MicroRNAs regulatory networks in cardiotoxicity

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Basel, den 22. Mai, 2012

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Prof. Dr. Martin Spiess

Dekan

„Alle Ding' sind Gift, und nichts ohn' Gift;

allein die Dosis macht, daß ein Ding kein Gift ist"

"All things are poison, and nothing is without poison;

only the dose permits something not to be poisonous."

(Paracelsus, ca. XVI Century)

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Foreword

This thesis work summarizes the main findings I obtained during the last 3 and half years (October 2008 to now), working in the laboratory of Genome Biology of the Discovery and Investigative Safety in the Pre-Clinical Safety department of NIBR. Our section is particularly interested in all aspects of molecular toxicology, in order to better define the safety profiles of drugs before clinical testing. Our department aims at elucidating the mechanisms of drug toxicity, predicting the impact on human subjects, and suggesting translatable safety indicators to monitor the toxic effects in the clinic.

We chose to start working on microRNAs since no previous work specifically covered their role in drug-induced cardiac toxicity, and because of the proven impact of disturbances in microRNAs homeostasis on cardiac physiology.

The main findings were arranged in 2 manuscripts, each covered in the central chapters of this thesis. Chapter 2 illustrates an example of drug-induced cardiac injury, and its impact on expression of microRNAs in rats' hearts. This manuscript has been submitted to PLoS ONE, and we are currently responding to the reviewers' questions. At the moment, we are running a few more functional experiments in cardiac myoblasts, and we could not fully incorporate these results in this thesis. Chapter 3 will describe an approach to determine the distribution of mRNAs and microRNAs in different cardiac structures in rat, dog and Cynomolgus monkey, since these are the most used model species in pharmaceutical research, and are still poorly annotated for mRNAs and microRNAs distribution. This manuscript will be submitted in the course of the next days to PLoS Genetics.

Importantly, only the material that was considered sensible for complete publication in peer reviewed journals will be reported in this thesis. With a few exceptions in Chapter 2, techniques validation, incomplete or inconclusive data

will not be shown. This is in line with the guidelines of the University of Basel that accepts "compilative" PhD thesis essentially composed of submitted/accepted manuscripts.

As for every PhD, there are many people whose contributions have been crucial throughout my time in Novartis and that have to be acknowledged here. From the professional side I would like to thank at first Philippe Couttet. As my direct mentor and thesis supervisor he constantly challenged me to develop a critical opinion, always made the time to discuss with me and kept the projects on track. I would also like to thank Olivier Grenet and Jonathan Moggs, who kept a careful eye on the quality of the science and provided strategic steering at all times. My gratitude also goes to Prof. Alex Odermatt for the great exchange of scientific ideas, and also for his impeccable supervision in going through all grad school requirements at the University of Basel.

The most special thanks go to Estelle Marrer, who devoted her personal commitment to this thesis and greatly contributed to the accomplishments obtained in the course of my PhD.

A great "thank you" goes of course to my colleagues from the lab: Magali, Gregory, Sarah, Tulipan and especially Manuela from whom I learnt a lot of tricks that have made my life much easier.

Particular thanks go to Yasmina Bauer, Chiara Buono-Dalton, André Cordier, Valerie Dubost, Bérengère Dumotier, Florian Hahne, Pierre Moulin and Philippe Scheubel, who contributed with their life-long experience on special topics. All the present and past members of the Molecular Toxicology and Molecular Pathology section deserve my special gratitude, as everyone contributed with precious advices and made the time to sit with me throughout the years.

My best acknowledgments go to all co-authors of my two manuscripts: in alphabetical Brian Berridge, Sandrine Bongiovanni, Kevin Gerrish, Hisham Hamadeh, Martin Letzkus, Jon Lyon, Ric Paules, François Pognan, Frank

Last but not least, I owe a great lot to my fabulous chiropractor Marie Claude. She was always ready to listen and shared many valuable advices and points of view accumulated during her challenging life.

Lastly, thanks to all of those who believed in me and gave me the extra encouragement when I most needed it: this is the product of your contribution and you must be very proud for making all this happen.

Summary

A long time and many steps are needed to bring valuable drugs to patients. Drugs not only have to be effective against the disease which they are intended for, but also safe. All drugs cause toxic effects in proportion to the dose (Paracelsus, ca. XVI cent.). Therefore, assessing carefully the optimal dose at the desired benefit/risk ratio (Stevens and Baker, 2009) and characterizing mechanisms of toxicity are essential for maintaining high safety standards. The experimental strategies used to predict drugs' adverse events are many, and include the use of non-invasive biomarkers that can ideally be translated from test species to humans. In particular, cardiac safety requires special attentions, as it causes the cessation of drug development in over the 30% of cases due to toxic liabilities in Phase III, and over 40% post-approval (Redfern *et al.*, 2011).

In 1993 Victor Ambros, Rosalind Lee and Rhonda Feinbaum (Lee *et al.*, 1993) described for the first time in *C. elegans* the role of short non-coding RNAs, called microRNAs. MicroRNAs pair with target mRNAs via sequence complementarity, and lead to mRNA degradation or translation inhibition (Filipowicz *et al.*, 2008; Guo *et al.*, 2010). MicroRNAs are implicated in many biological processes and in the evolution of the complexity of superior organisms, for review see (Bartel, 2004; Berezikov, 2011). Their role in cardiovascular development and diseases has been supported by an increasing number of publications, for review see (Kinet *et al.*, 2012; Small and Olson, 2011). Recently, tissue-specific microRNAs were found in a variety of body fluids following drug-induced tissue injury (Wang *et al.*, 2009). In particular, myocardial necrosis of different etiologies (such as acute coronary syndrome and myocardial infarction) caused an increase of heart enriched microRNAs in plasma/serum of patients, as reviewed by (McManus and Ambros, 2011).

This work is aimed at increasing our knowledge about cardiac microRNAs function in different toxicological contexts and across species.

Chapter 1 will give an overview of drug safety assessment focusing on the cardiovascular system, microRNAs biology and their potential as toxicity biomarkers.

In Chapter 2 I will illustrate an example of drug-induced cardiac injury, and its impact on expression of microRNAs in rats' hearts. Chronic treatment of rats with low amounts of doxorubicin (an anti-tumoral compound) caused myocardial vacuolation in ventricular tissue and a significant increase of specific microRNAs and genomic indicators of cardiomyopathy. We found that Sipa1 could be directly inhibited by miR-34c by using a luciferase assay. Notably, the phenotypic anchoring of microRNAs with histopathological read-outs in the tissue showed that over-expression of miR-216b in the heart preceded the rise of overt lesions.

Chapter 3 will describe an approach to determine the distribution of mRNAs and microRNAs in different cardiac structures in rat, dog and Cynomolgus monkey. We showed that microRNAs could discriminate the different heart structures as good as mRNAs. We also assessed the expected anti-correlation between microRNAs levels and their predicted target mRNAs, and showed that mRNAs of 4 genes implicated in cardiac diseases and muscular development could be inhibited at the post-transcriptional level by 4 distinct microRNAs. We propose that our mRNAs and microRNAs data sets could be used to assess the human relevance of preclinical findings in these 3 species, and to derive potential tissue and circulating biomarkers for drug-induced cardiac injury.

In conclusion, profiling microRNAs have the potential to help biomedical research in bringing drugs to patients, since they can shed light on toxicity mechanisms in the tissues (Taylor and Gant, 2008) and are a source of translatable non-invasive toxicity biomarkers in body fluids.

Chapter 1: Literature review on cardiac toxicity and microRNAs

1.1 Drug development and safety assessment

1.1.1 Impact of safety liabilities in drug development

The rise of safety concerns for patients in the past 10 years increased the attrition rate due to organ toxicity in drug development (Figure 1-1), and today between 25 and 30% of the causes of attrition can be attributed to pre-clinical and clinical toxicity (adverse drug reactions) (Guengerich, 2011).
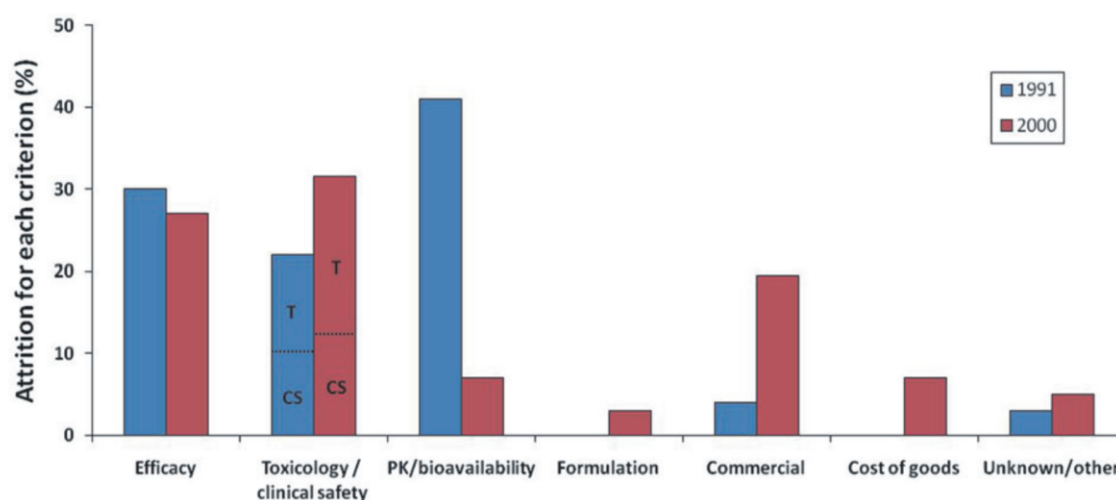
Figure 1-1: Reasons for drug attrition in 1991 and 2000. Rising safety concerns increased the number of drugs withdrawn from market or discontinued (Laverty *et al.*, 2011). Modified from (Kola and Landis, 2004).

Safety aspects contribute to decisions during the whole pharmaceutical R&D: from target characterization, to candidate and back-up compounds selection, lead optimization, pre-clinical and clinical phases. Pre-clinical and clinical tests are aimed at balancing predicted risk against expected benefit, and are tightly interconnected.

During all drug development pre-clinical findings influence clinical development and vice versa: animal studies are run after first-in-human stage, to better understand the toxic mechanisms observed, and to mitigate risks. Mandatory pre-clinical safety tests include genotoxicity (mainly *in vitro*), core safety pharmacology (SP) studies (*in vivo* cardiovascular SP, *in vitro* cardiac SP, respiratory SP and central nervous system SP) (Gad, 2008), and repeated dose *in vivo* toxicity studies. Animal studies that run in parallel with clinical phases include: repeated dose (13-26 weeks), *in vivo* genotoxicity, reproductive toxicity (segments I, II and III), immunotoxicity and carcinogenicity (2 years).

Factors influencing the definition of safety margins include the indication of the drug, foreseen co-medications and co-morbidities and relevance of the pre-clinical species used, among others. Integrated risk assessment and evaluation of pre-clinical safety profile are strategic elements for taking successful go/no-go decisions.

1.1.2 Terminology and definitions in safety assessment

1.1.2.1 <u>Safety Biomarker</u>

The term "biomarker" has several definitions, depending on the field of use. A drug safety biomarker is an objectively measured parameter that indicates whether a biological process is normal or pathological in response to a therapeutic intervention (Biomarkers Definitions Working Group, 2001).

Regulatory authorities require biomarkers to be:

‒ Validated, which includes establishing characteristics of the analytical assay, such as accuracy, precision, limit of quantification (LOQ), stability of analyte (Lee *et al.*, 2006).

‒ Qualified, which includes establishing the physiological, toxicological, pharmacological or clinical significance (Goodsaid and Frueh, 2007).

Ideally, biomarkers developed at the pre-clinical stage should be translatable for human use.

1.1.2.2 *Adverse effect*

An adverse effect is a harmful and undesired effect resulting from a medication.

1.1.2.3 *Toxicity endpoints*

A toxic endpoint is a parameter monitored during an *in vivo* study in animal or human, that reflects the toxicity of a compound. Data on endpoints are used to determine the No Observed Adverse Effect Level (NOAEL) and the Lowest Observed Adverse Effect Level (LOAEL). Toxic endpoints include, (but are not limited to):

− In life changes: food/water consumption, weight gain, behavior, reproductive rate, etc.

− Primary immunotoxicity: leukocytes count, hematology, coagulation, globulin and albumin levels, gross evaluation of thymus and spleen, etc.

− Histopathology: macroscopic and microscopic alteration of tissues and cells

− Gene expression changes: transcriptomics and proteomics in affected organs

− Metabolic changes: metabolomics and clinical chemistry

− Organ and tissue function: imaging techniques, hemodynamic, metabolic circulating markers

− Clinical chemistry biomarkers: circulating tissue injury indicators

Endpoints can be validated and qualified to become biomarkers.

1.1.2.4 *Adverse vs. non adverse effect*

When differences in selected endpoints are observed in a treatment group it is necessary to determine whether these a) are treatment related, and b) they are adverse.

Discriminating factors a):

− There is no obvious dose response

− Findings encountered in "outlier" animals

− Measurement of selected endpoint are inherently imprecise

− Values measured are within normal biological variation (historical control data)

− Lack of biological plausibility

Discriminating factors b)

− There is no alteration in the general function of the test organism or of the organ/tissue tested

− Secondary to other adverse effects

− Adaptive response

− Transient

− Limited severity (magnitude of change)

− Effect is isolated or independent and/or is not a precursor of changes known to progress and establish adverse effects

− A consequence of the experimental model

1.1.2.5 *NOAEL: No Observed Adverse Effect Level*

The highest exposure level at which there is no statistically significant increases in the frequency or severity of adverse effect between the exposed population and its appropriate control. Some effects may be produced at this level, but they are not considered to be adverse or precursors to adverse effects (Figure 1-2).

1.1.2.6 *LOAEL: Lowest Observed Adverse Effect Level*

The lowest exposure level at which there is a statistically or biologically significant increase in the frequency or severity of adverse effects between the exposed population and its appropriate control (Figure 1-2).

1.1.2.7 <u>MTD: Maximum Tolerated Dose</u>

The highest dose of a pharmacological treatment that will produce the desired effect without unacceptable toxicity (lethality is never an intended endpoint).
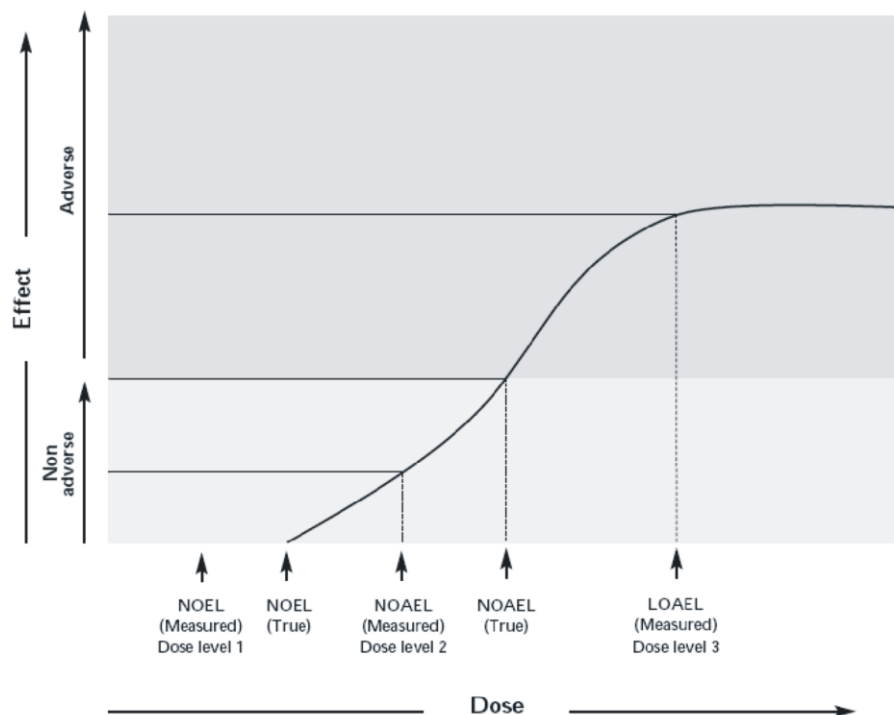
Figure 1-2: Dose escalation according to NOEL, NOAEL, and LOAEL. Doses used in a toxicity study should be chosen accordingly to observe no effects, no adverse effects, and some adverse effects. NOEL: no effect level; NOAEL: No Adverse Effect Level; LOAEL: lowest observed adverse effect level.

