through direct downregulation of the inhibitor PTEN by miR-21. Treatment of cells with pre-miR-21 alone was able to

induce the gene expression changes seen in EndMT of CD31 and eNOS downregulation and MMP2 and MMP9 upregulation.[46] In work by Xu et al.,[31] the proportion of p-Akt in endothelial cells was reduced in hypoxia but could be rescued by inhibition of miR-126a-5p which is upregulated under such conditions. However, the targets of this miRNA were not elucidated in this study as levels of PI3K remained stable and although miR-126a-5p shares 8 complementary bases with the TGFβ2 gene, again it was unable to significantly affect expression.[31] Zhang et al.,[29] similarly found PI3K activity to be reduced in response to TGFβ1 and attributed this to an increase in inhibition from PI3KR2. The reduction in miR-126 seen in these conditions was thought to be the effector for this change as PI3KR2 was found to be one of its direct targets. Furthermore, overexpressing miR-126 prevented this reduction in the proportion of p-Akt associated with TGFβ1 treatment.[29]

Clinical relevance

Some studies have demonstrated that existing treatments for diseases associated with fibrosis influence miRNA expression and so inhibit EndMT.[48,55,56] Specifically, atorvastatin was shown to act on the KLF4-miR-483-CTGF axis in Kawasaki's Disease[48] and the downregulation of miR-29 and let-7 observed in the diabetic mouse kidney was prevented by linagliptin[56] and imidapril[55] respectively. However, this body of research does not extend into the realms of allograft rejection.

MiRNA levels can be manipulated *in vivo*[21,46] to effectively alter a disease process. Expression can be increased with synthetic miRNA mimics[57] or vectors carrying a pre-miRNA sequence.[58] miRNA blockade is achieved with antagomirs (chemically modified antisense oligonucleotide sequences to a particular miRNA), miRNA sponge[59] to competitively bind miRNAs or miRNA mask[60] to competitively bind the target sequence. Promising progress is being made in the area of HCV where a miR-122 inhibitor (Miravirsen) effectively reduced HCV RNA levels beyond the duration of treatment in a phase II clinical trial, without developing viral resistance.[61] Drugs targeting miRNAs to treat cancer have also reached clinical development.[62]

Aside from therapeutics, understanding changes in miRNA levels could contribute to clinical care through diagnosis and monitoring of disease. At this stage, only a handful of papers discuss the potential for using miRNAs as biomarkers in CAD[37,63-69] but there is more work on acute allograft rejection. The stable nature of miRNAs make them an attrctive target to investigate as biomarkers. So far this quality has facilitated their study in various samples including tissue, urine, serum and plasma.[69]

It is worth considering also the implications of losing endothelial cell phenotype through EndMT, rather than purely the gain of mesenchymal features as contributing

to the disease process. A logical consequence of endothelial cells losing their phenotype in this way would be a reduction in the density of capillaries. Particularly in the kidney, capillary rarefaction is one of the main features of progressive fibrotic disease[70,71] in both native and transplanted organs. In targeting EndMT it may therefore be possible to reduce both gain of fibrotic tissue and loss of capillaries in order to improve organ function.

Concluding remarks and future perspectives

The predominant preclinical model used for fibrosis is diabetes (Table 2) and more is required at the preclinical level to specifically look at the process of CAD. Although levels of some miRNAs have been shown to be altered in serum,[48] other studies have suggested an autocrine or paracrine action.[43] If local mechanisms of action do predominate, a more invasive approach to human studies would be required using tissue samples rather than serum to detect changes.

Given the dearth of research into microRNA regulation of fibrosis in human CAD a key next step would be to investigate whether findings from animal models reliably translate into humans. As summarized in Tables 1 and 2 there is some variability in miRNA expression changes between *in vitro* and *in vivo* models depending on cell types used; particularly in the case of the let-7 which is perhaps the most widely studied miRNA in this field. It is therefore essential to correlate findings from preclinical models to those in human disease. Ultimately the goal should be for a better understanding of miRNA in fibrosis to enable early detection and treatment of CAD so improving transplant survival and patient quality of life.

