MicroRNAs as Biomarkers in Solid Organ Transplantation

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Abstract

Important progress has been made in improving short term outcomes in solid organ transplantation. However, long-term outcomes have not improved during the last decades. There is a critical need for biomarkers of donor quality, early diagnosis of graft injury and treatment response. MicroRNAs (miRNAs) are a class of small single stranded noncoding RNAs that function through translational repression of specific target mRNAs. MiRNA expression has been associated with different diseases and physiological conditions. Moreover, miRNAs have been detected in different biological fluids and these circulating miRNAs can distinguish diseased individuals from healthy controls. The noninvasive nature of circulating miRNA detection, their disease specificity, and the availability of accurate techniques for detecting and monitoring these molecules has encouraged a pursuit of miRNA biomarker research and the evaluation of specific applications in the transplant field. miRNA expression might develop as excellent biomarkers of allograft injury and function. In this minireview, we summarize the main accomplishments of recently published reports focused on the identification of miRNAs as biomarkers in organ quality, ischemia-reperfusion injury, acute rejection, tolerance and chronic allograft dysfunction emphasizing their mechanistic and clinical potential applications and describing their methodological limitations.

Keywords

Introduction

Critical advances have been made in the transplant field during the last decades. Most of this progress relates to short term outcomes as consequence of improved surgical techniques and the use of new and more powerful immunosuppressive therapies (1). Unfortunately, the progress achieved in short term outcomes does not translate to long term outcomes. This may be partially consequence of the lack of a robust gold standard to monitor graft function (1,2). Appropriate immunosuppression represents a challenging situation, including a

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delicate balance between rejection rates and chronic allograft dysfunction as well as immunological and non-immunological side effects (3, 4).

Allograft biopsy represents the gold standard for diagnosis of conditions like acute rejection (AR), disease recurrence and drug toxicity (5–8). However, allograft biopsy often relies on "subjective" measures, with high variability in results and reporting methods among pathologists or limited diagnostic accuracy associated with sampling error (5–8). Also, the lack of success in the implementation of biomarkers in clinical transplantation might be related to the absence of well-defined end-points, short follow-up times, and underpowered studies. In a recent publication evaluating current clinical endpoints in kidney transplantation, including the limitations of several ongoing studies and clinical trials, Schold *et al.* (9) concluded that the use of specific endpoints should lead to an improved discrimination and identification of important risk factors, an increased understanding of disease processes and an enhanced prospective application of research findings to clinical care.

There is a critical need for biomarkers for early diagnosis, treatment response, and outcome prediction in organ transplantation, with the final goal of predicting the individual's risk of allograft injury, leading to an individualized treatment. Advances in understanding the molecular basis of disease using genomics and proteomics technologies have provided new opportunities to develop genomic-based tools to diagnose, predict disease onset or recurrence, tailor treatment options, and assess treatment response(9–12). However, there is still no routine application of any of these markers in clinical transplantation.

Recently, microRNAs (miRNAs) have emerged as promising disease biomarkers. MiRNAs are a class of post-transcriptional regulators. They are short ~22 nucleotide (nt) RNA sequences that bind to complementary sequences in the 3′ UTR of multiple target messenger RNAs (mRNAs), usually resulting in their silencing. MiRNAs target ~60% of all transcribed genes, are abundantly present in all human cells and are able to repress hundreds of targets each (13–16). The discovery and characterization of miRNAs in the last decade is changing the understanding of gene regulation, cell differentiation, proliferation, apoptosis, metabolism and pathophysiology of many diseases. As result of their critical cellular roles, miRNA expression patterns are affected in many diseases (17, 18).

In this review, we focused on the evaluation of miRNAs as potential biomarkers in solid organ transplantation including a discussion of the most recent publications in the field and their expected impact in the clinical setting, in the upcoming years.

MiRNA Biogenesis

MiRNA genes are encoded within the genome, suggesting that their transcription might be coordinated with the transcription of other genes including the protein-coding genes that serve either as a source of miRNAs or as their targets. MiRNA biogenesis and mechanisms of action have been characterized in amazing detail (13–16, 18). Independently of the genomic location (approximately 10% of known miRNA genes are located within exons), generation of mature miRNAs occurs following a highly conserved mechanism that involves the processing of the primary miRNA transcript in the nucleus, to the mature product in the cell cytosol (19, 20). The primary miRNA transcripts (pri-miRNAs) present themselves to the processing machinery not merely as specific sequences but rather as particularly shaped structures. Primary miRNA transcripts are processed into approximately 70 nt stem–loop precursors (pre-miRNAs) by the Drosha–DGCR8 microprocessor complex and, after nuclear export, are cleaved into approximately 22 nt duplexes by the Dicer enzyme. One strand of the duplex is incorporated into a RNA-induced silencing complex (RISC) and

guides the complex to its cognate mRNAs. When miRNA–mRNA duplexes are near perfect, the target is cleaved by the RISC component called argonaute (Figure 1). MiRNA-guided regulation of gene expression has been implicated in almost every cellular pathway. In addition, each cell type expresses a specific subset of miRNAs to ensure that cell type specific mRNA profiles are established and maintained (13–16).