1.1.3 Impact of current strategies for organ toxicity assessment

Hepatic, neurological and cardiovascular adverse drug reactions rank in the top positions of reasons for development interruption or withdrawal due to toxicity, especially during the clinical phase as shown in Table 1-1 (Hamadeh *et al.*, 2002; Redfern *et al.*, 2011; Stummann *et al.*, 2009). It has been proposed that implementation of more predictive tests of liabilities in these organs already in the earliest research phases would significantly lessen the overall compound attrition due to toxicity concerns (Stevens and Baker, 2009).

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Table 1-1: Drug safety impact by target organ.

Phase	'Nonclinical'	Phase I	Phase I-III	Phase III/ Approval	Post-Approval	Post-Approval
Information:	Causes of attrition	Serious ADRs	Causes of attrition	ADRs on label	Serious ADRs	Withdrawal from sale
Source:	Car (2006)	Sibille et al. (1998)	Olson et al. (2000)	BioPrint® (2006)	Budnitz et al. (2006)	Stevens & Baker (2008)
Sample size:	88 CDs stopped	1,015 subjects	82 CDs stopped	1,138 drugs	21,298 patients	47 drugs
Cardiovascular:	27%	9%	21%	36%	15%	45%
Hepatotoxicity:	8%	7%	21%	13%	0%	32%
Haematology/BM:	7%	2%	4%	16%	10%	9%
Nervous system:	14%	28%	21%	67%	39%	2%
Immunotox; photosensitivity:	7%	16%	11%	25%	34%	2%
Gastrointestinal:	3%	23%	5%	67%	14%	2%
Reprotox:	13%	0%	1%	10%	0%	2%
Musculoskeletal:	4%	0%	1%	28%	3%	2%
Respiratory:	2%	0%	0%	32%	8%	2%
Renal:	2%	0%	9%	19%	2%	0%
Genetic tox:	5%	0%	0%	0%	0%	0%
Carcinogenicity:	3%	0%	0%	1%	0%	0%
Other:	0%	0%	4%	16%	2%	2%

0% ☐   1-9% ☐   10-19% ☐   >20% ☐

Reproduced with permission of W. Redfern (Redfern *et al.*, 2011). Information collated from published articles and from database BioPrint® (Budnitz *et al.*, 2006; Car, 2006; Krejsa *et al.*, 2003; Olson *et al.*, 2000; Sibille *et al.*, 1998; Stevens and Baker, 2009).

The choice of the species to use for *in vivo* pre-clinical studies is critical, and is conditioned by the relevance to human. It is noted that cardiovascular and oncology disease areas have the highest attrition rate due to poor predictivity of the animal models (Kola and Landis, 2004), and this can be applied to safety testing too. Crucial is to characterize the mechanisms of drug-induced toxicity, the target organ, whether it is an on-target, off-target, idiosyncratic or immunological mediated effect and evaluate the translatability of the findings to the patients. Accumulating data on biological processes and gene/protein expression in model organisms serve as basis for understanding the translatability of toxic findings to human. Of utmost importance is to be aware of similarities

and differences at the molecular level (single genes and pathways) between test species and human. For this purpose genomic techniques are being widely employed to describe molecular mechanisms of pathologies and diseases, and propose novel toxicity/efficacy biomarkers.

1.1.4 Additional Considerations

An earlier and more cost-effective evaluation of the safety profile is essential, in order to make drug development process less cost-intensive, and reduce the attrition of promising compounds at expenses of those at risk of liabilities once in the clinic. The combination of mechanism-based toxicity evaluations and availability of predictive translational biomarkers provides a safer ground for decisions regarding advancing compounds in development.

Knowledge generated by the progress in biological research is constantly taken in consideration throughout drug development, and safety assessment is not an exception. Novel molecular entities, and technologies, should be implemented in safety assessment, to provide new predictive endpoints, elucidation of drug-related molecular events and potential safety/efficacy biomarkers.

1.2 Drug-induced cardiac liabilities

1.2.1 Common drug-induced cardiovascular safety concerns

Drug-induced cardiac liabilities leading to withdrawal and research program discontinuation can be broadly divided into the following classes, according to their pathological outcome (Stummann *et al.*, 2009):

– Arrhythmias: a large spectrum of conditions induced by conduction abnormalities

– Contractility: interference with the ability of muscle fibers to contract (inotropic effects)

– Ischemia: restriction of blood supply to the heart, due to blood vessels (coronary) restrictions

– Secondary toxicity: caused by vascular effects

– Valve toxicity: impairment of valve function to keep unidirectional blood flow between chambers and vessels.

1.2.2 Routine cardiac safety studies and regulatory guidelines

A plethora of morphological, functional and biochemical parameters are used to diagnose classify and characterize these cardiopathies at the pre-clinical and clinical level.

EMEA/CHMP/SWP/488313/2007, ICH S4, ICH S7A and ICH S7B guidelines, issued by the International Conference on Harmonization (ICH), recommend the strategy for the design of cardiac safety pharmacology studies (International Conference for Harmonization, 2011). The requirement for an integrated safety assessment approach include a two-weeks repeated dose toxicity study in a rodent (rat) and a non-rodent species (dog or monkey) followed by histopathology, clinical chemistry parameters and functional endpoints. *In vitro* assays include I_{Kr} (hERG) patch-clamp inhibition assay, ADP studies in cardiac cellular animal preparation to evaluate the effect of the compound on voltage-gated ion channels. Inhibition of I_{kr}, L-type $Ca2^+$ and Na^+ currents lead to slower repolarisation and consequent elongation of QT phase in the electrophysiological track. A long QT syndrome (LQTS) is associated to Torsade de Pointes (TdP) and pro-arrhythmic potential of compounds. However, evidences suggest that a long QT measured *in vitro* within I_{Kr} (hERG) assays is not necessarily associated to cardiac liabilities in human (Redfern *et al.*, 2003). Conversely voltage-gated channel alterations are not causally implicated in other compound-induced cardiovascular pathologies, such dilating cardiomyopathy and valvular heart disease. The toolbox of

experimental techniques to tackle each type of cardiovascular toxicity is reviewed in Stumman C. *et al.* (Stummann *et al.*, 2009).

1.2.3 Impact and shortcomings of current pre-clinical cardiovascular safety strategy

Laverty *et al.* review the impact of pre-clinical strategies in place to prevent the risk of liabilities in patients (Laverty *et al.*, 2011). Arrhythmic potential (TdP) is tackled thanks to the systematic focus on QT interval in ECG investigations. Ischemic diseases however are often not detected, in part due to the use of young animals in pre-clinical studies and young healthy volunteers in Phase I, which do not represent populations at risk, especially elders with elevated blood pressure and atherosclerosis. It has been proposed that monitoring of T-waves may predict the risk of infarction in population at risk. Myocardial necrosis can be monitored via clinical chemistry endpoints like circulating cardiac troponin I (cTnI), creatinine kinase (CK-MB) and N terminal pro-natriuretic peptide (NT-pro BNP) (Marrer and Dieterle, 2010; Muller and Dieterle, 2009; Wallace *et al.*, 2004). However, these markers are elevated when the damage has already occurred and what are lacking are robust biomarkers to detect and monitor rather the onset of cardiac structural damage or changes in cardiomyocytes homeostasis (Buck *et al.*, 2008). Risk of heart failure can be determined by hemodynamic measurements, such as left ventricle ejection fraction (LVEF) and cardiac contractility. However these techniques are not routinely applied in all companies at the pre-clinical stage. Cardiac valves disorders are rather difficult to observe in animals: the incidence is quite low, no circulating biomarkers exist and only direct histological evaluation can confirm it. However fixed cardiac samples from animal studies for histopathology rarely contain the valves, and only a few slides per organ are evaluated.

Further needs identified by Laverty and colleagues include better understanding of the mechanisms of the drug-induced cardiac injury (DICI) at the cellular and structural level (Laverty *et al.*, 2011).

1.2.4 Reference compounds of cardiac toxicity

The use of compounds with known adverse cardiovascular effects may help to extensively characterize and classify new chemical entities, and establish alternative assays *in vitro* to be applied very early on during target discovery (Stevens and Baker, 2009).

Drugs belonging to the same pharmacological class, i.e. sharing the same target, tend to have similar liabilities pattern. Increase of focus on kinases as targets for oncology indications in the last 10 years also drag the attention on the safety liabilities of kinase inhibitors. While inhibiting mutated or over expressed kinases in tumors is a very promising strategy, kinases are pivotal genes in sustaining physiological activity in all organs. However, designing compounds that mimic ATP (Type I inhibitors, small molecules) or that target sites adjacent to the catalytical pockets leads to high probability of off-target toxicity. Nevertheless, Type III inhibitors with increased single kinase specificity achieved by target regions of the proteins far from the ATP binding pocket, do not fully prevent kinase mediated cardiotoxicity, as cardiac performances depend on multiple kinase-regulated pathways such as the ATP and Ca^{2+} dependent processes, as illustrated in Figure 1-3 (Force and Kolaja, 2011).

Drugs withdrawn from the market due to valvular dysfunction in the past were all 5-HT2B receptor agonists (serotonin receptors), which is highly expressed in valve leaflets (Bhattacharyya *et al.*, 2009; Elangbam, 2010). These include appetite suppressants (fenfluramine and dexfenfluramine), dopamine agonists indicated for Parkinson's disease (pergolide and cabergoline) and ergot alkaloids against migraine (methysergide and ergotamine). Highly selective agonists of other serotonin receptor isoforms are devoid of valve liabilities. The mechanism

of 5-HT receptors-induced proliferation implicates downstream activation of G protein coupled/ERK and phospholipase C-β/PKC. TGF-βR and Smad2/4 pathway is also activated. 5-HT2BR expression levels are similar between rat, monkey and human (Elangbam, 2010), suggesting these species are of practical use to establish animal models to predict drug-induced valvular liabilities.

1.2.5 Additional Considerations

It is widely understood that routine cardiac safety assessment is not fully predictive of findings in the clinics, and that mechanistic understanding is essential to distinguish physiological *vs.* pathological processes. Furthermore, physiological differences across test species and human patients should be clarified and taken in account. Both these aspects have to be taken in consideration for an integrated cardiovascular safety assessment.
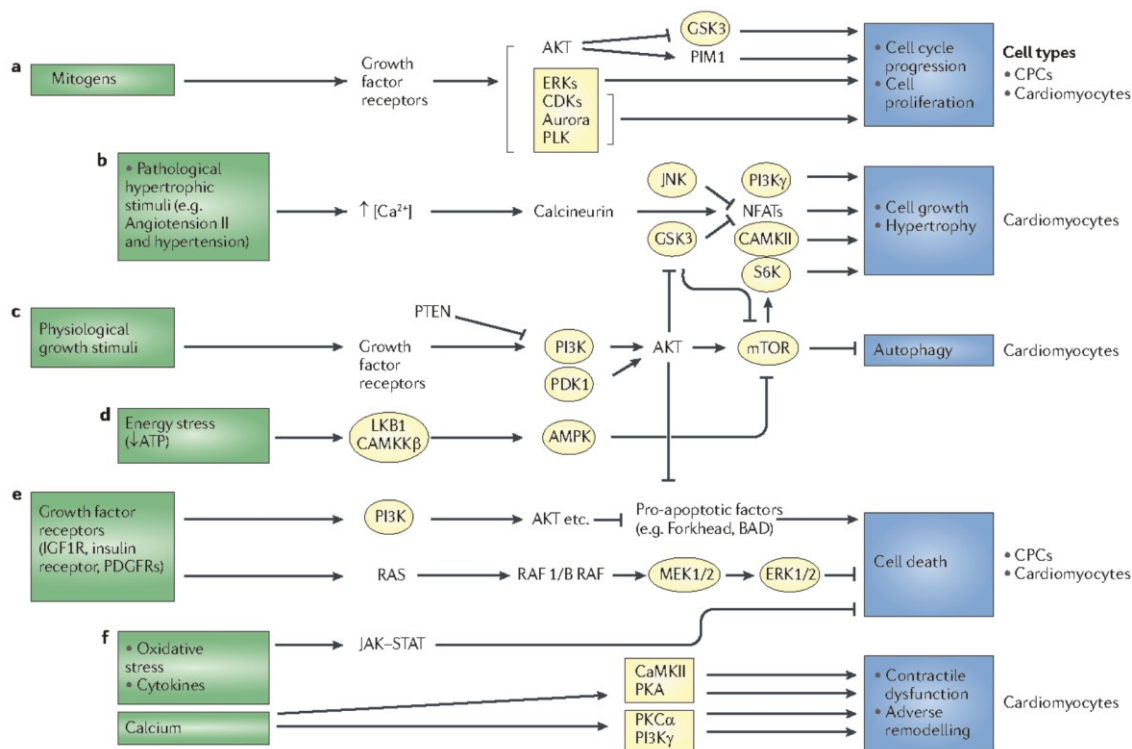
Figure 1-3: Crucial signaling pathways in the heart and consequences of their activation or inhibition (Force and Kolaja, 2011). Kinases are circled in light yellow ovals.

1.3 Impact of transcriptomics in drug safety assessment and biomarker discovery

1.3.1 Impact of toxicogenomics in drug safety assessment

Technical advancements of the post-genomic era are supported by high-throughput nucleic acid profiling platforms, such as RNA or DNA based microarray (Figure 1-4) that can quantify transcription changes at the global level, reveal association between diseases and genetic mutations, or characterize the epigenetic changes at the chromatin level. Although next-generation sequencing is becoming more affordable, microarray based profiling are routinely used in drug discovery during the preclinical safety assessment.
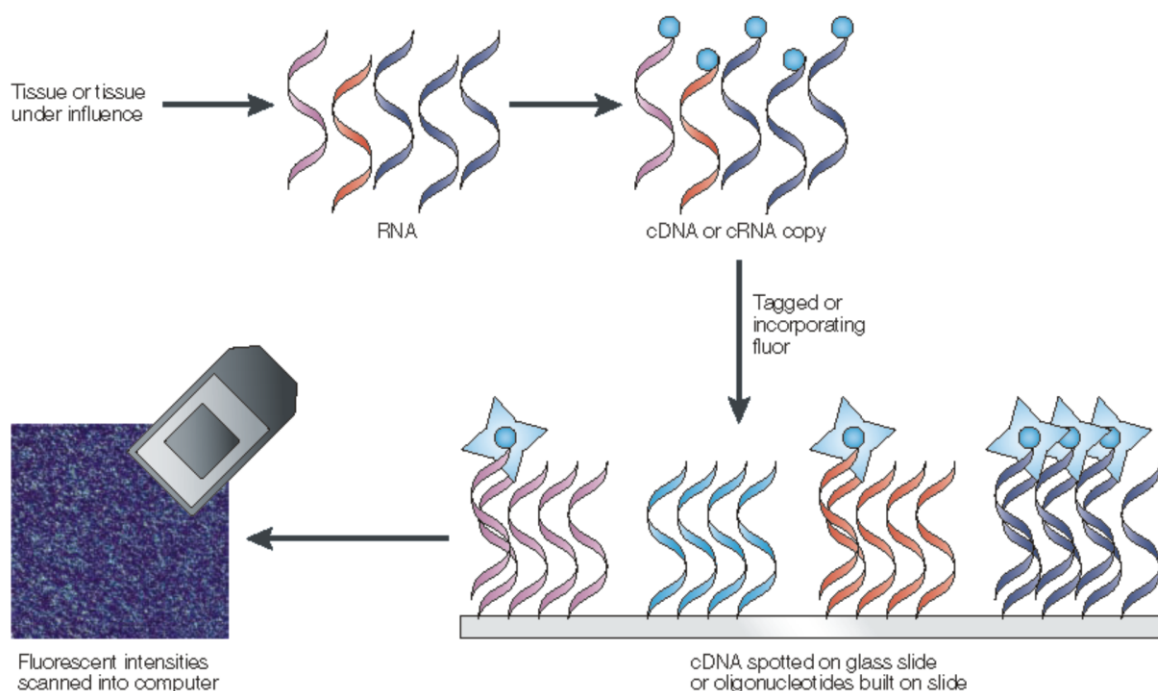
Figure 1-4: Experimental process of microarray hybridization experiments for gene expression determination (Butte, 2002).