References

1. Annual Report of the U.S. Organ Procurement and Transplantation Network and the Scientific Registry of Transplant Recipients: Transplant Data. In. Rockville, MD: U.S. Department of Health and Human Services, Health Resources and Services Administration, Healthcare Systems Bureau, Division of Transplantation; 2012.
2. Wilkes DS. Chronic lung allograft rejection and airway microvasculature: is HIF-1 the missing link? *J Clin Invest.* 2011;121(6):2155-2157.
3. Stegall MD, Cornell LD, Park WD, Smith BH, Cosio FG. Renal Allograft Histology at 10 Years After Transplantation in the Tacrolimus Era: Evidence of Pervasive Chronic Injury. *Am J Transplant.* 2017.
4. Studer SM, Levy RD, McNeil K, Orens JB. Lung transplant outcomes: a review of survival, graft function, physiology, health-related quality of life and cost-effectiveness. *Eur Respir J.* 2004;24(4):674-685.
5. Lodhi SA, Lamb KE, Meier-Kriesche HU. Solid organ allograft survival improvement in the United States: the long-term does not mirror the dramatic short-term success. *Am J Transplant.* 2011;11(6):1226-1235.
6. Yazdani S, Bansal R, Prakash J. Drug targeting to myofibroblasts: Implications for fibrosis and cancer. *Adv Drug Deliv Rev.* 2017.
7. Piera-Velazquez S, Mendoza FA, Jimenez SA. Endothelial to Mesenchymal Transition (EndoMT) in the Pathogenesis of Human Fibrotic Diseases. *J Clin Med.* 2016;5(4).
8. Medici D, Kalluri R. Endothelial-mesenchymal transition and its contribution to the emergence of stem cell phenotype. *Semin Cancer Biol.* 2012;22(5-6):379-384.

9. Perez L, Munoz-Durango N, Riedel CA, et al. Endothelial-to-mesenchymal transition: Cytokine-mediated pathways that determine endothelial fibrosis under inflammatory conditions. *Cytokine Growth Factor Rev.* 2017;33:41-54.
10. Friedman RC, Farh KK, Burge CB, Bartel DP. Most mammalian mRNAs are conserved targets of microRNAs. *Genome Res.* 2009;19(1):92-105.
11. Krenning G, Zeisberg EM, Kalluri R. The origin of fibroblasts and mechanism of cardiac fibrosis. *J Cell Physiol.* 2010;225(3):631-637.
12. LeBleu VS, Taduri G, O'Connell J, et al. Origin and function of myofibroblasts in kidney fibrosis. *Nat Med.* 2013;19(8):1047-1053.
13. Stempien-Otero A, Kim DH, Davis J. Molecular networks underlying myofibroblast fate and fibrosis. *J Mol Cell Cardiol.* 2016;97:153-161.
14. Zeisberg EM, Tarnavski O, Zeisberg M, et al. Endothelial-to-mesenchymal transition contributes to cardiac fibrosis. *Nat Med.* 2007;13(8):952-961.
15. Kanisicak O, Khalil H, Ivey MJ, et al. Genetic lineage tracing defines myofibroblast origin and function in the injured heart. *Nat Commun.* 2016;7:12260.
16. Almudever P, Milara J, De Diego A, et al. Role of tetrahydrobiopterin in pulmonary vascular remodelling associated with pulmonary fibrosis. *Thorax.* 2013;68(10):938-948.
17. Choi SH, Hong ZY, Nam JK, et al. A Hypoxia-Induced Vascular Endothelial-to-Mesenchymal Transition in Development of Radiation-Induced Pulmonary Fibrosis. *Clin Cancer Res.* 2015;21(16):3716-3726.
18. Hashimoto N, Phan SH, Imaizumi K, et al. Endothelial-mesenchymal transition in bleomycin-induced pulmonary fibrosis. *Am J Respir Cell Mol Biol.* 2010;43(2):161-172.

19. Wang Z, Han Z, Tao J, et al. Role of endothelial-to-mesenchymal transition induced by TGF-beta1 in transplant kidney interstitial fibrosis. *J Cell Mol Med.* 2017;21(10):2359-2369.

20. Chen PY, Qin L, Tellides G, Simons M. Fibroblast growth factor receptor 1 is a key inhibitor of TGFbeta signaling in the endothelium. *Sci Signal.* 2014;7(344):ra90.