MiRNAs and their potential as biomarkers

MiRNAs (often found in association with exosomes) are present in a stable form and can be found expressed in serum, plasma, urine, saliva, and other body fluids. Moreover, the unique expression patterns of these circulating miRNAs have been correlated with certain human diseases, including various types of cancer and represent potentially informative biomarkers (19–20).

Technical advances have permitted the accurate detection of miRNAs in different biological fluids. Table 1 describes the advantages and disadvantages of measuring miRNAs in different sample types. Currently, various applications are available to determine the abundance of miRNAs. The expression profiles of many different miRNAs in parallel can be measured by microarray analysis or deep sequencing, whereas Northern blotting, real-time RT-PCR, and in situ hybridization (ISH) can be used to determine the level of individual miRNAs(13). Deep sequencing uses massively parallel sequencing, generating millions of small RNA sequence reads from a given sample. Deep sequencing measures absolute abundance and allows for the discovery of novel miRNAs that have eluded previous cloning and standard sequencing efforts.

Selection of the more appropriate method for evaluation of miRNAs as biomarkers mainly relies on the specific characteristics and needs of the system to be evaluated. All the available methods have advantages and limitations (13, 21). Specifically, multiplex polymerase chain reaction and microarrays have been developed for profiling levels of known miRNAs. These methods are not able to identify novel miRNAs. However, RT-QPCR methods have the advantage of determine expression at a wide dynamic range. There are several commercially available assays for mature miRNAs; however, their high cost may discourage researchers from this profiling technique. Deep sequencing methods are providing suitable platforms for genome wide transcriptome analysis and have the ability to identify novel transcripts. However, these methods are time consuming, and non-cost effective. Perhaps the most challenging aspect of deep sequencing is the bioinformatics analysis required to select the small RNA sequences that are actually miRNAs versus other small RNAs (13, 21). A number of bioinformatics tools and expertise are now available to perform these analyses (21). Overall, applying multiple methods in parallel will increase the likelihood of proper reflection of the presence or regulation of a miRNA.

MiRNAs as free nucleic acids, thought to derive from cell turnover, are shaded into blood and urine. As a result, miRNAs have emerged rapidly as a major new area of biomedical research with relevance in different fields including transplantation (Figure 2). MiRNA expression has been shown to differ among organs as well as between different organ regions (14).

Evaluating miRNAs as new biomarkers

A number of developmental objectives must be accomplished before any biomarker, including miRNAs, can be considered useful for the clinical setting. For achieving these objectives, there are specific considerations that need to be included as part of the study design. The more critical aspects are appropriate sample size to assure powered results, study design including testing of the candidate markers in an independent set of samples/

patients, and evaluation by an independent laboratory to demonstrate the robustness of the assay among different groups. Discovery and validation of miRNAs as disease biomarkers, as most biomarker studies, are usually better fitted as a training-validation study design. Specifically, a training set represents the sample populations included in a well-defined cross-sectional, retrospective, or prospective study to identify differential variables statistically significant between or among well-selected study groups using a determined technology and or algorithm. Meanwhile, the validation set embodies a sample population with similar characteristics from the initial training set assigned to corroborate the statistical significance of those previous identified differential variables.

Another critical aspect of miRNA analysis is that miRNAs do not function through a single gene target. The combined regulation of many different genes determines the functionality of a miRNA. Studying miRNAs include the evaluation of mRNA target identification and functional studies for evaluation of regulatory mechanisms. Initial insight into miRNA targets can be obtained using bioinformatics tools through a number of freely available programs that predict potential mRNA targets for individual miRNAs (Figure 3). These programs only predict putative targets. As consequence, it is important to confirm these predictions using miRNA target validation techniques. There are multiple strategies already described and available for miRNA target validation with most commonly used being cloning the 3′ UTR of a predicted mRNA target into a luciferase reporter (22).

For evaluating regulatory mechanisms, the more appropriate way to study the functional relevance of a miRNA is by examining phenotypic changes in culture or within an organism in response to regulation of a miRNA. Recently, several strategies for gain- and loss-of-function studies for specific miRNAs both in vitro and in vivo have been developed (22).

Profiling miRNAs as markers of organ donor quality/ischemia reperfusion injury

The critical importance of donor organ quality in determining short and long-term graft function is becoming increasingly clear. More efficient and accurate tools to determine deceased-donor kidney quality could help optimize allograft management and decrease the risk of discarding viable organs, as well as, of avoiding the transplantation of poor quality organs.