Specific signatures of altered genes can be causally implicated into drugs toxicity in the tissue of interest (Buck *et al.*, 2008; Chen *et al.*, 2008a; Mori *et al.*,

2010). Goals of toxicogenomics in drug safety assessment screenings are multiple (Van and Sasaki, 2010): 1) formulate hypothesis on the molecular mechanism of undesired phenotypic changes, uncoupling them from the pharmacological effects, 4) give information on the organ structures mostly affected by the drug , 3) support the identification of biomarkers of the pathological process that can be used as screening endpoints for same class and backup compounds and 4) classify compounds in classes based on the toxicity outcome (Ruepp *et al.*, 2005; Steiner *et al.*, 2004).

1.3.2 Experimental approach of genomics studies for organ toxicity assessment

Upon stress stimuli, including xenobiotics exposure, a series of changes take place in the cells, ultimately leading to adaptation or functional impairment of the organ. Molecular alterations precede morphological changes and can be identified early on upon treatment, after only one or two doses of the drug, and in absence of overt toxicity (Hamadeh *et al.*, 2002). Data collection at multiple time points reveals cause-effect relationship between early and later gene expression (Buck *et al.*, 2008).

In a complex toxicity *in vivo* study including several doses and time points, data analysis still remains the bottle-neck, as the analyst has to take in consideration a plethora of accompanying data (histopathology, clinical pathology, and imaging) to carefully choose the most significant samples set to answer biological questions and enable critical compound prioritization and limit attrition as early as possible.

1.3.3 Data analysis strategy depends on the goal of toxicogenomics studies

A variety of approaches for analysis of large data sets are available, between supervised and unsupervised methods (Butte, 2002). Unsupervised approaches include principal component analysis, clustering and Bayesian networks. Supervised approaches range from the nearest-neighbor analysis and support

vector machine (machine-learning algorithms able to recognize patterns in analyzed data). It is important to decide *a priori* whether the outcome of the analysis is aimed at predicting toxicity only, or at obtaining a mechanistic interpretation of the molecular modifications involved. Gene signatures extracted from the analysis can be long (thousands of genes) in the former case, but must be much shorter (few tens of genes) in the latter case (Natsoulis *et al.*, 2005). In any case, transcriptomics results must be anchored to ancillary data, such as histopathology, in order to establish a temporal evolution of the findings, and propose relevant predictive toxicity indicators that would be noted earlier than overt lesions.

1.3.4 Toxicogenomics in biomarker discovery

Transcriptomics analysis can support discovery and validation of molecular indicators (biomarkers) of toxicity 1) in the tissue (pre-clinical only) and 2) in body fluids (pre-clinical and clinical), i.e. if a certain gene is translated to a released protein product. Elevated levels of these molecules can be measured in the peripheral circulation of patients, and specifically reflect leakage of cell content due to tissue injury. Ideally, biomarkers should be translatable across species, including preclinical species, and restricted in expression to the tissue of interest (Decristofaro and Daniels, 2008).

Since the mRNA expression levels may not match in all cases with the level of functional protein translated, proteomics investigations may be needed to confirm the functional outcome of altered gene expression.

1.3.5 Examples of transcriptomics studies for drug-induced cardiac toxicity (DICI)

In the case of drug-induced cardiac injury (DICI) histopathological readouts are limited to few qualitative observations (cardiomyocytes degeneration, vacuolation or necrosis) and do not provide explanations of the molecular events causing the injury (Buck *et al.*, 2008), since similar adverse phenotypes can be caused by

different mechanisms. Hemodynamic measurements supported by electro- and echocardiography are operationally intensive and detect the damage when the affected structure is functionally compromised. Therefore transcriptomics investigations bear the great potential of unveiling the mechanistic progression of toxicity. To date the vast majority of literature on toxicogenomics studies available in the public domain regard drug-induced liver toxicity (DILI) (Van and Sasaki, 2010), while cardiovascular toxicants have not received such systematic attention. Two examples of gene expression studies applied to cardiac safety are illustrated below.

Mori and colleagues illustrated the effects on gene expression of 3 known cardiotoxicants: a pesticide (carbofuran), a beta-adrenergic agonist (isoproterenol) and a DNA intercalator (doxorubicin, DOX) (Mori *et al.*, 2010). Several genes were consistently up-regulated after 1 dose only of the three compounds even earlier than histopathological findings, suggesting similar molecular mechanism may be involved in the toxic outcome for all the 3 compounds. These include Spp1, Fhl1, Timp1, Ccl7 and Reg3b genes. However, dosage and administration scheme did not reflect a real accidental exposure situation. Furthermore, those changes may be transient and not be maintained after repeated dosing.

Thompson and colleagues used a different approach and focused on early molecular events induced by DOX that are maintained after repeated administration of low doses in the rats, more closely resembling the clinical setting. The authors determined the gene expression changes after 2 weeks of chronic DOX administration and identified lists of gene significantly altered (Thompson *et al.*, 2009). These were either associated with the cellular response to oxidative stress, encoding mitochondrial proteins or calcium homeostasis. These observations were consistent with the molecular mechanism of proposed DOX-induced cardiac injury due to reactive oxygen species production (Chen *et*

al., 2007). Further cardiotoxicity aspects associated to anthracyclines chemotherapy are reviewed in the following chapter.

1.3.6 Additional Considerations

In addition to transcriptomics, metabolite profiling and proteomics approaches are often used to characterize various aspects toxic liabilities, such as biomarker discovery and characterization of off-target effects upon drug pull-down in protein extracts. The emerging fields of epigenetics and non-coding RNAs are increasingly integrated in mechanistic interpretations of xenobiotics response. Relevance of microRNAs in cardiac physiology and toxicity are reviewed in the next paragraphs.

1.4 MicroRNAs biology and function regulation

1.4.1 MicroRNAs biogenesis and function

MicroRNAs are ~22 nucleotides long non-coding RNAs, genomically encoded and conserved throughout evolution in all superior organisms. They are transcribed by DNA Pol II either as independent singular or clustered transcriptional units, or originate from hosting gene during intron splicing (Bartel, 2004; Goymer, 2007; Ruby *et al.*, 2007). Maturation of microRNAs involves sequential steps that include the generation of a primary hairpin, trimmed to a precursor-microRNA by Drosha, which is then exported to the cytoplasm where Dicer cuts the loop, releasing a short double stranded RNA. The duplex is separated to single-stranded RNA filaments, incorporated into an Ago2-RNA Induced Silencing Complex (RISC) (Figure 1-5). Regulation of microRNA expression can take place at all stages of biogenesis, and their function is influenced by several factors such as RISC-complex regulators, RNA binding proteins (RBP) and target mRNA conformation, among others (Krol *et al.*, 2010).
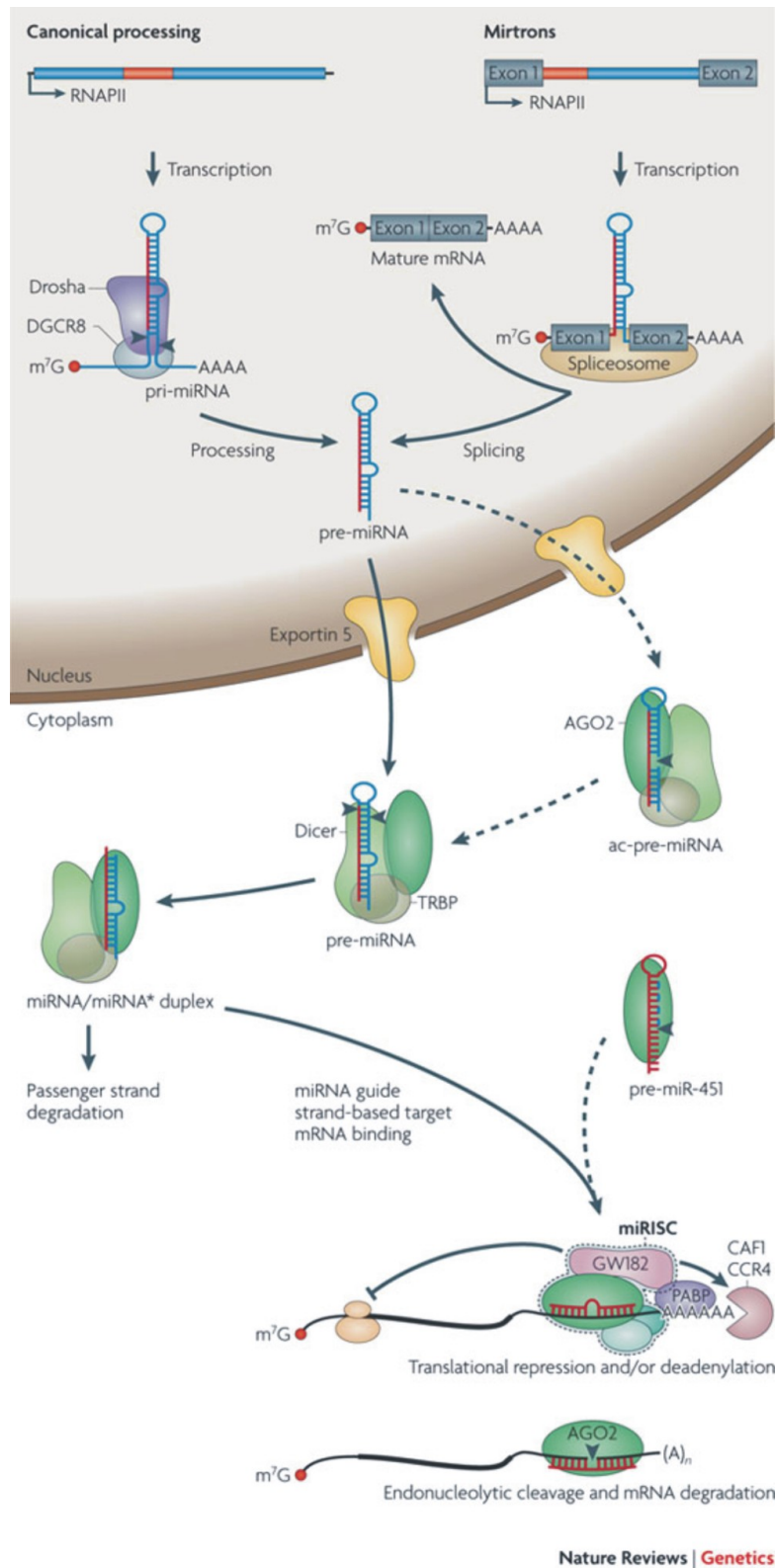
Figure 1-5: MicroRNAs biogenesis. Adapted from (Krol *et al.*, 2010).

Increasing evidences implicate non-coding RNAs as central players of cellular homeostasis by tuning the expression level of hundreds of mRNAs (Lim *et al.*, 2005; Stark *et al.*, 2005; Wang *et al.*, 2011a; Wu and Belasco, 2008). Although microRNAs were initially described as fine regulators of gene expression, it is now believed that single microRNAs can have dramatic effect on genome homeostasis, especially in disease state.

1.4.2 MicroRNAs role in transcriptome homeostasis

MicroRNAs are gene expression inhibitors, acting at the post-transcriptional level by directing the RISC complex to target mRNAs via imperfect pairing with its 3' untranslated regions (UTR) (Bartel, 2004; Guo *et al.*, 2010). MicroRNAs embedded in the Ago2-miRISC serves as a guiding strand for RISC towards target mRNAs, and exert their inhibitory function inducing translational inhibition, mRNA de-adenylation and decay (Figure 1-5). RISC-mediated microRNAs inhibition requires an almost perfect complementarity between the first 7-8 nucleotides at the 5' end of the microRNA and the mRNA target, in a region called seed (Grimson *et al.*, 2007). MicroRNA target sequences lie mostly within the 3' UTR of mRNAs, and multiple sites can often be found. Globally, sites in the coding region appeared to be generally less effective at repressing mRNAs, and accumulating evidences suggest that these may escape evolutionary pressure (Fang and Rajewsky, 2011). The RISC-mediated direct cleavage of the endogenous mRNA is a common mechanism of plant microRNAs, thanks to the almost perfect complementarity with their target. In animals, however, the complementarity is mostly limited to the seed and additional 3' sites, and mRNAs target decay is induced by a different mechanism. MicroRNAs-loaded RISC recruits factors promoting de-adenylation like CCR4–NOT via GW182-dependent (Chekulaeva *et al.*, 2011). Loss of the poly-A tail causes the mRNA to be degraded (Couttet *et al.*, 1997), consistently with the observation that knock out/in of specific microRNAs revealed a detectable variation in mRNAs dependent on

the presence of target sequences (Baek *et al.*, 2008; Guo *et al.*, 2010; Lim *et al.*, 2005).

MicroRNAs inhibit cap-dependent translation at the initiation step via interference with the cap recognition process. Notably microRNAs failed to inhibit IRES-dependent translation, which does not rely on the m7Gppp cap to recruit the translational machinery (Fabian *et al.*, 2010; Filipowicz *et al.*, 2008). Whether microRNAs can inhibit translation at the elongation, as proposed by Maroney and colleagues, and at the termination step is controversial (Maroney *et al.*, 2006).

1.4.3 Finding the targets of specific microRNAs

1.4.3.1 In silico *predictions and gene expression analysis*

A few rules apply to the interaction between microRNAs and their targets. Pairing at the seed region must be perfect, and comprising at least nucleotides 2 to 8. The presence of bulges and imperfect match in the central portion of the duplex precludes the cleavage of the target mediated by Ago, however additional complementarity at the 3' end increases the repressive efficiency of the microRNAs, when the seed interaction is below 8 nucleotides (Grimson *et al.*, 2007).

The reduced length and the presence of variable degree of complementarity hinder the possibility of exact target prediction via *in silico* sequence analysis.

A variety of algorithms exist for identification of microRNAs target genes (Table 1-2). These take into account the degree of complementarity to the miRNA seed sequence, conservation of the microRNA seed element within the target gene across different species, free energy of the miRNA-mRNA duplex binding, and accessibility of the target site. Predictions based on evolutionary conserved seed-matching identified hundreds of potential targets for each microRNA (Lewis *et*

al., 2005). The extent of false-rate prediction is difficult to assess, and oscillates between 30% and 50% (Alexiou *et al.*, 2009; Thomas *et al.*, 2010).

It has to be kept in mind that the function of individual microRNAs depends on the molecular context: each cell type expresses a limited repertoire of genes that can be under the control of single microRNAs.

Additional *in silico* strategies include the mining of extensive gene expression databases in order to identify mRNAs profiles compatible with microRNAs targeting (Alexiou *et al.*, 2010; Antonov *et al.*, 2009; Le Brigand *et al.*, 2010; van Dongen *et al.*, 2008).

Table 1-2: List of *in silico* microRNAs target prediction tools. (Yokoi and Nakajima, 2011)

DIANA microT	http://diana.cslab.ece.ntua.gr/microT/
EMBL-Target Gene Prediction	http://www.russelllab.org/miRNAs/
MicroCosm	http://www.ebi.ac.uk/enrightsrv/microcosm/htdocs/
MicroRNAdb	http://bioinfo.au.tsinghua.edu.cn/micrornadb/
miRanda	http://www.microrna.org/microrna/home.do
miRBase	http://microrna.sanger.ac.uk/
miRGator	http://genome.ewha.ac.kr/miRGator/
mirnaviewer	http://cbio.mskcc.org/mirnaviewer/
miRWalk	http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/
PicTar	http://pictar.org/
PITA	http://genie.weizmann.ac.il/pubs/mir07/index.html
RNA22	http://cbcsrv.watson.ibm.com/rna22.html
RNAhybrid	http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/
TargetRank	http://hollywood.mit.edu/targetrank/
TargetScan	http://www.targetscan.org/

1.4.3.2 *Experimental identification of microRNAs targets*

A variety of experimental approaches have been set up in order to identify the real and biologically relevant targets of microRNAs, as reviewed by Thomson and colleagues (Thomson *et al.*, 2011).