21. Chen PY, Qin L, Barnes C, et al. FGF regulates TGF-beta signaling and endothelial-to-mesenchymal transition via control of let-7 miRNA expression. *Cell Rep.* 2012;2(6):1684-1696.

22. Correia AC, Moonen JR, Brinker MG, Krenning G. FGF2 inhibits endothelial-mesenchymal transition through microRNA-20a-mediated repression of canonical TGF-beta signaling. *J Cell Sci.* 2016;129(3):569-579.

23. Zhang H, Hu J, Liu L. MiR-200a modulates TGF-beta1-induced endothelial-to-mesenchymal shift via suppression of GRB2 in HAECs. *Biomed Pharmacother.* 2017;95:215-222.

24. Widyantoro B, Emoto N, Nakayama K, et al. Endothelial cell-derived endothelin-1 promotes cardiac fibrosis in diabetic hearts through stimulation of endothelial-to-mesenchymal transition. *Circulation.* 2010;121(22):2407-2418.

25. Wermuth PJ, Li Z, Mendoza FA, Jimenez SA. Stimulation of Transforming Growth Factor-beta1-Induced Endothelial-To-Mesenchymal Transition and Tissue Fibrosis by Endothelin-1 (ET-1): A Novel Profibrotic Effect of ET-1. *PLoS One.* 2016;11(9):e0161988.

26. Ghosh AK, Bradham WS, Gleaves LA, et al. Genetic deficiency of plasminogen activator inhibitor-1 promotes cardiac fibrosis in aged mice:

involvement of constitutive transforming growth factor-beta signaling and endothelial-to-mesenchymal transition. *Circulation.* 2010;122(12):1200-1209.

27. Srivastava SP, Shi S, Kanasaki M, et al. Effect of Antifibrotic MicroRNAs Crosstalk on the Action of N-acetyl-seryl-aspartyl-lysyl-proline in Diabetes-related Kidney Fibrosis. *Sci Rep.* 2016;6:29884.
28. Ghosh AK, Nagpal V, Covington JW, Michaels MA, Vaughan DE. Molecular basis of cardiac endothelial-to-mesenchymal transition (EndMT): differential expression of microRNAs during EndMT. *Cell Signal.* 2012;24(5):1031-1036.
29. Zhang J, Zhang Z, Zhang DY, Zhu J, Zhang T, Wang C. microRNA 126 inhibits the transition of endothelial progenitor cells to mesenchymal cells via the PIK3R2-PI3K/Akt signalling pathway. *PLoS One.* 2013;8(12):e83294.
30. Li Z, Jimenez SA. Protein kinase Cdelta and c-Abl kinase are required for transforming growth factor beta induction of endothelial-mesenchymal transition in vitro. *Arthritis Rheum.* 2011;63(8):2473-2483.
31. Xu YP, He Q, Shen Z, et al. MiR-126a-5p is involved in the hypoxia-induced endothelial-to-mesenchymal transition of neonatal pulmonary hypertension. *Hypertens Res.* 2017;40(6):552-561.
32. Zhao Y, Qiao X, Tan TK, et al. Matrix metalloproteinase 9-dependent Notch signaling contributes to kidney fibrosis through peritubular endothelial-mesenchymal transition. *Nephrol Dial Transplant.* 2017;32(5):781-791.
33. Ying R, Wang XQ, Yang Y, et al. Hydrogen sulfide suppresses endoplasmic reticulum stress-induced endothelial-to-mesenchymal transition through Src pathway. *Life Sci.* 2016;144:208-217.

34. Guo Y, Li P, Bledsoe G, Yang ZR, Chao L, Chao J. Kallistatin inhibits TGF-beta-induced endothelial-mesenchymal transition by differential regulation of microRNA-21 and eNOS expression. *Exp Cell Res.* 2015;337(1):103-110.

35. Xu X, Tan X, Hulshoff MS, Wilhelmi T, Zeisberg M, Zeisberg EM. Hypoxia-induced endothelial-mesenchymal transition is associated with RASAL1 promoter hypermethylation in human coronary endothelial cells. *FEBS Lett.* 2016;590(8):1222-1233.

36. Guo H, Ingolia NT, Weissman JS, Bartel DP. Mammalian microRNAs predominantly act to decrease target mRNA levels. *Nature.* 2010;466(7308):835-840.