A recent study showed that miRNAs retrieved from preservation solution are more predictive of graft quality than the evaluation of the same miRNA expression in liver tissue (23). As part of the study, perfusate flushes of 29 heart beating and 10 non-heart beating liver grafts were collected at the end of the cold ischemia time. Cell-free solutions were analyzed for the presence of hepatocyte abundant miRNAs (miR-30e and miR-296) and cholangiocyte-abundant miRNAs (miR-30e and miR-148a) by RT-QPCR. Levels of miRNAs in the perfusates were compared to levels of the same miRNAs in liver biopsies retrieved at same time. Perfusates of liver grafts showed that miRNAs were differentially released during liver transplantation depending on the donor type (heart beating *versus* non-heart beating). Furthermore, miRNAexpression was positively correlated with a longer cold ischemia time and increased serum GT in serum post -transplantation. In this initial pilot study, the authors propose to measure miRNA levels as a non-invasive method to asses graft quality in liver transplantation. Due to organ shortage, non-heart beating grafts are increasingly used for liver transplantation. As a consequence, these interesting initial findings might have critical impact in organ utilization. Nevertheless, further validation in larger sample sets as well as longer follow-up studies to validate the utility of these biomarkers in the evaluation of organ quality at pre-implantation is required.

To examine the possibility that changes in miRNA expression could be used as a biomarker for ischemia reperfusion injury (IRI), Shapiro *et al.*(24) evaluated changes in miRNA expression in C57BL/6 mice on days 1, 3, 5, 7, 14, 21 and 30 after warm ischemia (IRI) or sham surgery. The results showed that IRI leads to lymphocyte independent alterations in miRNA expression profiles, showing that changes in miRNA expression could be used as a biomarker of renal injury resulting from ischemia and subsequent reperfusion. The same authors previously reported that miR-21, miR-20a, miR-146a, miR-199a-3p, miR-214, miR-192, miR-187, miR-805 and miR-194 were differentially expressed in C57BL/6 mice undergoing IRI compared to the expression observed in mice undergoing a sham procedure (25). Even when the study did not evaluate how alterations in the differentially expressed miRNAs are functionally involved in the kidney's response to the injury; the authors suggest that the observed miRNA expression profile observed after renal IRI reflects a survival response.

To investigate changes in intrahepatic miRNA expression in response to IRI, Faird *et al.* (26) analyzed 45 biopsy samples from liver grafts one hour after reperfusion. Liver graft tissue biopsy samples (n = 45) were analyzed for hepatocyte-abundant miRNAs (miR-122, miR-148a, and miR-194) using RT-QPCR. The levels of miR-122 and miR-148a (but not miR-194) showed a significant inverse correlation with the length of the warm ischemia time in the studied liver graft biopsy samples. However, the authors did not find a significant correlation between the miRNA levels and cold ischemia time, concluding that graft injury associated with longer warm ischemia times reduced the levels of specific hepatocyte-abundant miRNAs.

The promise of using antimiRs(miRNA inhibitors) to pharmacologically manipulate miRNAs *in vivo* anticipates interesting therapeutics opportunities for the near future. However, the biological understanding of how miRNAs alter gene expression in ischemic tissues remains incomplete, and additional mechanistic studies to identify miRNA targets that could be used to treat ischemia and reperfusion will be essential to benefit from taking advantage of such pharmacological approaches.

MiRNA profiling associated with acute rejection in transplant patients

The introduction of powerful immunosuppressant therapies in the past three decades reduced the incidence of AR in transplant recipients. However, the lack of noninvasive biomarkers of rejection precludes an optimization of antirejection therapy. The use of protocol biopsies has provided insights into the pathogenesis of many renal allograft diseases (3–7). Nevertheless, the histological evaluation of biopsies remains subjective, associated with some degree of variability depending on the pathologist evaluating the tissue sample as was previously mentioned and described (3–7). Moreover, despite the minimal risk involved in obtaining a renal allograft biopsy, it stills an invasive procedure.

Studies to identify noninvasive biomarkers of rejection and its underlying molecular events have increased significantly during the last years (10, 11), but new accurate markers are still lacking. Accumulating evidence underlines a critical function for miRNAs in the modulation of innate and adaptive immune responses (27–29).

Sui *et al*(30) described the first comparison between miRNA expression profiles of AR and the controls. Through microarray analysis and RT-QPCR confirmation, the authors identified 20 miRNAs differently expressed in AR after renal transplantation (8 up-regulated and 12 down-regulated). Their data indicated that miRNAs are potentially involved in the pathogenesis of AR. However, the sample size was an important limitation of this initial study (a total of 6 patients (3 AR and 6 normal)), mainly because of the fact that samples

were pooled together (all RNAs from each sample group were merged). Recently, Anglicheau *et al*(31) identified several miRNAs predictive of AR of human renal allografts. The authors used a 2-step approach to develop miRNA signatures predictive of AR. First, they evaluated intragraft expression patterns of 365 mature human miRNAs in 7 human renal allograft biopsies classified as AR or normal. In the second step, modified TaqMan miRNA assays was used to determine absolute copy numbers of miRNAs in 26 additional renal allograft biopsies. Intragraft levels of miR-142–5p, -155, -223, -10b, -30a-3p, and let-7c were proposed as having diagnostic value for AR, with miR-142–5p, miR-155, and miR-223 each predicting AR with >90% sensitivity and specificity. Up-regulated miR-142-5p, -155, and miR-223 were strongly linked to intragraft levels of CD3 and CD20 mRNA, suggesting the altered expression of miRNAs in AR biopsies might be due to graft-infiltrating immune cells.