Over expression or knock down of specific microRNAs coupled with microarray analysis allowed to observe the changes induced by that microRNA in model cell lines (Lim *et al.*, 2005). However these strategies have several caveats, in fact not all biologically relevant targets may be expressed in the cell line of choice. The analysis of seed matching sequences in down regulated genes helped to discriminate potential direct from indirect targets, however in most cases it was not assessed whether the observed effects were mediated directly by the microRNA, or were rather a result of unspecific regulation.

Conversely, biochemical approaches involving the co-immunoprecipitation of mRNAs with RISC components combined with over expression of a specific microRNA (cell re-programming) could in principle identify direct microRNAs targets (Hendrickson *et al.*, 2008; Karginov *et al.*, 2007; Matkovich *et al.*, 2011; Tan *et al.*, 2009). However these procedures are difficult to set up and rely on sufficiently stable interactions between the mRNAs and the RISC components.

Cross-linking aided protocols have been developed to overcome this potential drawback, such as the high-throughput sequencing of RNA isolated by crosslinking immunoprecipitation HITS-CLIP (Chi *et al.*, 2009) and the photoactivatable-ribonucleoside-enhanced crosslinking and immunoprecipitation (PAR-CLIP) (Hafner *et al.*, 2010). However it is not clear which percentage of the pulled down targets are specific for a single microRNA.

In order to address this problem Orom and Lund have successfully identified the direct targets of miR-10a by using a biotinilated synthetic microRNA as bait for their pull down experiment in (Orom and Lund, 2007) in *Drosophila* and

human cells. However whether the biotin group had an effect on the microRNA functionality was not elucidated.

1.4.4 Alternative mechanisms of microRNA function

Noteworthy, microRNAs not only inhibit gene expression, but also mediate translational activation of a few target genes in specific cell state, such as quiescent cells in G0, germline differentiation, ribosomal protein, HCV RNA etc. (Vasudevan, 2011). These effects can be sequence-dependent (i.e. microRNA-mediated recruitment of activator RNPs), or sequence-independent, (i.e. no direct interaction between the microRNA and the transcriptionally activated/stabilized mRNA). A number of microRNAs were shown to have direct stimulatory activity on the translation of their target mRNAs: miR-122 and miR-10a possess translation activation properties on specific transcripts by pairing at the 5'UTR of respectively HCV and ribosomal genes (Henke *et al.*, 2008; Orom *et al.*, 2008). Others, such as miR-125b and miR-466l mask the binding site for the ARE-decay factor tristetraprolin (TTP) on their respective targets κB-RAS2 and IL10 (Figure 1-6C) (Ma *et al.*, 2010; Murphy *et al.*, 2010). Generic features of direct transcriptional activation mediated by microRNA/mRNA direct interaction include the modification of RNPs recruited by microRNAs/Ago2 complex, such as lack of GW182/PABP complex, recruitment of activator proteins FXR1-iso-a (Figure 1-6A) or interference with regulatory AU-rich elements, shortened or absent polyA (Figure 1-6B) and binding to a 5'UTR site in closer proximity to the cap (Figure 1-6B). MicroRNAs can also act as decoy for repressive proteins and relieve the repression on their targets in a sequence independent manner, such as the case of pre-miR-328 interacting with the RNA inhibitory hnRNP. This rescues the repression of Cebpa mediated by hnRNP in chronic myelogenous leukemia via the miR-328 C-rich cluster, similar to the one present in the 5' UTR of the Cebpa mRNA, shown in Figure 1-6D (Eiring *et al.*, 2010).
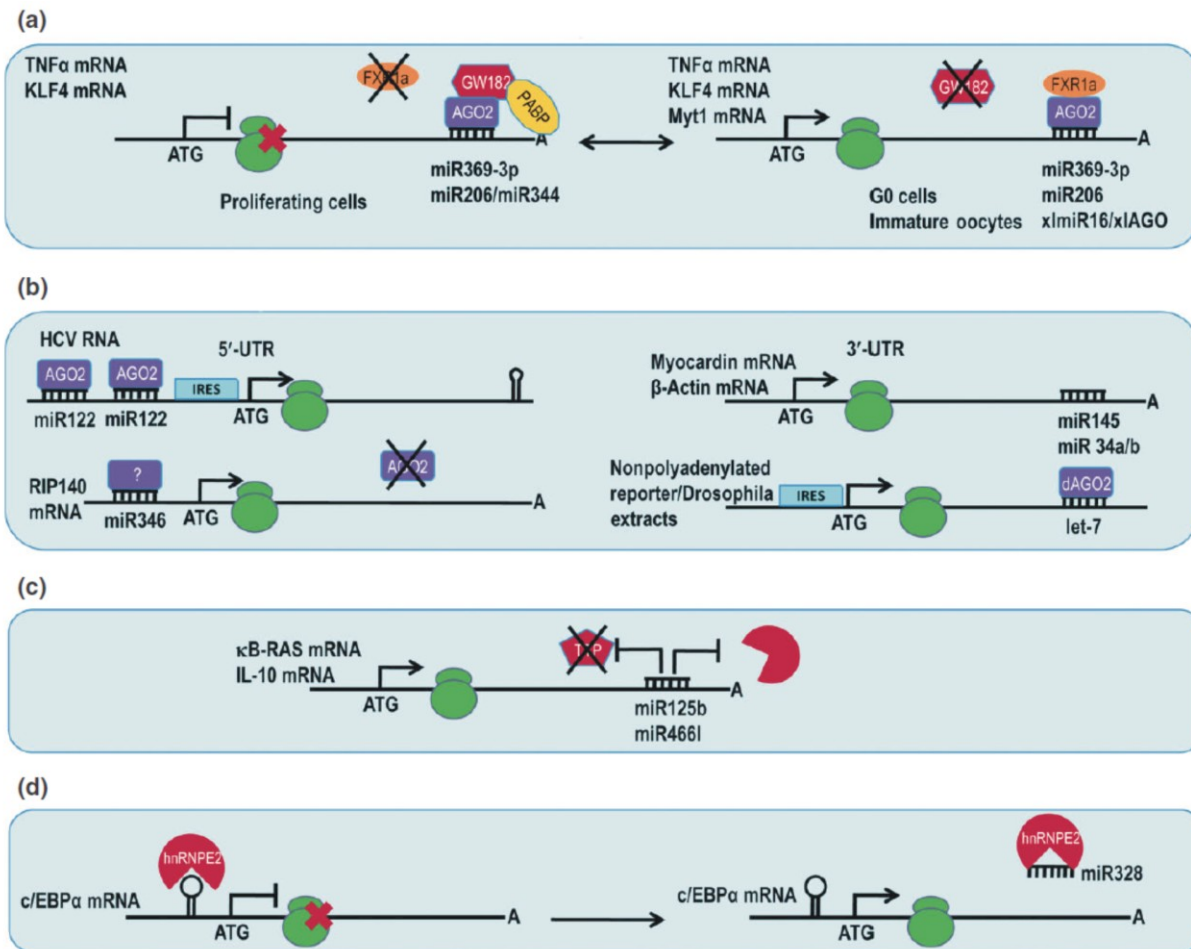
Figure 1-6: Mechanisms for microRNAs-mediated post-transcriptional activation (Vasudevan, 2011).

Some non-functional pseudo-genes have been shown to possess a buffer activity on their functional homolog, and accumulating evidences suggest that these conserved microRNA targets sequence would be under evolutionary pressure in order to release the microRNA-mediated inhibition on their homolog gene (Cesana *et al.*, 2011; Poliseno *et al.*, 2010; Sumazin *et al.*, 2011).

MicroRNAs were also found in the nucleus of cells, but their function is at present not fully understood.

The splicing regulator MBNL1 is implicated in myotonic dystrophy (MD), and it has been recently shown to bind to pre-miR-1 in the nucleus and promote its maturation in normal conditions. In diseased conditions, MBNL1 is sequestered

and as a consequence the levels of mature miR-1 are decreased, suggesting that miR-1 could be an effector of the cardiac defects observed in MD patients via its targets (Rau *et al.*, 2011). This also suggests that microRNAs could bind to transcriptional regulators in the nucleus, and have downstream effects on transcriptional gene regulation. Another possibility is that nucleus compartmentalization of either the mature or precursor microRNAs could be a further layer of post-transcriptional regulation of microRNAs activity.

Evidences exist that Ago2 recruitment at the promoter of specific genes, driven by complementarity with guide microRNA, is a functional mechanism in mammals (Li *et al.*, 2006; Younger and Corey, 2011), and was suggested that microRNAs can direct transcriptional activation or inactivation of genes with complementary sequences in their promoters or regulatory regions (Kim *et al.*, 2008; Place *et al.*, 2008). Whether microRNAs-mediated gene regulation at the transcriptional level is a physiological and common layer of gene regulation in animals, similar to the RNAi-induced transcription silencing (RITS) characterized in yeast, is a hypothesis that opens complete new avenues of investigations for the role of non-coding RNAs (Li *et al.*, 2006; Woolcock *et al.*, 2011; Younger and Corey, 2011).

1.4.5 Additional Considerations

In order to minimize the risks and perform predictive experiments, researchers must be confident about the human relevance of the experiments performed in test animals. Unfortunately the test species useful for drug development (rat, canine and non-human primates) are not thoroughly characterized: extensive genomic annotation, detailed proteomics data, gene expression, epigenetic marks and transcription factors profiles are lacking. Given the proven impact of microRNAs in multiple layers of gene regulation, it makes sense to accumulate knowledge on

their role in species of interest for biomedical research and, in our case, in cardiovascular safety.

1.5 MicroRNAs detection and quantification

In the last ten years all high-throughput techniques available for transcriptomics have been adapted for microRNAs detection. These include quantitative PCR (qPCR), hybridization-based techniques and next-generation sequencing (NGS) (Table 1-3).

Table 1-3: MicroRNAs detection platforms. AQ: absolute quantification; RQ: relative quantification

		Single assay	Whole genome	AQ	RQ	*A priori* knowledge	Input material	Analysis costs
qPCR	Single assays	x		x	x	yes	Low	Low
	Pre-spotted plates		x	?	x	yes	Medium	Low
Hybridization	MicroArrays		x	?	x	yes	Medium	Medium
	Panomics	x		x	x	yes	High	Low
	Nanostring	x		x	x	yes	Low	Low
	Northern blot	x			x	yes	Low	-
	ISH	x				yes	-	-
NGS	DGE		x	?	x	no	Medium	Intense

1.5.1 Quantitative PCR (qPCR)

Advantages of qPCR-based microRNA profiling include relative ease of use, low demands for amount of starting material, high sensitivity, straightforward protocol for sample preparation and data analysis. Since microRNAs are too short to design a regular couple of primers, different strategies illustrated in Figure 1-7A-C can be used to increase the length of the amplicon *via* ligation, or reverse-transcription. Then, a microRNA specific primer is mixed with a universal primer that recognizes the extended 3' tail during the PCR reaction. Differentially regulated candidates obtained by high-throughput qPCR can be further confirmed by other means, or justified by biological consistency (i.e. co-regulation with host

genes in the case of intronic microRNAs). However, the specific stem-loop primer used to extend microRNAs length during reverse transcription can fail to recognize length variants (Figure 1-8), decreasing the qPCR sensitivity. When length variants could be present in the sample, the use of a mixture of degenerated RT primers can help to reverse transcribe all microRNAs irrespectively of their 3' end.
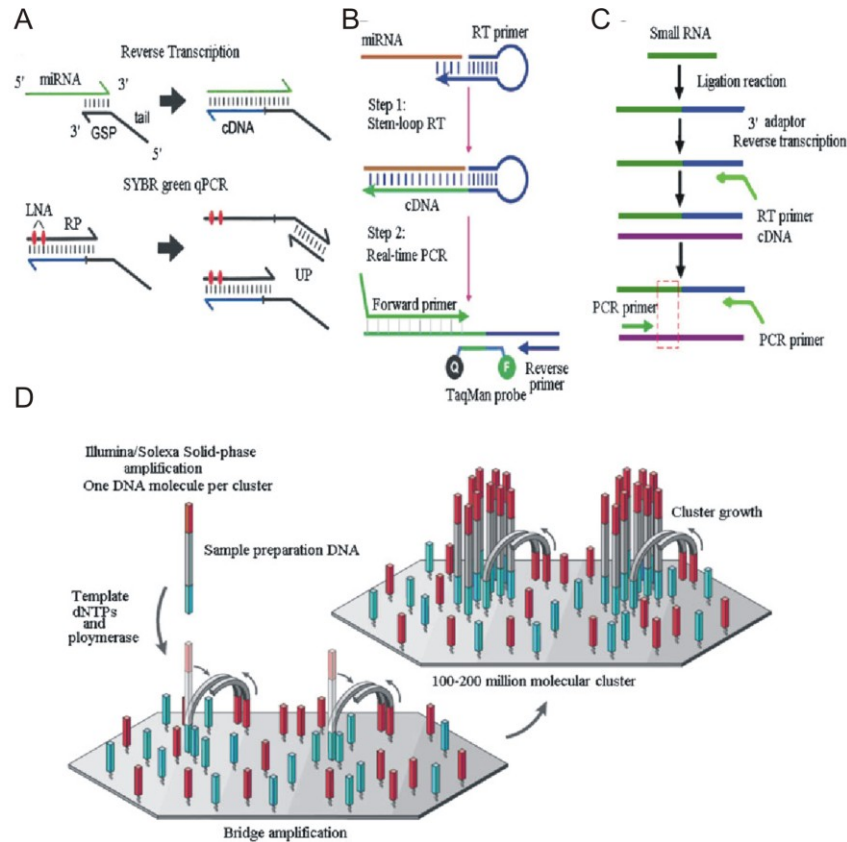
Figure 1-7: MicroRNAs detection. Schemes of three kinds of RT-PCR methods: Primer extension (A), stem-loop primer (B), and 3' adapter ligation (C). Solid-phase amplification of Illumina small RNA libraries (D). Adapted from (Huang *et al.*, 2011).
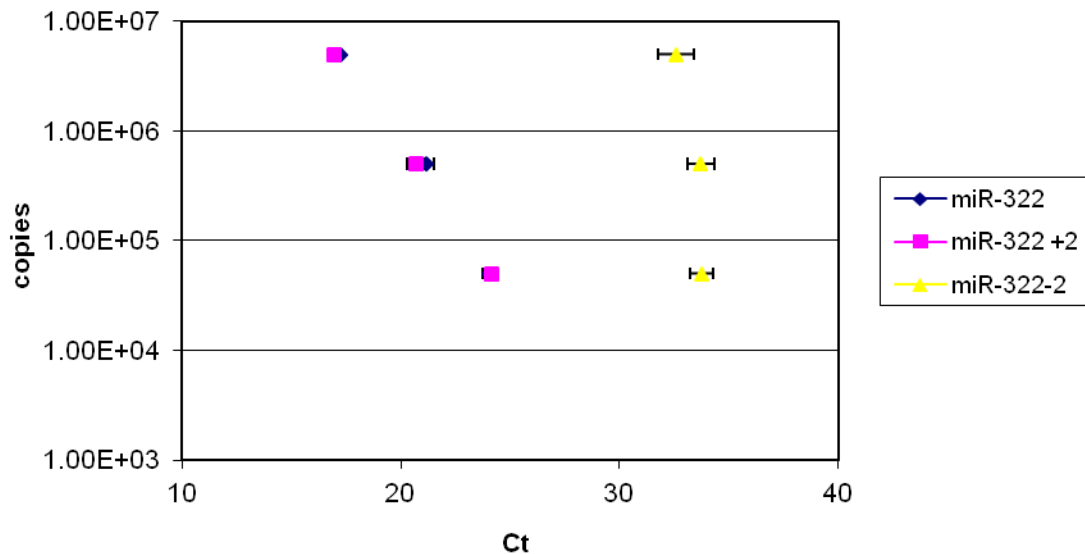
Figure 1-8: Sensitivity of Taqman single assay RT and qPCR on miR-322 length variants. Shorter microRNAs variant may not be efficiently reverse transcribed by a single microRNAs specific RT primer.