37. Bruneau S, Wedel J, Fakhouri F, et al. Translational implications of endothelial cell dysfunction in association with chronic allograft rejection. *Pediatr Nephrol.* 2016;31(1):41-51.

38. Bartel DP. MicroRNAs: target recognition and regulatory functions. *Cell.* 2009;136(2):215-233.

39. Fabian MR, Sonenberg N, Filipowicz W. Regulation of mRNA translation and stability by microRNAs. *Annu Rev Biochem.* 2010;79:351-379.

40. Xiang Y, Zhang Y, Tang Y, Li Q. MALAT1 Modulates TGF-beta1-Induced Endothelial-to-Mesenchymal Transition through Downregulation of miR-145. *Cell Physiol Biochem.* 2017;42(1):357-372.

41. Wang J, He W, Xu X, et al. The mechanism of TGF-beta/miR-155/c-Skiregulates endothelial-mesenchymal transition in human coronary artery endothelial cells. *Biosci Rep.* 2017.

42. Feng B, Chakrabarti S. miR-320 Regulates Glucose-Induced Gene Expression in Diabetes. *ISRN Endocrinol.* 2012;2012:549875.

43. Feng B, Cao Y, Chen S, Chu X, Chu Y, Chakrabarti S. miR-200b Mediates Endothelial-to-Mesenchymal Transition in Diabetic Cardiomyopathy. *Diabetes.* 2016;65(3):768-779.

44. Kwon OS, Kim KT, Lee E, et al. Induction of MiR-21 by Stereotactic Body Radiotherapy Contributes to the Pulmonary Fibrotic Response. *PLoS One.* 2016;11(5):e0154942.

45. Bijkerk R, de Bruin RG, van Solingen C, et al. MicroRNA-155 functions as a negative regulator of RhoA signaling in TGF-beta-induced endothelial to mesenchymal transition. *Microrna.* 2012;1(1):2-10.

46. Kumarswamy R, Volkmann I, Jazbutyte V, Dangwal S, Park DH, Thum T. Transforming growth factor-beta-induced endothelial-to-mesenchymal transition is partly mediated by microRNA-21. *Arterioscler Thromb Vasc Biol.* 2012;32(2):361-369.

47. Geng H, Guan J. MiR-18a-5p inhibits endothelial-mesenchymal transition and cardiac fibrosis through the Notch2 pathway. *Biochem Biophys Res Commun.* 2017.

48. He M, Chen Z, Martin M, et al. miR-483 Targeting of CTGF Suppresses Endothelial-to-Mesenchymal Transition: Therapeutic Implications in Kawasaki Disease. *Circ Res.* 2017;120(2):354-365.

49. Chen S, Puthanveetil P, Feng B, Matkovich SJ, Dorn GW, 2nd, Chakrabarti S. Cardiac miR-133a overexpression prevents early cardiac fibrosis in diabetes. *J Cell Mol Med.* 2014;18(3):415-421.

50. Zununi Vahed S, Poursadegh Zonouzi A, Ghanbarian H, et al. Differential expression of circulating miR-21, miR-142-3p and miR-155 in renal transplant

recipients with impaired graft function. *Int Urol Nephrol.* 2017;49(9):1681-1689.

51. Zununi Vahed S, Omidi Y, Ardalan M, Samadi N. Dysregulation of urinary miR-21 and miR-200b associated with interstitial fibrosis and tubular atrophy (IFTA) in renal transplant recipients. *Clin Biochem.* 2017;50(1-2):32-39.
52. Van Aelst LN, Summer G, Li S, et al. RNA Profiling in Human and Murine Transplanted Hearts: Identification and Validation of Therapeutic Targets for Acute Cardiac and Renal Allograft Rejection. *Am J Transplant.* 2016;16(1):99-110.
53. Di Carlo S, Rossi E, Politano G, et al. Identification of miRNAs Potentially Involved in Bronchiolitis Obliterans Syndrome: A Computational Study. *PLoS One.* 2016;11(8):e0161771.
54. Li L, Kim IK, Chiasson V, Chatterjee P, Gupta S. NF-kappaB mediated miR-130a modulation in lung microvascular cell remodeling: Implication in pulmonary hypertension. *Exp Cell Res.* 2017.
55. Nagai T, Kanasaki M, Srivastava SP, et al. N-acetyl-seryl-aspartyl-lysyl-proline inhibits diabetes-associated kidney fibrosis and endothelial-mesenchymal transition. *Biomed Res Int.* 2014;2014:696475.
56. Kanasaki K, Shi S, Kanasaki M, et al. Linagliptin-mediated DPP-4 inhibition ameliorates kidney fibrosis in streptozotocin-induced diabetic mice by inhibiting endothelial-to-mesenchymal transition in a therapeutic regimen. *Diabetes.* 2014;63(6):2120-2131.
57. Wang Z. The guideline of the design and validation of MiRNA mimics. *Methods Mol Biol.* 2011;676:211-223.