This study's relevance mainly relates on being the first attempt to discover miRNA signatures associated with AR including an appropriate study design, with a group of patients used as training set and a second set of independent samples for validation. Also, some target validation analyses were performed and correlations among miRNA and predicted target mRNA were identified. However, the limited sample size and the cross-sectional study design were some limitations of the study.

In a recent study, Asaoka *et al.* (32) evaluated the expression of 384 mature miRNAs during intestinal acute cellular rejection in a total of 79 cases of intestinal mucosal biopsy specimens from 52 recipients. Also, the expression of 280 miRNAs associated with immune, inflammation and apoptosis processes were evaluated and correlated with the miRNA signatures. From the analysis of the data, 28 miRNAs and 58 mRNAs were identified as differentially expressed providing a potential miRNA signature associated with intestinal acute cellular rejection. Intragraft expression levels of miR-142-3p, miR-132, miR-886-3p, miR-16, miR-19a, miR-194 and miR-375 were quantified in an independent set of 53 intestinal biopsies. ROC analyses revealed that miR-886-3p had the highest AUC of 0.88, followed by miR-19a with an AUC of 0.84. A positive correlation between the intragraft expression levels of three miRNAs (miR-142-3p, miR-886-3p and miR-132) and 17 mRNAs including CTLA4 and GZMB was found. However, positive Pearson's correlation between miRNA and mRNA expression does not offer definitive proof of these molecular interactions. Overall, the results suggest that miR-142-3p, miR-886-3p and miR-132 could be closely involved in balancing the allograft's immune effector and regulatory cell populations.

Limitations of this study are the sample size and the number of assayed miRNAs. However, an important additional remark from this study is the use of formalin fixed embedded paraffin tissue as study samples, showing the stability of miRNAs in archival samples and the opportunity for evaluating big sets of samples previously collected from unique patients. Furthermore, the study was very well designed and included (in addition to training and validation set), miRNA/mRNA study associations as well as co-detection of miRNAs and protein markers related to miRNAs expression on a specific cell type.

The hypothesis that urinary cell and/or peripheral blood cell miRNA expression profiles are predictive, diagnostic and/or prognostic biomarkers of allografts is very attractive for non-invasive biomarker discovery and deserves further evaluation(27). MiRNAs have been proposed as stable biomarkers in urine samples (29, 33). In a recent study (34), Lorenzen *et al.* tested the hypothesis that urinary miRNAs of transplant patients with AR have a characteristic miRNA signature and moreover, might serve as biomarkers and predictors of long-term allograft function. The miRNAs were measured in urine of 62 patients with AR, including 19 patients before the rejection episode and after successful antirejection therapy

and 19 transplant patients with stable kidney function without the evidence of rejection. Thirteen stable transplant patients with urinary tract infection served as additional disease controls. The use of urine samples for miRNA studies in patients with AR was validated with inclusion of miRNA stability using different sample storage conditions. The initial global signature was evaluated using pooled samples (N=10) and 21 miRNAs were identified as significantly differentially expressed. During the independent validation using RT-QPCR (N=88 samples), miR-210 was identified as the only specific urinary biomarker of acute cellular rejection. Contrarily to miR-10a and -10b, miRNA-210 discriminated between stable control transplant patients and other pathologies. Furthermore, the study included urine samples of 19 patients before or after acute cellular rejection (12 patients before the occurrence of rejection and 7 patients after successful antirejection therapy). MiR-210 expression was significantly lower in urine samples of patients with AR compared to the patients before rejection highlighting the utility of the marker in monitoring response to the treatment. This study represents the first clinical evaluation of miRNAs in urine samples from patients with AR. However, the study lacks functional/validation studies to evaluate the biological significance of the identified biomarkers in the development of AR.

Even when there are still few studies published in the field, the potential of miRNAs as biomarkers for diagnosis of AR and response to therapy in non-invasive monitoring might have critical impact in the transplant field in the immediate future.