1.5.2 Hybridization based techniques

Hybridization-based methods have been used since many years, and microarray technology adapted to microRNAs is offered by a variety of providers (Affymetrix, Agilent, Illumina, Exiqon, Toray). These technologies suffer from unspecificity issues intrinsic of all hybridization-based technologies (non-uniform Tm, normalization strategies, controversial specificity at the single nucleotide level, GC content), exacerbated by the fact that microRNAs are not amenable to multiple probe sets design, differently from mRNAs. Furthermore, the restricted number of microRNAs analyzed in each chip and the lower signal intensity due to inferior labeling efficiency require special attentions during quality control, between-chips normalization and analysis algorithms (Sarkar *et al.*, 2009). Pradervand *et al.* and Sato *et al.* tested the performances of different microRNAs microarray providers (Affymetrix, Agilent, Ambion, Exiqon, Invitrogen and Toray) and concluded that Agilent had a superior performance, reproducibility

and comparability within same platform and with other platforms (qPCR and NGS) (Pradervand *et al.*, 2010; Sato *et al.*, 2009).

Sample preparation can be a cause of biases, due to enzyme-based tagging or pre-amplification, and Nanostring and Panomics technologies are promising in that the isolated small RNAs are directly assessed without any further sample preparation step and at high sensitivity. However, these technologies may suffer from specificity issues common to virtually all hybridization-based platforms.

Northern blot is the ultimate and traditional technique for single microRNAs quantification, but it is a targeted approach and requires previous knowledge of the microRNA of interest. It is often used in confirmatory follow-up experiments.

In situ hybridization with either fluorescence or chromogenic detection is used for specific localization of relevant microRNAs within a fixed or frozen tissue section (Nuovo, 2008, 2010; Nuovo *et al.*, 2009; Obernosterer *et al.*, 2007). This technique is still time consuming, requires extensive troubleshooting via optimization of conditions, and cannot be used as absolute quantification method. Furthermore sample prep conditions (fixation), age of animal, reagents used etc can introduce a potentially high variability of results depending on.

1.5.3 Next-generation sequencing

The current method of choice to investigate small RNA expression is the NGS approach, also called digital expression profiling (DGE). In contrast to other methods, NGS profiling is hybridization-independent, can discriminate microRNA family members with single nucleotide differences, and capture 5′/3′ end variability (for example, isoMirs). Furthermore, sequencing does not require *a priori* information, and can be used to simultaneously detect known and discover new microRNAs (Linsen *et al.*, 2009). Small RNA NGS is based on high-throughput DNA sequencing of libraries captured and ligated with adaptors necessary for building clusters inside the flow-cells (Illumina), for recognition of the sequencing beads (in the case of SOLiD) and for priming the synthesis (Figure

1-7D). Mammalian cells express a few hundreds of microRNAs at any given time. This is a relatively small number compared to a standard gene expression profile, and multiplexing of microRNAs samples guarantees a sufficient coverage. This is achieved by indexing (or barcoding) the adapters used for each sample with additional nucleotide sequences that can unequivocally assign the reads, allowing simultaneous analysis of a pool of samples in the same flowcell. However, normalization and data analysis is computationally expensive and requires specific bioinformatics knowledge. Sample preparation is time consuming and requires manual steps, like band excision from gel, which can easily be source of batch-to-batch differences.

1.5.4 Additional Considerations

As reviewed in (Linsen *et al.*, 2009) it is controversial whether qPCR, arrays or NGS are suited for absolute quantification. The extensive sample preparation that includes enzyme based reactions (ligation, reverse transcription, starting material amplification, gel purification) can bias the population of microRNAs measured, as exemplified in the Supplementary Results of Chapter 2. However there is general good concordance of relative expression across the different platforms, and especially between qPCR and NGS, also reported in Chapter 3 (Pradervand *et al.*, 2010).

1.6 MicroRNAs in cardiac physiology and diseases

1.6.1 Implications of cardiac microRNAs in organ development and diseases

MicroRNAs are required for correct cardiovascular development and function, and *Dicer* conditional deletion causes heavy developmental defects and spontaneous dilated cardiomyopathy mice (Chen *et al.*, 2008b; da Costa Martins *et al.*, 2008; Rao *et al.*, 2009). Among the most abundant microRNAs in the heart,

miR-1 and miR-133 are encoded from the same precursor (miR-1-2/miR-133a-1).
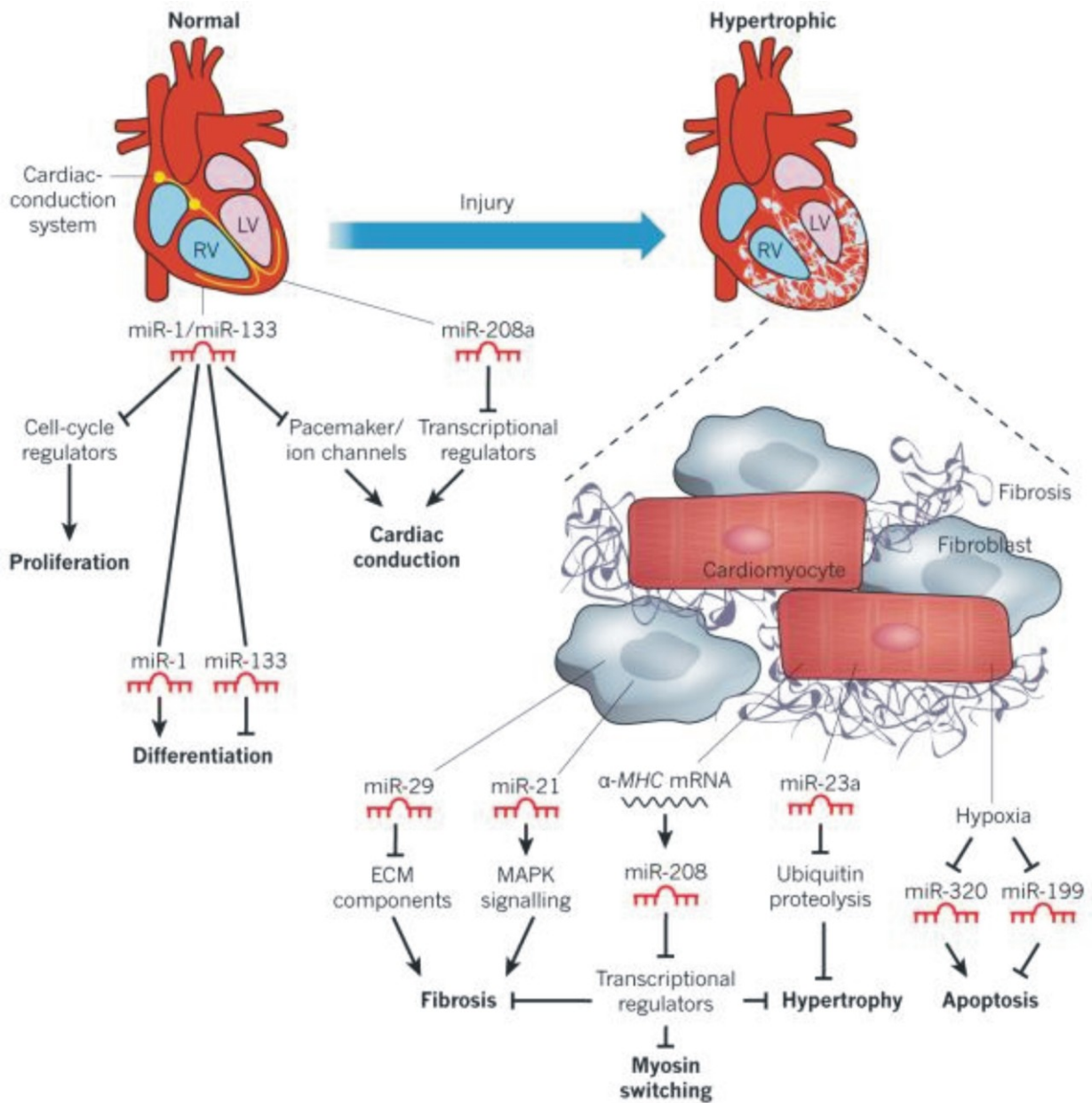
Figure 1-9: Functional role of microRNAs in normal and diseased heart. MicroRNAs are depicted as bulged red ribbons. Arrows indicated activation, while blunted lines indicate inhibition of the downstream elements. Cardiomyocytes and fibroblasts are represented. Adapted from (Small and Olson, 2011).

They are however under the control of transcription enhancers responsive to muscle differentiation factors that include Srf, MyoD and Mef2 (Liu *et al.*, 2008; Liu *et al.*, 2007; Zhao *et al.*, 2007; Zhao *et al.*, 2005).

MiR-1 and miR-133 have opposing effects in the cardiac lineage fate: miR-1 induces differentiation, while miR-133a stimulates proliferation (Ivey *et al.*, 2008). Although their knock-down caused heavy developmental defects, none of the two microRNAs were absolutely required, as 50% of heart-specific knock down mice are viable (Liu *et al.*, 2008; Zhao *et al.*, 2007).
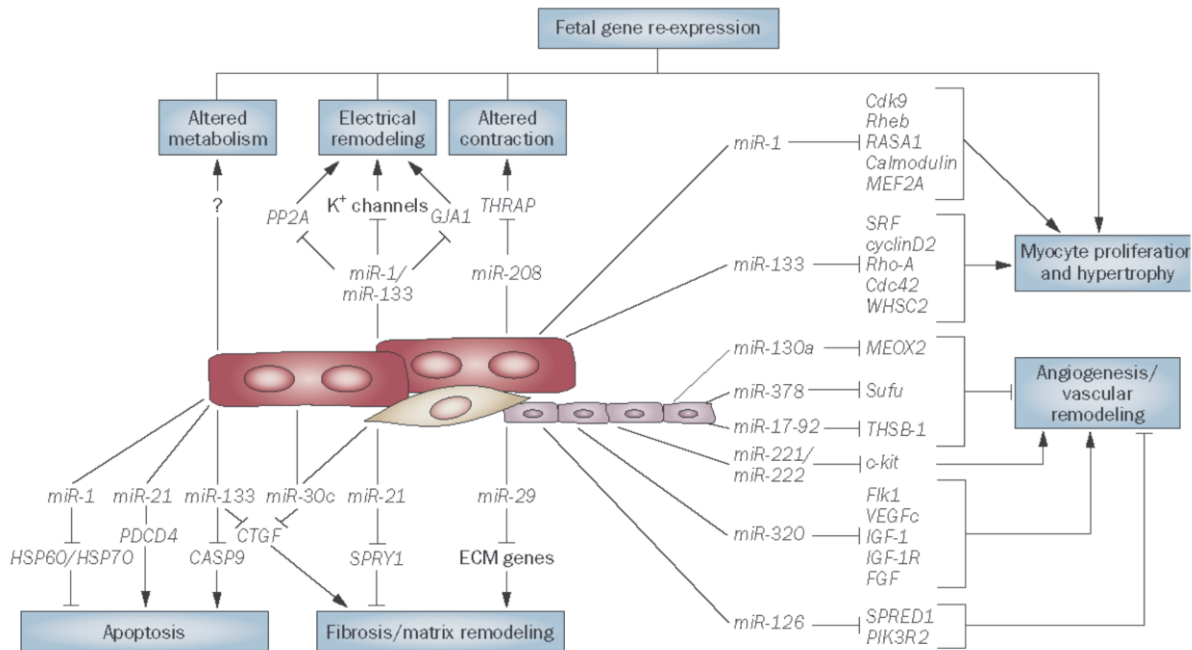
Figure 1-10: MicroRNAs and genes implicated in cardiac diseases. Different cell types involved in cardiac remodeling are indicated: cardiomyocytes (squared red), fibroblasts (speared beige), and endothelial cells (small pink). Adapted from (Latronico and Condorelli, 2009).

Moreover, microRNAs are implicated in cardiac diseases of different etiologies (Catalucci *et al.*, 2009; Kinet *et al.*, 2012; Latronico and Condorelli, 2009).

Several microRNAs have the ability to influence cardiac remodeling through specific effects on signaling pathways (mir-133, mir-1, miR-9, miR-98, mir-199b, miR-23a), sarcomere organization (miR-208a/b, miR-499) and inter-cellular communication (miR-29 and mir-21) (Small and Olson, 2011) (Figure 1-9). Genes that are actively targeted by cardiac microRNAs include regulators of metabolism, electrical conduct, proliferation/hypertrophy and angiogenesis

(Figure 1-10). MiR-1 was described as a potential arrhythmogenic since it can target connexin 43 (GJA1) (Figure 1-10) and the K+ channel subunit Kir2.1 (KCNJ2), and its deletion ameliorates recovery in post-infarction rodent models (Yang *et al.*, 2007).

Induction of miR-208a was sufficient to induce arrhythmias, and this microRNAs was implicated in cardiac performance and maintenance of conduction via targeting of two regulators of muscle growth and hypertrophy, namely thyroid hormone-associated protein 1 (Thrap1) and myostatin (Mstn) (Figure 1-10). Mir-21 and miR-29 were shown to be induced by fibrotic stimuli (i.e. after myocardial infarction) and mediated extra-cellular matrix (ECM) deposition (Thum *et al.*, 2008; van Rooij *et al.*, 2008).

In addition, genetic polymorphism in pre-mir-499 and pre-miR-196a-2 are associated to human dilated cardiomyopathy (Lu *et al.*, 2008).

1.6.2 Cardiac microRNAs and pathological heart remodeling

Pathological (irreversible) cardiac hypertrophy is caused by a variety of stimuli (i.e. hypertension, valvular heart disease, infarction, obesity, exaggerated pharmacology) and may cause heart failure. The disease progression is complex and involves several stages of molecular and histological remodeling (distinct from physiological hypertrophy due to exercise and pregnancy). At first, hypertrophic growth of cardiomyocytes and thickening of ventricular walls compensate for the increased demands (with or without chambers' restriction), however sustained pathological stimuli can lead to a functional decompensation due to progressive thinning of the ventricular walls (Hill and Olson, 2008; Hoshijima and Chien, 2002; van Rooij and Olson, 2007). The transition from physiological to pathological is accompanied by re-expression of fetal genes and a switch in myosin isoforms (in rats Myh6 is down-regulated and Myh7 is up-regulated). Interestingly, three microRNAs called myomiRs (miR-208a, miR-208b and mir-499) are co-transcribed within the introns of myosin genes. In

particular, a variety of stressors, including pressure overload and thyroid hormone cause activation of the fetal Myh7 (and miR-208b) transcription in adult rodents in a miR-208a dependent manner (van Rooij *et al.*, 2009). Another cardiac microRNA, miR-133a was linked to cardiac hypetrophy: inhibition of miR-133 via infusion of an antisense construct *in vivo* caused sustained cardiac hypertrophy in rats. The authors identified the GDP-GTP exchange factor RhoA, the kinase Cdc42 and a cardiopoietic nuclear factor Whsc2 as direct targets of miR-133a (Carè *et al.*, 2007) (Figure 1-10).

A calcineurin-dependent transcriptional pathway has been shown to decrease apoptosis in a mice model being therefore beneficial for heart failure recovery (De Windt *et al.*, 2000). However, the calcineurin-dependent nuclear factor NFATc3 can induce the over-expression of the pro-hypertrophic miR-23a upon β-adrenergic hypertrophic stimulation. The knock down of miR-23a was sufficient to relieve the isoproterenol induced enlargement (Lin *et al.*, 2009).

1.6.3 Additional Considerations

Accumulating evidences strongly implicate microRNAs as key mechanistic players in cardiac pathways and regulatory circuits in both development and pathogenesis, and suggest microRNAs could be considered as provocative therapeutic targets (van Rooij and Olson, 2007). Interference with regulatory pathways can have multiple faceted effects, and microRNAs can be involved in the tight regulation required for proper homeostasis (i.e. miR-23 and calcineurin pathway mentioned above).

An important caveat in microRNAs research in cardiac diseases is the assumption that disease-related microRNAs would be found either over- or down-regulated in a diseased state. Jentzsch *et al.* performed a functional screening of pro-hypertrophic microRNAs in rat neonatal cardiomyocytes, by transfecting a library of human microRNAs and measuring the cell size by high content microscopy. They found that most of microRNAs that induced cardiomyocytes

hypertrophy (miR-212, miR-365, miR-22, miR-30c, miR-30d) were never found dysregulated in previous works (Jentzsch *et al.*, 2011). This intriguing observation will have to be further confirmed with mechanistic studies, since potentially not all transfected microRNAs could have been be functional in the cells tested and that an isolated cell model often does not recapitulate *in vivo* findings.