58. Meng L, Liu C, Lu J, et al. Small RNA zippers lock miRNA molecules and block miRNA function in mammalian cells. *Nat Commun.* 2017;8:13964.

59. Ebert MS, Neilson JR, Sharp PA. MicroRNA sponges: competitive inhibitors of small RNAs in mammalian cells. *Nat Methods.* 2007;4(9):721-726.

60. Wang Z. The principles of MiRNA-masking antisense oligonucleotides technology. *Methods Mol Biol.* 2011;676:43-49.

61. Janssen HLA, Reesink HW, Lawitz EJ, et al. Treatment of HCV Infection by Targeting MicroRNA. *New England Journal of Medicine.* 2013;368(18):1685-1694.

62. Rupaimoole R, Slack FJ. MicroRNA therapeutics: towards a new era for the management of cancer and other diseases. *Nat Rev Drug Discov.* 2017;16(3):203-222.

63. Iwasaki K, Yamamoto T, Inanaga Y, et al. MiR-142-5p and miR-486-5p as biomarkers for early detection of chronic antibody-mediated rejection in kidney transplantation. *Biomarkers.* 2017;22(1):45-54.

64. Xu Z, Ramachandran S, Gunasekaran M, et al. MicroRNA-144 dysregulates the transforming growth factor-beta signaling cascade and contributes to the development of bronchiolitis obliterans syndrome after human lung transplantation. *J Heart Lung Transplant.* 2015;34(9):1154-1162.

65. Scian MJ, Maluf DG, David KG, et al. MicroRNA profiles in allograft tissues and paired urines associate with chronic allograft dysfunction with IF/TA. *Am J Transplant.* 2011;11(10):2110-2122.

66. Mas VR, Le TH, Maluf DG. Epigenetics in Kidney Transplantation: Current Evidence, Predictions, and Future Research Directions. *Transplantation.* 2016;100(1):23-38.

67. Mas VR, Dumur CI, Scian MJ, Gehrau RC, Maluf DG. MicroRNAs as biomarkers in solid organ transplantation. *Am J Transplant.* 2013;13(1):11-19.

68. Li JY, Yong TY, Michael MZ, Gleadle JM. Review: The role of microRNAs in kidney disease. *Nephrology (Carlton).* 2010;15(6):599-608.

69. Hamdorf M, Kawakita S, Everly M. The Potential of MicroRNAs as Novel Biomarkers for Transplant Rejection. *J Immunol Res.* 2017;2017:4072364.

70. Afsar B, Afsar RE, Dagel T, et al. Capillary rarefaction from the kidney point of view. *Clinical Kidney Journal.* 2017:sfx133-sfx133.

71. Kramann R, Dirocco DP, Maarouf OH, Humphreys BD. Matrix Producing Cells in Chronic Kidney Disease: Origin, Regulation, and Activation. *Curr Pathobiol Rep.* 2013;1(4).

72. Zeisberg EM, Potenta SE, Sugimoto H, Zeisberg M, Kalluri R. Fibroblasts in kidney fibrosis emerge via endothelial-to-mesenchymal transition. *Journal of the American Society of Nephrology : JASN.* 2008;19(12):2282-2287.

73. Kato T, Sekiguchi A, Sagara K, et al. Endothelial-mesenchymal transition in human atrial fibrillation. *J Cardiol.* 2017;69(5):706-711.

74. Willis BC, duBois RM, Borok Z. Epithelial origin of myofibroblasts during fibrosis in the lung. *Proc Am Thorac Soc.* 2006;3(4):377-382.