MiRNA signatures in chronic allograft dysfunction

Chronic allograft dysfunction (CAD) is pleomorphic with a mixed histology and pathophysiology and differential rates of progression, and should be thought of as a non-specific end-pathway of tubulointerstitial, microvascular and glomerular damage resulting from a variety of insults to the transplanted kidney, reflecting a limited repertoire of tissue response (35). It seems likely that transition of epithelial cells to mesenchymal cells contributes to tubular atrophy (TA) and interstitial fibrosis (IF), explaining why TA and IF are so strongly linked (35). Transition of epithelial to mesenchymal cells is recognized as a substantial contributor to the development of kidney fibrosis (36). Epithelial mesenchymal transition (EMT) describes a reversible series of events during which epithelial cells undergo morphological changes and acquire mesenchymal characteristics. Several studies have now found that EMT is regulated by miRNAs, notably the miR-200 family and miR-205 (37, 38).

Our group recently published the first miRNA signature associated with CAD (39)in allograft tissue and paired urine samples. We aimed to evaluate miRNA signatures in CAD with IF/TA and appraise correlations with paired urine samples and potential utility in the prospective evaluation of graft function. MiRNA signatures were established between CAD with IF/TA *versus* normal allografts using microarrays. Fifty-six miRNAs were identified in samples with CAD-IF/TA. Five miRNAs were selected for further validation by RT-QPCR using an independent set of samples and prospective evaluation of urine samples. Differential expression was detected for miR-142-3p, miR-204, miR-107 and miR-211and miR-32. Furthermore, differential expression of miR-142-3p, miR-204 and miR-211 was also observed between patient groups in urine samples. A characteristic miRNA signature for IF/TA that correlates with paired urine samples was identified. These results support the potential use of miRNAs as noninvasive markers of CAD with IF/TA and for monitoring graft function. Prospective evaluation of these markers is ongoing in a larger set of patients evaluated at multiple time points during 36 months post-transplantation.

MiRNA patterns in transplant tolerance

Achieving drug-free tolerance or successfully using only small doses of immunosuppression is a major goal in organ transplantation. The most extreme approach at long-term immunosuppression minimization is the complete withdrawal of immunosuppressive drugs. This has been described to be particularly frequent in liver transplantation, where approximately 20% of selected recipients enrolled in drug withdrawal trials are able to discontinue all drugs (40). The patients able to maintain stable graft function off immunosuppressive drugs without clinically significant detrimental immune responses and/ or immune deficits are conventionally termed operationally tolerant (OT).

In a recent study, Danger *et al.* (41) reported on the modulation of expression of eight miRNAs in peripheral blood mononuclear cells (PBMCs) from kidney graft recipients with drug-free OT compared with patients with stable graft function under immunosuppression. Global miRNA profile in PBMCs from kidney transplant recipients using miRNA Taqman low-density arrays that target 381 mature human miRNAs was performed. For the training set, a total of 9 OT patients and 10 patients with stable graft function under immunosuppression were tested. Eight miRNAs were identified as significantly differentially expressed between groups (4 were overexpressed (miR-450b-5p, miR142-3p, miR-876-3p, and miR-106b) and 4 were underexpressed (miR508-3p, miR-148b, miR-324-5p, and miR-98). The authors focused their analysis on miR142-3p based on the fact that this miRNA was highly differentially expressed between groups and its already described association with the hematopoietic lineage and possible role in B cell functions (41). A significant overexpression of miR-142-3p in the B-lymphocyte subset of OT compared with patients with stable allograft function was observed. Moreover, this report showed that the expression of miRNA-142-3p was not modulated by immunosuppressive treatment in tolerant kidney transplant recipients or when purified B cells from human healthy volunteers were cultured with cyclosporine A in vitro, indicating that its overexpression in OT kidney transplant recipients was not just a consequence of the absence of immunosuppression. Further investigations are still needed to evaluate whether this overexpression of miR-142-3p in B cells contributes to controlling inflammatory responses and tolerance maintenance or is only a consequence of the tolerance state.

Future directions and challenges

There are several major challenges in exploring the role of miRNAs in solid organ transplantation, including among others: 1) there are yet many unanswered questions regarding miRNA biology; 2) the mechanism of regulation of miRNA production is not completely clear; 3) while many miRNAs are located within introns of host genes, their expression does not always correlate perfectly with that of host genes suggesting further, post-transcriptional, regulation; 5) specific targets for most miRNAs remain unclear and even when bioinformatics analyses have predicted many thousands of miRNA-target pairs, only a small proportion of the predictions had been validated experimentally. As consequence of the cited challenges, the immediate clinical benefits are likely to result from the identification of miRNAs and/or miRNA signatures that can be used as reliable biomarkers for diagnosis, prognosis and response to therapy, in both diagnosis and prediction of allograft disease and function post-transplantation.

As a positive remark, miRNAs have the potential of being reliable biomarkers because they are tissue specific, stable in different biological fluidics (including archival samples), relate with clinical conditions, and can be measured using cost-effective technology. In addition, further discovery of the association between miRNAs and diseases would provide potential

targets for novel therapy in transplantation. Indeed, strategies have already been developed to regulate specific miRNA expression both *in vitro* and *vivo* (42).