1.7 MicroRNAs in toxicology

MicroRNAs are considered important genome regulators, and their modulation may be prodromal to gene expression changes. This is of particular interest in early detection of toxic liabilities and ultimately to explain the mechanisms of undersired effects in the affected tissues.

Systematic toxicological studies involving microRNAs were performed mainly with hepato- and neuro- toxicants and carcinogens, while cardiac toxicity was not thoroughly investigated. A collection of toxicological studies has been illustrated by Lema and Cunningham (Lema and Cunningham, 2010) (Table 1-4).

Table 1-4: microRNAs and toxicology. (↑) up-regulation; (↓) down-regulation. (Lema and Cunningham 2010)

Organism	Toxicant	miRNA	Target	Biologic effect	Reference
Mouse	Wy-14,643, peroxisome proliferator-activated receptor alpha (PPARα) agonist	↑let-7C	Transcription factor, c-myc	Hepatocyte proliferation	Shah et al. (2007)
Mouse	Ethanol	↓miR-21, -335, -153	Notch-receptor ligand, Jagged-1, brain-specific regulator of RNA stability, ELAVL2 (embryonic-lethal abnormal vision, Drosophila-like 2)	Induction of cell cycle and stem cells maturation	Sathyan et al. (2007)
Mouse	Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), environmental contaminant	↑let-7, miR-15, -16, -26, -181; ↓miR-10b	Cell cycle regulators	Regulation of tumor pathogenesis	Zhang and Pan (2009)
Mouse	Hexahydro-1,3,5-	↑miR-206, -30,	Brain-derived	Neurotoxicity	Zhang and Pan

Organism	Toxicant	miRNA	Target	Biologic effect	Reference
	trinitro-1,3,5-triazine (RDX), environmental contaminant	-195	neurotrophic factors (BDNFs)		(2009)
Rat	Tamoxifen	↑miR17-92 cluster, -106a, -34	Cell cycle regulators, chromatin modifiers and expression regulators	Hepatocarcinogenesis	Pogribny et al. (2007)
Rat	Cigarette smoke	↑miR-294	Transcriptional repressor genes	Altered gene expression	Izzotti et al. (2009)
Rat	Cigarette smoke	↓let-7c, miR-34c, -222	Genes involved in stress response and cell proliferation	Carcinogenesis	Izzotti et al. (2009)
Rat	Acetaminophen; Carbon tetrachloride	↓miR-298, -370	Thioredoxin reductase 3	Hepatotoxicity	Fukushima et al. (2007)
Rat	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), tobacco carcinogen	↓miR-126	Cytochrome P450 (CYP) 2A3	Tumorigenesis	Kalscheuer et al. (2008)
Human	1alpha,25-dihydroxyvitamin D(3) (1,25(OH)(2)D(3)), major vitamin D metabolite	↑miR-125b	Endogenous vitamin D receptor (VDR)	Augmentation of the antitumor effects of 1,25(OH)(2)D(3)	Mohri et al. (2009)
Human	Sodium arsenite	↓miR-210	Cellular methyl-donor pool	Alterations of genomic methylation	Marsit et al. (2006)
Human	Cigarette smoking	↑miR-218	Transcription factor, MAFG (v-maf musculoaponeurotic fibrosarcoma oncogene homolog G)	Expression of MAFG targets	Schembri et al. (2009)
Human	Iron- and aluminum-sulfate	↑miR-9, -125b, -128	Regulatory and pathogenic genes	Neurotoxicity	Lukiw and Pogue (2007)
Human	5-fluorouracil (5-FU), antineoplasic drug	↑miR-200b	Protein tyrosine phosphatase (PTPN12)	Inactivation of oncogenes products (c-Abl, Src or Ras)	Rossi et al. (2007)

1.7.1 MicroRNAs role in cytochrome toxicity

Cytochromes and nuclear receptors are pivotal genes in pharmacodynamics and toxicology. Recent works suggest a number of these genes may be regulated by microRNAs (Figure 1-11) (Yokoi and Nakajima, 2011). MiR-27b, miR-298, miR-

24, miR-34a and miR-148a were implicated in either direct or Hnf4/Pxr-mediated regulation of Cyp3a4 (Pan *et al.*, 2009; Takagi *et al.*, 2010; Takagi *et al.*, 2008). Interestingly, Cy1b1 and RXRα were also found to be modulated by miR-27b (Ji *et al.*, 2009a; Tsuchiya *et al.*, 2006). Cyp2e1, the most abundant cytochrome in human liver, is over expressed in diabetes and obesity and down-regulated by insulin treatment. The capability of miR-378 to decrease Cyp2e1 was first described by Mohri and colleagues, and is likely to have toxicological relevance (Mohri *et al.*, 2010). Cyp2a3 is responsible for the metabolic activation of the nicotine-derived pro-carcinogen nitrosamine ketone (NNK) α-hydroxylation. Kalscheuer and colleagues found that one of the microRNAs down regulated by NNK chronic administration in rat lungs, miR-126*, was also directly targeting Cyp2a3 (Kalscheuer *et al.*, 2008), suggesting the implication of miR-126* in NNK carcinogenic potential. Cy24a1 and vitamin D receptor are involved in calcium homeostasis of solid tumors and are directly inhibited by miR-125b, which is itself modulated in a variety of tumors (Komagata *et al.*, 2009; Mohri *et al.*, 2009).
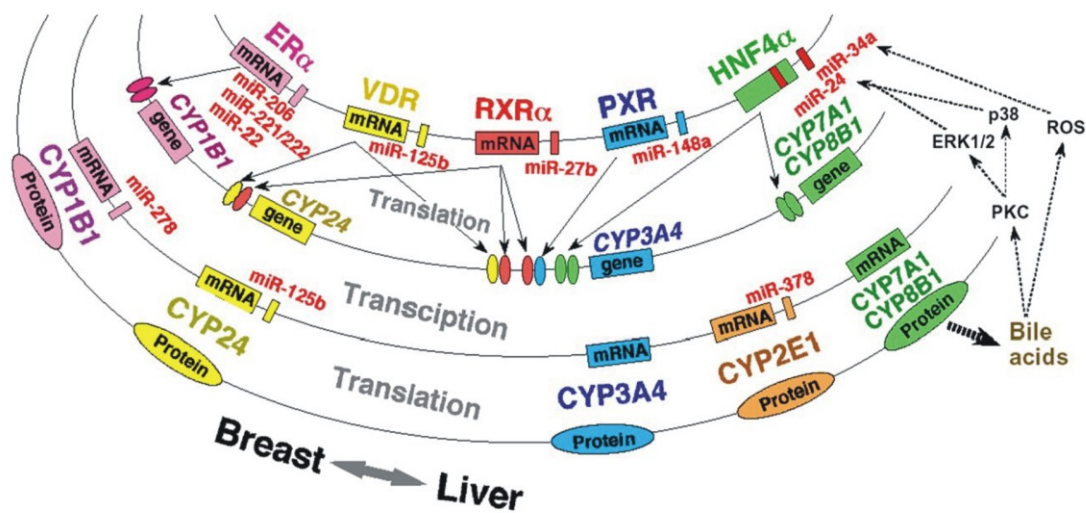
Figure 1-11: MicroRNA-related networks of cytochrome P450 and nuclear receptors (Yokoi and Nakajima 2011).

1.7.2 Xenobiotics-induced carcinogenesis

MicroRNAs expression was found altered upon xenobiotics administration, including cigarette smoke, tamoxifen, acetaminophen, ethanol, hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), dioxin, arsenite and carbon tetrachloride (Fukushima *et al.*, 2007b; Izzotti *et al.*, 2010; Marsit *et al.*, 2006b; Pogribny *et al.*, 2007; Sathyan *et al.*, 2007; Schembri *et al.*, 2009; Yoshioka *et al.*, 2011; Zhang and Pan, 2009), however the impact of such changes on toxicity was often not investigated.

Carcinogenesis is a common toxic outcome of xenobiotics exposure (Pogribny *et al.*, 2007). Pparα signaling has been implicated in hepatocytes proliferation and tumor development. Shah and colleagues reported that let-7c was down regulated by administration of a PPARα agonist. Let-7c modulated c-myc cascade by directly inhibiting the c-myc oncogene via 3'UTR targeting. Thus let-7c appeared to be critical in PPARα -induced liver proliferation (Shah *et al.*, 2007).

Arsenic is known to induce bronchial epithelium carcinoma, via not completely understood mechanism. Wang *et al.* found that miR-200b was heavily down-regulated in epithelial cells undergoing arsenic-induced epithelial-to-mesenchimal transition (EMT). Re-expression of miR-200b relieved the carcinogenic phenotype in vitro and tumorigenicity of arsenic exposed cells in a xenograft model (Wang *et al.*, 2011c).

Another cause of malignant bronchial epithelial cells transformation is exposure to benzopyrene. Silencing of miR-106a suppressed benzopyrene-induced cell proliferation, promoted apoptosis and cell cycle arrest. Retinoblastoma1 (Rb1) was confirmed as miR-106a target by luciferase assay. These results suggest miR-106a may work as an oncogene in chemical carcinogen transformation (Jiang et al., 2011).

1.7.3 Additional Considerations

As for transcriptomics, confirming causal implication of microRNAs in tissue injury requires careful *in vivo* study design. Drug-induced expression changes of microRNAs should be monitored at early stages, before overt histopathological readouts are present. Also, dosing scheme and duration should mimic foreseen applications in patients. Including several time points and a washout phase can help observing microRNAs being affected earlier than others, or that persist altered even after discontinuation from the treatment.

Noteworthy, lack of extensive characterization of microRNAs expression in different cell types and their multiplicity of downstream effects, hinder the possibility to build up cell type or functional lists of microRNAs, as it is instead possible with regular genes (expression atlas and gene ontology). Systematic target discovery/validation and detailed expression profiling of tissues cell types will enable a direct interpretation of changes in a network of microRNAs and mRNAs.

In conclusion, the implication of microRNAs in cardiotoxicity is still to be investigated.

1.8 Circulating microRNAs: from intercellular signaling and organ injury biomarkers

1.8.1 The discovery and function of microRNAs in body fluids

In recent years, microRNAs were detected and characterized in several cell-free fractions of mammalian blood, including vesicles (exosome, microparticles and apoptotic bodies), but also in protein associated fractions, and globally called circulating microRNAs (Figure 1-12).

1.8.1.1 *Exosomes, microparticles and apoptotic bodies*

The presence of microRNAs in extracellular exosomes (50-90 nm) was first described by Valadi and collaborators in 2007 (Valadi *et al.*, 2007). The same study revealed the extreme stability and resistance of cell-free microRNAs to enzymatic degradation. Further studies identified microRNAs in microparticles (> 100 nm) and apoptotic bodies. In fact, studies conducted on mammalian cell lines suggest that extracellular and intracellular microRNAs pools are clearly distinct, since preponderant cytoplasmatic microRNAs are not the most abundant in the respective supernatant (Collino *et al.*, 2010; Ohshima *et al.*, 2010; Pigati *et al.*, 2010). Furthermore respiratory chain inhibitors (i.e. rotenone) alter the microRNAs release kinetic pointing to a potential energy-dependent microRNAs secretion pathway (Wang *et al.* 2010b).

Microvesicles are important mediators of cell-to-cell communications, as they contain protein and chemokines (Ratajczak *et al.*, 2006). In general, release of microRNAs form both exosomes and microparticles appear to be an actively regulated process, raising the possibility that vesicle-enclosed microRNAs may work as long-distance inter-cellular communication and regulation (Kosaka *et al.*, 2010). Several studies documented how monocyte-secreted microvesicles contain high levels of miR-150, and can deliver it to different cells and inhibit miR-150 target c-Myb (Xiao *et al.*, 2007; Zhang *et al.*, 2010). Pegtel *et al.* found that an Epstein-Barr virus specific microRNA could accumulate in non-infected MoDC cells when co-cultured with EBV-infected B cells, and that this process is mediated by exosome transfer (Pegtel *et al.* 2010). Recently, Hergenreider *et al.* showed how vesicles encapsulated miR-143/145 cluster produced by HUVEC can control targets in co-cultured human smooth muscle cells, opening new avenues for atherosclerosis treatment (Hergenreider *et al.*, 2012).

In addition to secreted vesicles, pools of microRNAs reflecting their cellular origin are also found in apoptotic bodies. Notably, miR-126 contained in

endothelial cells apoptotic bodies could mediate CXCL12 production in recipient vascular cells, thus mitigating atherosclerosis via incorporation of stem cell antigen-1 positive progenitor cells in atherosclerotic plaques (Figure 1-12) (Zernecke *et al.*, 2009).

1.8.1.2 *Membrane-free microRNAs complexes*

More recent works proposed that microRNAs are present in membrane-free fraction, mainly in association with protein complexes or high-density lipoproteins (HDL). Ago2/miRNA complexes were identified in cell culture media (Turchinovich *et al.*, 2011) and plasma (Arroyo *et al.*, 2011). Protein-associated microRNAs originate mainly from cell death by-products and are stable in the bloodstream. This is partially attributed to further association of particle-free microRNAs to nucleophosmin (NPM1). Intrestingly, specific microRNAs, such as miR-16 and miR-92a, preferentially associated with vesicle fractions, while others, like let-7a, rather co-purified with protein complexes (Wang *et al.*, 2010c). Human HDL was shown to contain microRNAs. Native HDL could incorporate microRNAs and deliver genetic material to recipient cells. RhoB and Efna1 mRNAs were decreased upon HDL-mediated miR-223 delivery in human cultured hepatocytes. HDL-microRNAs transfer was dependent on the known HDL receptor scavenger receptor class B type I (SR-BI). Furthermore, HDL obtained from familial hypercholesterolemia (FH) patients, contained a pattern of microRNAs that differed from control subjects, raising the possibility of using HDL-associated microRNAs as diagnostic markers (Vickers *et al.*, 2011).
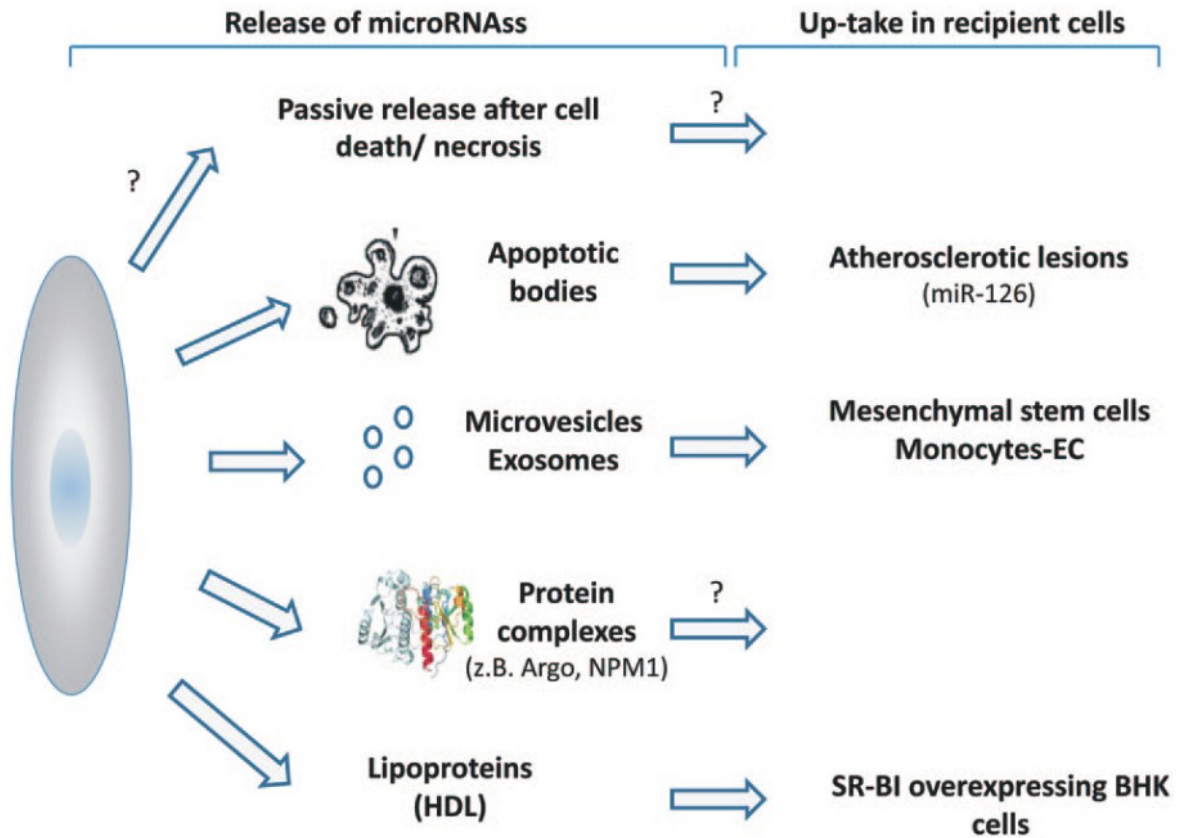
Figure 1-12: Circulating microRNAs. Mechanisms protecting microRNAs from degradation. EC indicates endothelial cells; HDL, high-density lipoprotein; NPM-1, nucleophosmin-1; SRB1, scavenger receptor class B member 1; BHK, baby hamster kidney (Fichtlscherer *et al.*, 2011).