75. Chen B, You WJ, Liu XQ, Xue S, Qin H, Jiang HD. Chronic microaspiration of bile acids induces lung fibrosis through multiple mechanisms in rats. *Clin Sci (Lond).* 2017;131(10):951-963.

76. Humphreys BD, Lin SL, Kobayashi A, et al. Fate tracing reveals the pericyte and not epithelial origin of myofibroblasts in kidney fibrosis. *Am J Pathol.* 2010;176(1):85-97.

77. Ladak SS, Ward C, Ali S. The potential role of microRNAs in lung allograft rejection. *J Heart Lung Transplant.* 2016;35(5):550-559.

78. Massagué J, Chen Y-G. Controlling TGF-β signaling. *Genes & Development.* 2000;14(6):627-644.

79. Srivastava SP, Koya D, Kanasaki K. MicroRNAs in kidney fibrosis and diabetic nephropathy: roles on EMT and EndMT. *Biomed Res Int.* 2013;2013:125469.

80. Bartel DP. MicroRNAs: Genomics, Biogenesis, Mechanism, and Function. *Cell.* 2004;116:281-297.

81. Servier. Servier Medical Art. Medical images. Available at: http://smart.servier.com/, 2017.

MiRNAs	Upregulated	Downregulated
let-7	Mouse cardiac endothelial cells[28]	Mouse transplanted aorta[21] Mouse diabetic kidney[55] HUVEC[21] HUAEC[21] HMEC[27]
18a-5p		HAVEC[47] Mouse diabetic heart[47]
20a		HUVEC[22]
21	Irradiated mouse lung[44] Human pulmonary endothelial cells[44] HUVEC[34,46] Mouse cardiac endothelial cells[28] Mouse TAC operated hearts[46]	
29		HUVEC[46] HMVEC[27,56] Mouse diabetic kidney[27,56]
30b	Mouse cardiac endothelial cells[28]	
122a		Mouse cardiac endothelial cells[28]
125b	Mouse cardiac endothelial cells[28]	
126	Chronic hypoxic rat lung[31] RPMEC[31]	EPC[29]
127		Mouse cardiac endothelial cells[28]

130a	Lung microvascular endothelial cells[54]	
133a		Mouse diabetic heart[49]
155	MEEC[45], HCAEC[41]	
195	Mouse cardiac endothelial cells[28]	
196		Mouse cardiac endothelial cells[28]
200a		HAEC[23]
200b		Mouse diabetic heart [43] Primary mouse heart endothelial cells[43]
216a	HUVEC[46]	
320		HUVEC[42]
375		Mouse cardiac endothelial cells[28]
483		HUVEC[48]

Table 1: miRNAs identified to be upregulated or downregulated in EndMT in different studied cells and tissues

Fibrosis model		**MiRNAs**
Transplantation	Mouse	**Down:** Let-7[21] (aorta)
Transverse aortic constriction	Mouse	**Up:** miR-21[46] (heart)
Diabetes	Mouse	**Down:** miR-200b[43] (heart), miR-18a-5p[47] (heart), miR-133a[49], let-7[27,55] (kidney), miR-29[27,56] (kidney),
	Rat	**Down:** miR-320[42] (kidney)
Radiation	Mouse	**Up:** miR-21[44] (lung)
Monocrotaline-induced pulmonary hypertension	Rat	**Up:** miR-130a[54] (lung)
Chronic hypoxia-induced pulmonary hypertension	Rat	**Up:** miR-126a-5p[31] (lung)

Table 2 Differentially expressed miRNAs in EndMT in different *in vivo* animal models of fibrosis

Upregulated **Up**. Downregulated **Down**. Tissue studied in brackets.