It is expected that the research focused in miRNAs as diagnosis and predictive markers will increase in the transplant field during the next years with high potential to be successfully translated into the clinical setting. However, large-scale multi-centered clinical studies are needed before they can be used in clinical practice. Evaluation of tissue graft and/or circulating miRNA profiles may accelerate the use of new biomarkers in guiding the diagnostic, therapeutic, and prognostic strategies that associates with over-immunosuppression, organ toxicity, and graft rejection or loss.

Acknowledgments

ABBREVIATIONS

AR	acute rejection
CAD	chronic allograft dysfunction
eGFR	estimated glomerular filtration rate
IF	interstitial fibrosis
IRI	ischemia reperfusion injury
mRNA	messanger RNA
miRNA	microRNAs
nt	nucleotide
RT-QPCR	Real-time quantitative-PCR
TA	tubular atrophy

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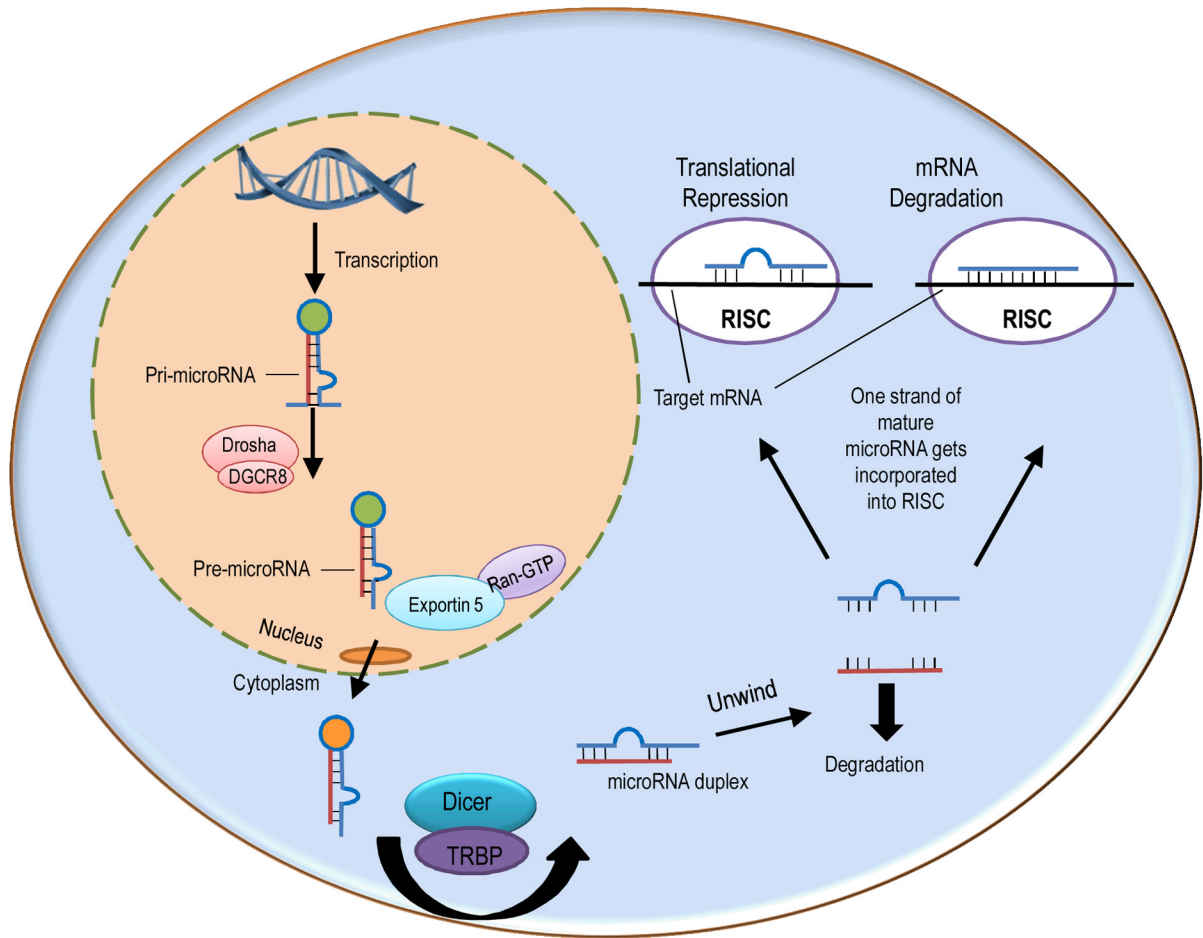
Figure 1. Schematic representation of biogenesis of MicroRNAs and mechanism of miRNA gene regulation in animal cells
Mature functional microRNAs of approximately 22 nucleotides are generated from long primary microRNA (pri-microRNA) transcripts. First, the pri-microRNAs, are processed in the nucleus into stem-loop precursors (pre-microRNA) of approximately 70 nucleotides by the RNase III endonuclease Drosha and its partner Pasha. The pre-microRNAs are then actively transported into the cytoplasm by exportin 5 and Ran-GTP and further processed into small RNA duplexes of approximately 22 nucleotides by the Dicer RNase III enzyme and its partner Loqacious (Loqs). The functional strand of the microRNA duplex is then loaded into the RNA-induced silencing complex (RISC). Finally, the microRNA guides the RISC to the cognate messenger RNA (mRNA) target for translational repression or degradation of mRNA.
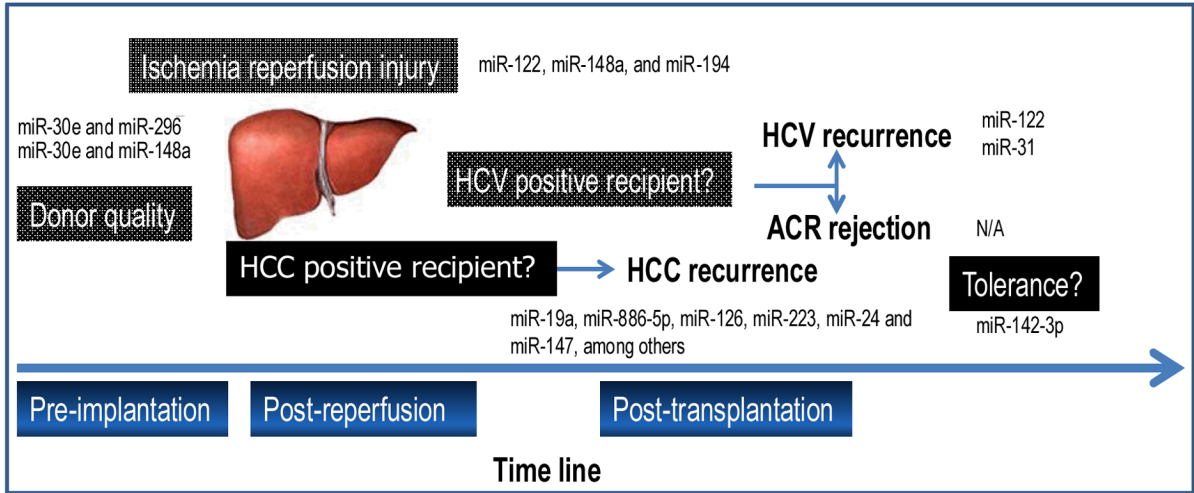
Figure 2. Potential applications of miRNAs as biomarkers in different conditions involved in graft function in liver transplantation