1.8.2 Circulating microRNAs as tissue injury biomarkers

Circulating microRNAs have been proposed to be non-invasive, sensitive and specific tissue injury biomarkers, thanks to their relative stability in the blood stream and the easiness of quantification via qPCR. Upon injury of different etiologies (infectious, pharmacological, etc.) microRNAs are released in the circulation in apoptotic bodies, or in protein complexes, where are usually stable (Laterza *et al.*, 2009). A number of microRNAs are expressed in a tissue specific manner and can be relatively stable if released in body fluids. Profiling of circulating microRNAs as indicators of drug-induced tissue injury may help to reinforce histopathology and clinical chemistry endpoints. However not very

many microRNAs are known to be strictly tissue specific. MiR-122 is mostly expressed in the liver, miR-124 in the brain and miR-133a in heart and skeletal muscle (Laterza *et al.*, 2009). Furthermore tissues are constituted by a heterogeneous population of cells assembled in distinct histological structures, characterized by different molecular expression.

1.8.2.1 *Biomarkers of cardiovascular diseases*

A growing field for application of circulating microRNAs as tissue injury biomarkers concerns the cardiovascular system, summarized in Table 1-5 (Fichtlscherer *et al.*, 2011). Myonecrosis associated with acute myocardial infarction, acute coronary syndrome and viral myocarditis caused increase of miR-1, miR-133, miR-499 and/or miR-208 in the plasma of patients, as documented by several publications (Adachi *et al.*, 2010; Ai *et al.*, 2010; Cheng *et al.*, 2010; Corsten *et al.*, 2010; D'Alessandra *et al.*, 2010b; De Rosa *et al.*, 2011; Kuwabara *et al.*, 2011; Wang *et al.*, 2010b; Widera *et al.*, 2011). Notably, miR-208 is the most cardiac specific microRNA, since its expression is restricted to the myocardial tissues, whereas miR-1, miR-133 and miR-499 are also expressed in skeletal muscles (Ji *et al.*, 2009b). Furthermore, miR-208 was released in blood of rats after coronary artery ligation, while miR-1, miR-133a and miR-499 were increased in the circulation of sham operated animals, in respect to non operated animals (Wang *et al.*, 2010b). None of the usual cardiac microRNAs was released in the circulation of patients suffering from congestive heart failure (CHF) (Adachi *et al.*, 2010). However, in one case researchers observed an increase of circulating miR-423-5p in a 30 CHF patients cohort (Tijsen *et al.*, 2010). Conversely, acute heart failure was associated with detectable increase of miR-499, reflecting a myocardial damage (Corsten *et al.*, 2010).

Table 1-5: Circulating microRNAs in patients with cardiac disease, adapted from (Fichtlscherer *et al.* 2011). AMI: acute myocardial infarction; ACS: acute coronary disease; CHF: congestive heart failure; HF: heart failure; CAD: coronary artery disease

Clinical Study Cohort	Groups and Numbers of Patients Studied	Major Findings	Association/ Correlation	RNA Isolation	miRNA Detection	Reference
AMI	33 pts with AMI 33 pts with stable CAD/other cardiovascular disease 30 healthy controls	miR-208a, miR-1, miR-133a, miR-499↑	ROC curve: miR-208a shows highest sensitivity and specificity, comparable to TnI	Plasma, TRI Reagent BD, Spiking with cel-miR-39	qPCR TaqMan (ABI)	(Wang et al. 2010a)
AMI	33 pts with STEMI 17 healthy controls	miR-1, miR-133a, miR-133b, miR-499-5p↑	Time course of upregulated miR associated with TnI	mirVana PARIS isolation kit (Ambion, Austin, TX)	qPCR TaqMan (ABI) (normalized to miR-17)	(D'Alessandra et al. 2010)
AMI unstable angina	9 pts with STEMI 5 pts with unstable angina 10 healthy controls	miR-499↑ in STEMI (within 48 h)	Correlation with CKMB	Vana PARIS Kit (Ambion) Spiking with small RNA	qPCR TaqMan (ABI)	(Adachi et al. 2010)
AMI	93 pts with AMI 66 healthy controls	miR-1↑	Association with QRS duration	mirVana PARIS (Ambion) protocol	qPCR	(Ai et al. 2010)
AMI	31 pts with AMI 20 healthy controls	miR-1↑	Association with CKMB levels	miR Isolation Kit (RNA Bioscience)	qPCR, primer ABI, Roche Light cycler	(Cheng et al. 2010)
AMI	29 pts with ACS 42 pts without ACS	miR-1, miR-133↑	Correlation with TnT	TRIzol LD (Invitrogen)	qPCR, TaqMan (ABI)	(Kuwabara et al. 2011)
AMI	32 pts with AMI 36 pts with chest pain but normal angiogram	miR-208b, miR-133a, miR-499↑ miR-223↓	miR-208b and miR-499 correlate with TnT	mirVana PARIS kit (Ambion)	qPCR, MiScript primers (Qiagen), BR SYBR Green (Quanta Biosciences)	(Corsten et al. 2010)
ACS	444 pts with ACS	In pts with ACS: miR-1, miR-133a/b, miR-208b↑	miR-133a and miR-208b associated with risk of death	miRNeasy RNA isolation kit (Qiagen, Inc.)	qPCR, TaqMan (ABI)	(Widera et al. 2011)

Clinical Study Cohort	Groups and Numbers of Patients Studied	Major Findings	Association/ Correlation	RNA Isolation	miRNA Detection	Reference
ACS	7 pts non-CAD 31 pts stable CAD 19 pts ACS	In pts with ACS: miR-133, miR-499, miR-208↑	Transcoronary gradients document cardiac release of miR-133 and miR-499	miRNeasy RNA isolation kit (Qiagen, Inc.)	qPCR, TaqMan (ABI)	(De Rosa et al. 2011)
CHF	30 pts with heart failure 20 pts with dyspnea (non-HF) 39 healthy controls	miR-423-5p↑	ROC showing that miR-423-5p is a predictor of heart failure	mirVana PARIS kit (Ambion)	qPCR High Resolution Melting Master (Roche)	(Tijsen et al. 2010)
CHF	15 pts CHF (II and III) 10 healthy controls	miR-499 below detection limit of the PCR in CHF pts and healthy controls		mirVana PARIS kit (Ambion)	qPCR TaqMan (ABI)	(Adachi et al. 2010)
CHF	33 pts with ischemic HF 17 controls	miR-126↓	miR-126 negatively correlated with age, BNP and NYHA class	mirVana PARIS kit (Ambion)	qPCR TaqMan (ABI)	(Fukushima et al. 2007)
Acute HF	33 pts with acute HF 34 controls	Acute Heart failure: miR-499↑		mirVana PARIS kit (Ambion)	qPCR, MiScript primers (Qiagen), BR SYBR Green (Quanta Biosciences)	(Corsten et al. 2010)
Viral myocarditis	14 pts with acute viral myocarditis 20 pts post-viral myocarditis 20 aged-matched controls	Acute viral myocarditis: miR-208, miR-499↑	Course of viral myocarditis	mirVana PARIS kit (Ambion)	qPCR, MiScript primers (Qiagen) BR SYBR Green (Quanta Biosciences)	(Corsten et al. 2010)

1.8.1 Technical aspects of microRNAs quantification in body fluids

Technically speaking, microRNAs quantification in plasma/serum is affected by high variability. Many factors, including withdrawal protocol, preparation of samples, age of the subject, diet and plasma components can alter the outcome of microRNAs profiling from human samples (Kim *et al.*, 2012). Obtaining plasma from whole blood requires addition to the test tubes of anti-coagulant agents, such as heparin, EDTA, Na-citrate or NaF/KOx. These can have inhibitory effect on either the RNA isolation or reverse transcription and qPCR (Kim *et al.*, 2012). Insufficient blood centrifugation, inaccurate supernatant withdrawal and hemolysis can lead to confounding cellular microRNAs to be co-purified and quantified. Therefore, standardized methods and accurate removal of contaminants (proteins, lipids and enzyme inhibitors) from body fluids is essential for quantification of microRNAs by qPCR.

1.9 Contribution to the field

In this thesis I will illustrate our strategies to generate data that can support elucidation of the molecular mechanisms of cardiac function and drug-induced cardiomyopathy. First, we identified a microRNAs signature altered by an anti-tumoral agent known for its cardiotoxicity (doxorubicin), and proposed a link with concomitant transcriptome variation, and we could identify a novel direct microRNAs-mRNAs interaction (miR-34/Sipa1). Second, we have uncovered the spatial distribution of microRNAs and mRNAs in 8 cardiac structures of rat, dog and Cynomolgus monkey, with a level of detail never achieved before in these model species. A subset of microRNAs with a patterned expression across cardiac structures was further characterized and 3 novel targets relevant for cardiac biology were validated (Timp3, Rbm24, Csnk2a2).

Ideally, our findings will contribute to develop more predictive safety models for drug development, especially for cardiac toxicity, as much needed in the field (Stummann *et al.*, 2009).

Chapter 2: Perturbation of microRNAs in rat heart during chronic doxorubicin treatment

2.1 Abstract

Anti-cancer therapy based on anthracyclines (DNA intercalating Topoisomerase II inhibitors) is limited by adverse effects of these compounds on the cardiovascular system, ultimately causing heart failure. Despite extensive investigations into the effects of doxorubicin on the cardiovascular system, the molecular mechanisms of toxicity remain largely unknown. MicroRNAs are endogenously transcribed non-coding 22 nucleotide long RNAs that regulate gene expression by decreasing mRNA stability and translation and play key roles in cardiac physiology and pathologies. Increasing doses of doxorubicin, but not EPS (a Topoisomerase II inhibitor devoid of cardiovascular toxicity), specifically induced the up-regulation of miR-208b, miR-216b, miR-215, miR-34c and miR-367 in rat hearts. Furthermore, the lowest dosing regime (1 mg/kg/week for 2 weeks) led to a detectable increase of miR-216b in the absence of histopathological findings or alteration of classical cardiac stress biomarkers. *In silico* microRNA target predictions suggested that a number of doxorubicin-responsive microRNAs may regulate mRNAs involved in cardiac tissue remodeling. In particular miR-34c was able to mediate the DOX-induced changes of Sipa1 mRNA (a mitogen-induced Rap/Ran GTPase activating protein) at the post-transcriptional level and in a seed sequence dependent manner. Our results show that integrated heart tissue microRNA and mRNA profiling can provide valuable early genomic biomarkers of drug-induced cardiac injury as well as novel mechanistic insight into the underlying molecular pathways.

2.2 Introduction

Doxorubicin (DOX), a potent drug used in cancer chemotherapy, intercalates with DNA and stabilizes a ternary complex with Topoisomerase II (Top2) thus preventing replication of DNA and subsequent cell proliferation(Momparler *et al.*, 1976).

Despite its beneficial therapeutic effects, acute administration of high DOX doses causes severe kidney damage, whilst toxic cardiomyopathy is observed when DOX is chronically administered for several weeks at doses devoid of severe nephrotoxicity (Bardi et al., 2007; Chen et al., 2007; Jones et al., 2006; Storm et al., 1989). The exact mechanism of its cardiac toxicity remains largely unknown. In a clinical setting, the occurrence of DOX-induced cardiomyopathy is mitigated by prophylactic treatment with dexrazoxane (DZR), a Top2 inhibitor and iron-chelating agent which limits the amount of metal ions interacting with DOX and thus reduces oxidative stress (Jones et al., 2006) . The cardioprotective mechanism of DZR is at present not fully understood (Hasinoff and Herman, 2007).

Administration of DOX ranging from 1 to 3 mg/kg/week to rats during several weeks recapitulates the cardiac symptoms observed in humans (Gianni et al., 2008; Herman and Ferrans, 1998). Similarly, combined treatment with DZR lessens the cardiac symptoms in rats treated with DOX (Thompson et al., 2009).

MicroRNAs are a class of genomically encoded non-translated short RNAs, about 22 nucleotides long, discovered in plants in 1993 (Lee et al., 1993). They are evolutionarily conserved throughout higher eukaryotes, from C.elegans to human, and their function is exerted via the mammalian RNA interference machinery (He and Hannon, 2004). They play a role in the post-transcriptional regulation of target genes, via imperfect base complementarities, and control gene expression mainly via translation inhibition and mRNA degradation (Filipowicz

et al., 2008; Guo et al., 2010; Wu and Belasco, 2008). MicroRNAs are involved in a diverse range of physiological and pathological processes including organism development (Marson et al., 2008), cancer (Croce, 2009) and immunity (Cullen, 2006; Rodriguez et al., 2007; Umbach and Cullen, 2009). In particular, microRNAs are key players in the development, physiology and pathology of the heart (Latronico and Condorelli, 2009; Thum et al., 2007; van Rooij et al., 2006). MicroRNAs have been shown to be differentially expressed in response to various signals resulting in post-transcriptional regulation of their predicted target genes. This extra level of regulation is critical during heart development and important in the cardiac response to stress (Callis et al., 2009; van Rooij et al., 2007).

Recent studies investigating DOX-induced changes in gene expression at the mRNA level in rat cardiac tissue have successfully identified genomic biomarkers whose molecular functions are consistent with proposed toxicity mechanisms (Thompson et al., 2009). However the emerging importance of microRNAs for the regulation of cardiac physiology and pathologies (Latronico and Condorelli, 2009; Thum et al., 2007; van Rooij et al., 2006), together with their potential to provide novel insight into mechanisms of xenobiotics-induced toxicity (Taylor and Gant, 2008), suggest that the integration of mRNA and microRNA profiling may provide new opportunities for elucidating mechanisms of DOX-induced cardiotoxicity.

In the present study, we report that the chronic myocardial toxicity induced by DOX in rats was associated with the modulation of microRNAs, and some of these can be phenotypically anchored to histopathology findings. Alteration of miR-216b could be detected earlier than overt myocardial damage. Furthermore, by integrating DOX-inducible microRNAs with mRNA profiles generated from the same cardiac tissue samples, the putative target genes of the DOX-induced microRNA signature were highlighted, including a number of genes that have not previously been described in DOX-induced cardiomyopathy. Finally, evidence for

the direct targeting of Sipa1 mRNAs by miR-34c was provided for the first time, and this microRNA-mRNA interaction could potentially play a role in the molecular response to DOX in the rodent heart.