MiRNA	Factors modulating miRNA levels	Actions of miRNA on pathway
FGF and TGFβ		
let-7[21,27]	Upregulated by FGF signaling which is inhibited by TNFα, IFNϒ and IL1β in fibrosis	Direct inhibition of TGFβR1 expression[21] upregulates FGFR1 and miR-29[27]
miR-20a[22]	Upregulated by FGF signaling	Inhibits TGFβ signaling: directly targets ALK5 (TGFβR1), TGFβR2 and SARA
miR-29[27]	Upregulated by FGFR1 signaling	Directly inhibits IFNϒ expression Upregulates FGFR1 and let-7
miR-133a[49]		Inhibits expression of endothelin-1 which acts synergistically with TGFβ. Prevented TGFβ-induced increased in pSmad2 and pERK1/2 expression.
miR-145[40]	TGFβ1 signaling reduces processing of pre-miR-145 into mature form by upregulating MALAT1 which inhibits DICER expression	Directly inhibits MALAT1 expression and TGFβR2 and Smad3 expression to inhibit TGFβ signaling
Ras/RhoA		

miR-200a[23]	Downregulated by TGFβ signaling	Directly inhibits growth factor receptor bound protein 2 (GRB2) expression. GRB2 is downstream of TGFβ and activates Ras to GTP-bound form.
NFκB		
miR-130a[54]	Upregulated by NFκB downstream of TGFβ	Promotes NFκB expression so part of a positive feedback loop
PI3K		
miR-21[46]		Directly inhibits PTEN, an inhibitor of PI3K, to increase PI3K activity downstream of TGFβ. Downregulated CD31 and eNOS and upregulated MMP2 and MMP9.
miR-126a-5p[31]		Prevents fall in proportion of phosphorylated Akt seen in hypoxia. Exact target is not known.
miR-126[29]	Downregulated by TGFβ1 signaling	Prevents TGFβ-induced fall in proportion of phosphorylated Akt by directly inhibiting expression of PI3KR2, a PI3K inhibitor.
DPP-4		
miR-29[56]		Directly inhibits DPP-4 expression
Acetylation		

miR-133a[49]		Prevents upregulation of the acetylating transcription coactivator P300
miR-200b[43]		Prevents upregulation of the acetylating transcription coactivator P300
Angiotensin		
Let-7[55]	Levels restored by increasing AcSDKP directly or through administration of ACE inhibitor	
miR-133a[49]		Prevents increase in angiotensinogen mRNA expression
Notch		
miR-18a-5p[47]		Directly inhibits Notch2 expression
KLF/CTGF		
miR-183[48]	Upregulated by KLF which is downregulated in inflammation	Directly inhibits CTGF (connective tissue growth factor) expression

Table 3 Actions of miRNAs on pathways implicated in regulation of EndMT

Pathways indicated by subtitles. MiRNAs that are downregulated in EndMT or fibrosis are listed in italics, those that are upregulated are in normal script. The second column lists factors and pathways that have been shown to influence miRNA expression. miRNAs affecting FGF and TGFβ signaling pathways have been grouped together as a reciprocal inhibitory interaction exists between these two pathways which is at least partly mediated by interaction with miRNAs.21,22,27

Figure 1 Allograft injury results in release of cytokines, chemokines and increase in inflammatory and immune cells in the allograft which induce differentiation of different cells, including tissue-resident fibroblasts[15], bone marrow progenitor cells[14], endothelial cells[14,18,24,72,73], epithelial cells[74,75] and pericytes[76], into myofibroblasts. Myofibroblasts produce collagen and collagen deposition results in fibrosis associated with chronic allograft failure.[6,11,77]

Figure 2 Summary of main signalling pathways involved in changing gene expression in EndMT and how microRNAs that are upregulated (orange) or downregulated (purple) interact with these pathways. Red arrows indicate inhibition. Green arrows indicate activation.[7,9,21-23,27-29,31,34,41-49,54-56,78]

Figure 3 In the nucleus, the miRNA gene is transcribed by RNA polymerase II into the double stranded hairpin-shaped pri-miRNA.[79,80] The enzyme Drosha RNase III endonuclease works with cofactor DGCR8 (DiGeorge syndrome critical region 8) to cleave both strands of pri-miRNA near the base of the primary stem loop to produce a shorter 60-70 nucleotide hairpin-shaped precursor known as pre-miRNA[79,80] Pre-miRNA is then actively exported into the cytoplasm via Exportin5 channel. Here, a second RNase III endonuclease, Dicer, cuts both strands of pre-miRNA near the base of the stem loop to leave a duplex of the mature miRNA and a similar sized complementary fragment of the opposing arm (miRNA*). The duplex is separated by incorporation of miRNA strand into RNA-induced silencing complex (RISC) by binding to argonaute proteins[38,79,81]