The liver transplantation model is used in the present Figure to show the most important applications of miRNAs as biomarkers. The miRNAs included in the Figure are the result of several publications and have been found to behave as disease-associated markers. This Figure depicts the many opportunities for evaluating these markers (e.g., conditions like AR (with or without HCV) have not yet been evaluated). Moreover, most of these results lack mechanistic studies exploring the role of the miRNAs in the disease.
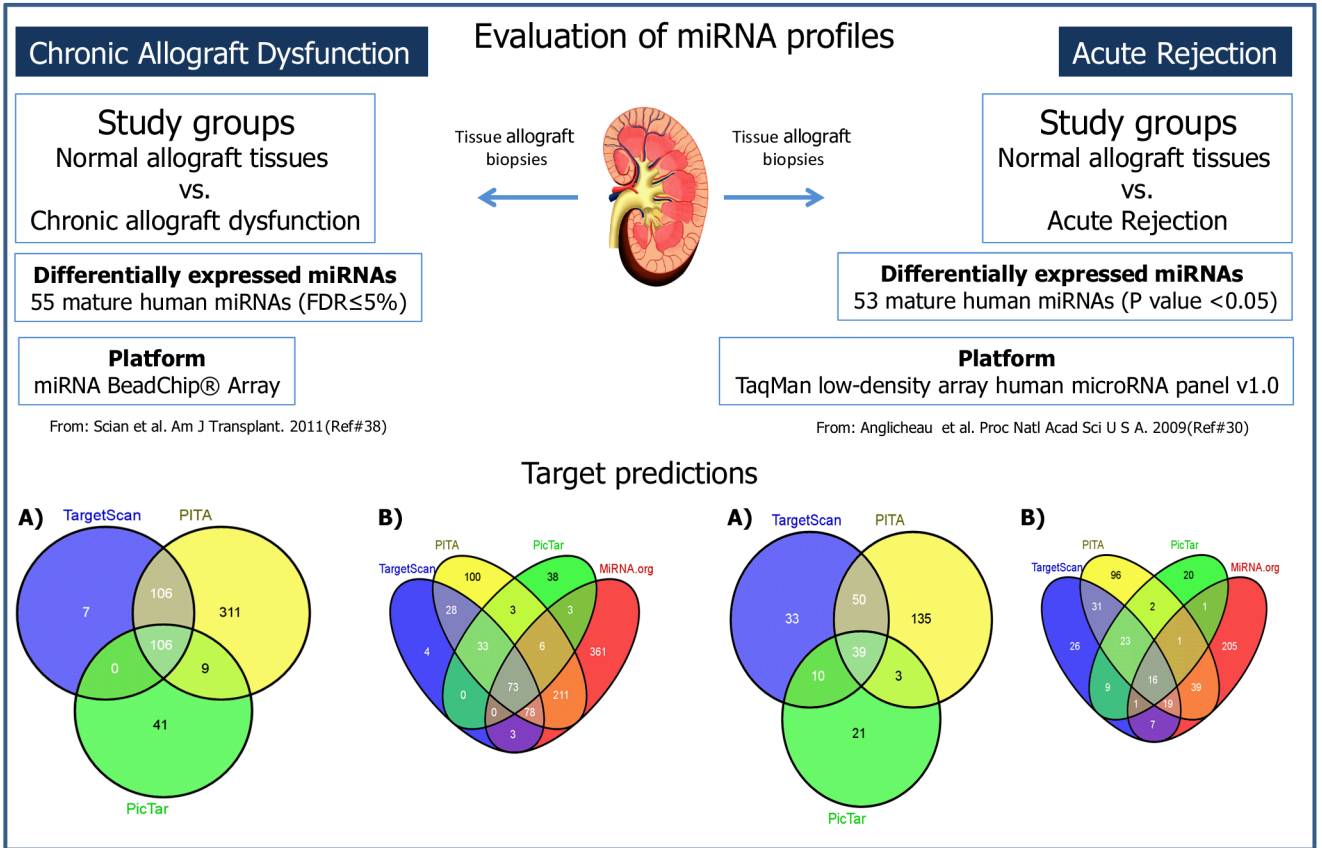
Figure 3. Combining mRNA and miRNA expression profiles for an accurate target prediction

It is now well established that the formation of a double-stranded RNA duplex through the binding of miRNA to mRNA in the RNA-induced silencing complex (RISC) triggers either the degradation of the mRNA transcript or the inhibition of protein translation. However, experimental identification of miRNA targets is not a straightforward process, and in the last few years, many computational methods and algorithms have been developed to predict miRNA targets. The first step in the prediction procedure requires the identification of potential miRNA binding sites in the mRNA 3′UTR according to specific base-pairing rules. The second step involves the implementation of cross-species conservation requirements. Among the most popular prediction algorithms, PicTar, TargetScan, PITA, MIRNA.org and miRanda are frequently used. Each algorithm has a define rate of both false positive and false negative predictions. It is currently accepted that more than one algorithm should be used to make reliable predictions about a particular miRNA:mRNA interaction. The Figure shows two examples of conditions affecting kidney allografts (CAD and AR). MicroRNAs were used from two published articles to predict mRNA targets (References 38 and 30, respectively). For each of these conditions, initially three different algorithms we used for the initial miRNA analysis (three-way Venn diagrams)(A) and then, for a more restricted secondary analysis, four algorithms were used (four-way Venn diagrams)(B). Conventionally, common elements found on the intersection of these Venn diagrams are selected for further validation and mechanistic studies.

Table 1

Advantages and disadvantages of sample type used as a source of miRNAs for biomarker validation

Sample type	Advantages	Disadvantages
Allograft tissue	• direct representation of profiles associated with the disease • techniques for miRNA isolation better optimized • more standardized endogenous controls • formalin embedded fixed tissues can also be tested	• invasive strategy • heterogeneity in tissue samples • genetic and environmental variations • variability in experimental techniques
Plasma	• non-invasive strategy • the wide range of sources of circulating miRNAs makes it possible for circulating miRNAs to reflect every aspect of human physiological status and therefore, provides an advantage for them to serve as better biomarkers than other circulating molecules, such as DNAs and RNAs. • allow for diagnosis and prediction • useful for continuous monitoring disease progression as well as response to treatment	• standardization of assays for miRNAs and endogenous controls, • validation of normal ranges of expression of miRNAs in larger diverse groups, • understanding of the mechanisms by which miRNAs are released into the plasma
Urine	• non-invasive strategy • allow for diagnosis and prediction • useful for continuous monitoring disease diagnosis/progression as well as response to treatment	• not clear yet the benefit between the use of urine pellets *versus* exosomes • tissue specificity of the markers proper RNA stabilizer in the clinical sample • timing between collection and sample processing is critical and need to be standarized
Peripheral blood mononuclear cells	• non-invasive strategy • allow for diagnosis and prediction • useful for monitoring disease progression as well as response to treatment	• heterogeneity of the sample • fluctuation depending on collection time • specificity of the markers • dependence on cell account