2.3 Materials and methods

2.3.1 Rat in vivo study and histopathology evaluation

This in vivo study was conducted in compliance with the Animal Welfare Act, and the Office of Laboratory Animal Welfare, after review and approval by the Covance site (Vienna, Virginia) Institutional Animal Care and Use Committee. Doxorubicin (Adriamycin, Lot No. 86G23FY, CAS# 23214-92-8), a generous gift from Adria Laboratories Inc. (Columbus, OH, USA), etoposide (ETOPOPHOS® Lot No. 5E04155, CAS# 117091-64-2) was purchased from Bristol-Myers Squibb (Princeton, NJ, USA) and dexrazoxane (Zinecard® Lot No. ADR074B, CAS# 24584-09-6) purchased from Pharmacia/Pfizer (Kalamazoo, MI, USA) were administered once weekly as follows: 11 week old male Crl:CD(SD) rats received DOX or EPS via intravenous injection once a week for 2, 4, or 6 weeks according to Supplementary table 2-1 at a dose volume of 2 mL/kg. Animals in the DZR groups were given a 10 mL/kg intraperitoneal injection of DZR followed (30±2 minutes later) by a 2 mL/kg IV injection of 0.9% sodium chloride or DOX. The intravenous (iv) route of administration for DOX and EPS and the intraperitoneal (ip) route for DZR respectively, were selected to match the clinical route of administration in humans. More details are provided in the Supplementary Material and Methods and in a forthcoming publication from Gerrish et al. The experimental strategy is summarized in Figure 2-1.

2.3.2 Heart tissue RNA extraction

Total RNA was obtained from 50-100 mg frozen cardiac tissue homogenized in Trizol, according to manufacturer instructions (Invitrogen, Life Technologies, Carlsbad, CA). Long and short RNA fractions were separated via affinity resin column during clean up, according to the manufacturer instructions (miRNeasy Mini Kit, Qiagen, MD). Short RNA was quantified by absorbance at 260 nm using a Nanodrop (Thermo Scientific, Wilmington, DE), and the quality and integrity were determined using Small RNA chips (Agilent Technologies, Santa Clara, Ca) and stored at -80°C until analysis.

2.3.3 Gene expression profiling and analysis

GeneChip experiment was conducted in the BMD Genechip laboratories (Novartis, BS) on Rat Genome Rat230 2.0 Array (Affymetrix, Inc.). Target preparation was performed with a starting amount of approximately 1 μg of total RNA unless otherwise specified using the Affymetrix GeneChip HT One-Cycle Target Labelling and control reagent according to manufacturer's instruction (Affymetrix, Inc.). cRNA size distribution (before and after fragmentation) was confirmed by agarose-gel electrophoresis. An amount of biotinylated cRNA of approximately 10 μg for each sample was hybridized for approximately 16 hours at 45°C on an array. The array was washed and stained on Affymetrix Fluidics Workstation 450 and scanned on Affymetrix Scanner 3000 according to manufacturer's technical manual. The scanned image was converted into numerical values of the signal intensity (Signal) and into categorical expression level measurement (Absolute Call) using the Affymetrix MAS 5.0 software. The software scaled the average intensity of each chip to a target intensity of 150. The Genechip data will be uploaded in ArrayExpress.

2.3.4 Amplification of microRNAs via quantitative PCR

Using the Taqman Low Density Array (TLDA) technology (Applied Biosystems, Life Technologies, Carlsbad, CA), about 500 rodent microRNAs were profiled without any pre-amplification step, using 700 ng of small RNA according to the manufacturer instructions (Applied Biosystems, Life Technologies, Carlsbad, CA). Sufficient RNA amount for the TLDA cards was obtained for 3 out of 6 animals receiving DOX 3 mg/kg/week for 4 weeks, and for 6 out of 6 receiving DOX 3 mg/kg/week for 2 weeks. An arbitrary cut-off at 35 Ct was set in order to exclude microRNAs out of the linear range of detection. Mammalian snRNA U6 was used as housekeeping gene for its expression was not affected by DOX treatment. In order to confirm the results of the TLDA, a subset of microRNAs were quantified using single Taqman assays in the 3 remaining animals treated with DOX 3 mg/kg/week for 4 weeks. All procedures were carried out according to manufacturer instructions (Applied Biosystems, Life Technologies, Carlsbad, CA) using a 7900HT Fast Real-Time PCR System.

2.3.5 Data Analysis

MicroRNA data obtained from the TLDAs were quality checked and analyzed using Statminer plugin (Integromics, Madrid, Spain) for TIBCO Spotfire (TIBCO Software, Palo Alto, CA). Average fold changes and standard deviations were calculated and plotted using GraphPad Prism 5.00 (GraphPad Software, San Diego California USA). Lists of putative targets for each DOX regulated microRNA were obtained from Microcosm (Griffiths-Jones *et al.*, 2008). Raw intensity values for each probe set were generated using RMA algorithm, using Genespring GX 7.3 (Agilent Technologies, Santa Clara, CA). Gene lists were filtered for raw expression (>80 raw signal in at least 50% of arrays) and anti-correlation with the microRNA (absolute fold change >1.3 or 1.5 in animals receiving DOX 3 mg/kg for 4 weeks *versus* control treated). Candidates passing

this cut-off were used to build networks of up- or down-regulated genes with their putative cognate microRNAs. P values were generated via unpaired, two-tailed Student t-test and were considered significant below 0.05. Phenotypic anchoring and Spearman correlation analysis were realized by using TIBCO Spotfire (TIBCO).

2.3.6 Cell culture and DOX treatment

HEK 293 (CRL-1573, ATCC) and rat myoblastic cells H9c2 (CRL-1446, ATCC) were cultured in MEM and DMEM respectively, supplemented with 10%v/v heat-inactivated fetal bovine serum (Invitrogen). DOX was purchased from Sigma, diluted in DMSO to a stock concentration of 10 mM and stored at -20°C. DOX treatment of H9c2 cells (n= 3) was independently repeated 3 times (total of 9 replicates). DOX was added cells transfected with microRNA reagents after 4 hours, buy replacing transfection medium with fresh growth medium supplemented with DOX at the indicated concentrations.

2.3.7 MicroRNA target luciferase reporter assay

Two-hundred nucleotides surrounding the microRNA predicted seed site in the 3'-UTRs of either murine Tnni3k or Sipa1 were synthesized (GeneArt, Invitrogen, Carlsbad, CA) and cloned into a pmiR-GLO vector (Promega, Madison, WI) downstream of the luciferase gene via a XbaI-SacI restriction site to generate the pmiR-GLO-Sipa1 wt expression vector. The pmiR-GLO expression vector contains both the luciferase and the Renilla genes under the control of different promoters. Complete insert sequences are listed in Supplementary Material and Methods section. Stratagene "Quick change" kit was used to introduce a 6 nt mutation in the miR-34c predicted seed site to generate the pmiR-GLO-Sipa1 mutant and the pmiR-GLO-Tnni3k mutant expression vectors.

Sipa1 mutagenesis Primer1: 5'- CTG CGC TGA GGC GCG TCT TAG **GGA ATT** CCC CTC TTC CCA GCC CAT TTG -3'. Sipa1 mutagenesis Primer 2: 5'- CAA ATG GGC TGG GAA GAG GGG **AAT TCC** CTA AGA CGC GCC TCA GCG CAG -3'.

2.3.8 Transfection of HEK 293

HEK 293 cells were plated in 24 wells plates 24 hours prior to co-transfection in antibiotic-free medium. Cells ($2x\ 10^5$ per well) were co-transfected with the reporter constructs (20ng/well) and synthetic mmu-miR-34a, b or c (40 or 80 nM) mimics (Dharmacon, Lafayette, CO) in serum-free medium, using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). A *C. elegans* microRNA mimic with no affinity for mammalian targets was used as negative control. Dual-GLO Luciferase Assay System (Promega, Madison, WI) was used to assess luciferase relative luminescence, according to manufacturer instructions.

2.4 Results

2.4.1 Doxorubicin induced cytoplasm vacuolation and dysregulation of tissue genomic cardiomyopathy indicators

Male rats were treated with DOX according to the scheme reported in

Figure 2-1A. Macro-and micro-vesicular vacuolation of individual cardiac myocytes was observed in atria and ventricles of DOX-treated animals. Representative histopathological image is shown in Figure 2-1B and C. Atrial and ventricular vacuolation occurred with increasing frequency and severity over time, with or without continued weekly exposure to drug (Supplementary table 2-2). The vacuoles could be caused by autophagic vesicles accumulation (Kobayashi et al., 2010); however, a hierarchical clustering using the expression of 31 autophagy-related genes could not discriminate between vehicle and DOX treated cardiac samples (data not shown). Given the small proportion of

cardiomyocytes undergoing vacuolation within the whole tissue it is possible that the expression changes of a large list of genes would be diluted by background signals. The treatment of DOXO at 1, 2 or 3 mg/kg/week for 2, 4 and 6 weeks showed a statistically significant increase of Ambra1 mRNA, a positive autophagy regulator (Fimia et al., 2007) but did not affect the level of other markers (Figure 2-6A).
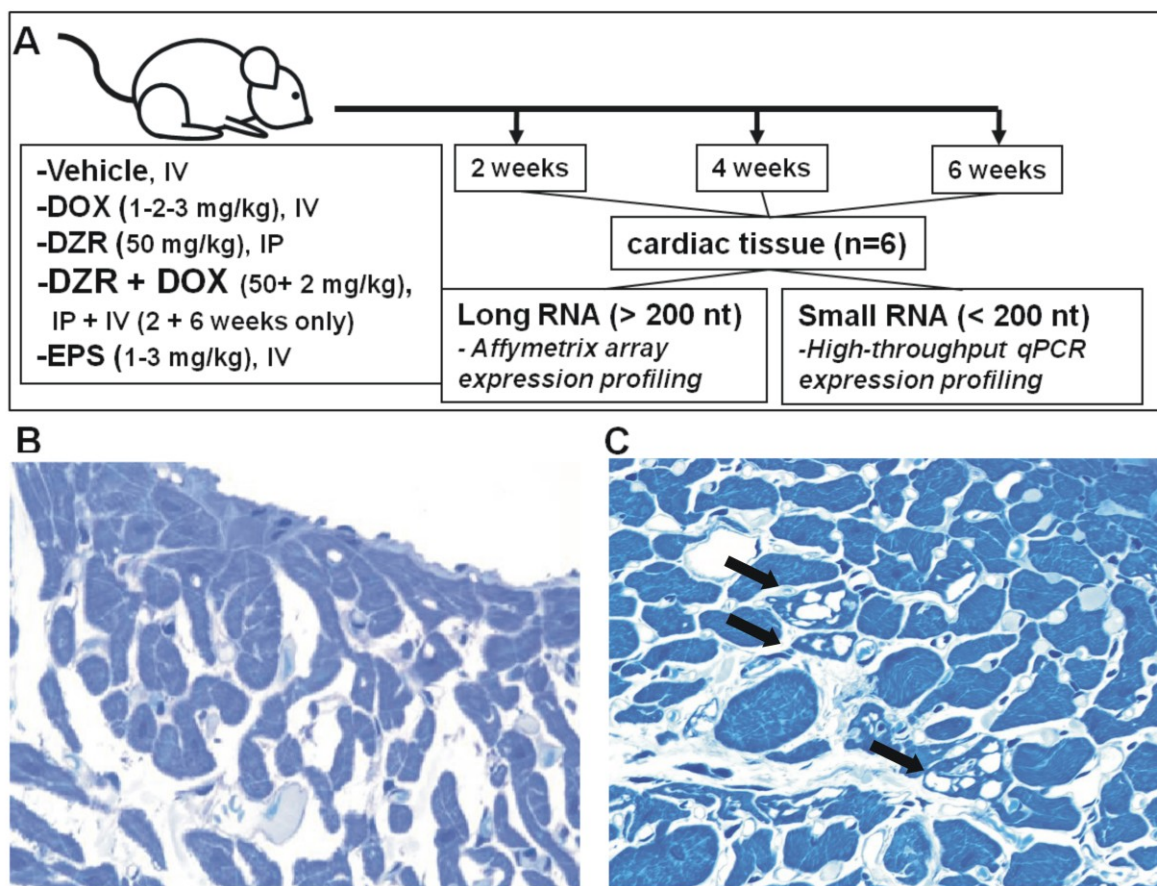
Figure 2-1: Study design and representative micrograph showing DOX-related vacuolation in the myocardium.(A) Six adult male rats were injected with the indicated doses of vehicle, doxorubicin (DOX), dexrazoxane (DZR), etoposide (EPS) or a combination of DOX and DZR for 2, 4 or 6 weeks. Cardiac tissue was excised and deep frozen for gene expression and microRNA profiling experiments. A representative micrograph of a toluidine blue stained myocardial section of acontrol (B) and of a DOX treated animal (C). Black arrows indicate sarcoplasmic micro- and macro- vacuolation of cardiomyocytes.

The administration of DOX at 3 mg/kg/week induced time-dependent changes in the expression of mRNAs that have previously been associated with molecular responses underlying cardiac pathologies (de Bold et al., 1996; Dorn et al., 1994; Mikhailov and Torrado, 2008) (Figure 2-2).
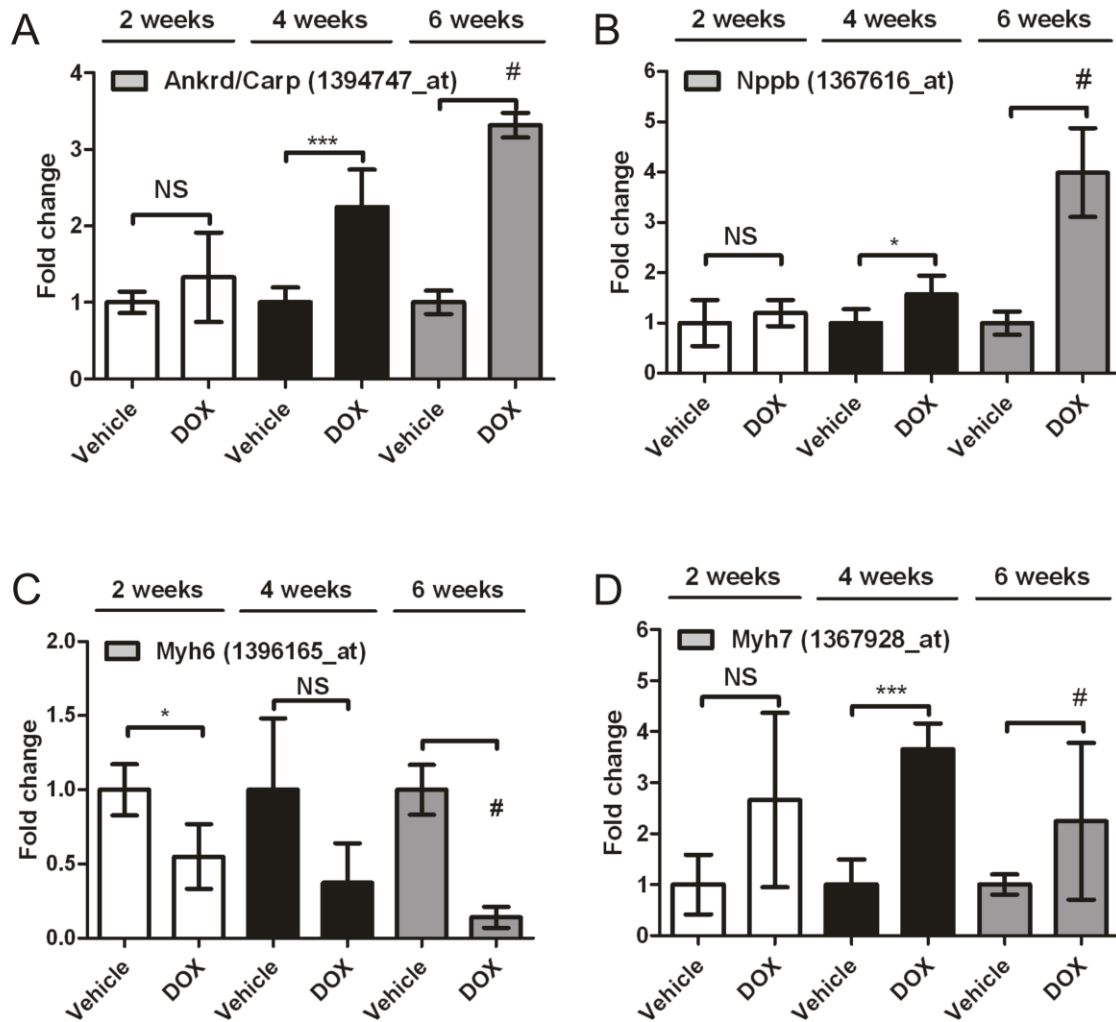
Figure 2-2: DOX 3mg/kg/week altered levels of genomic cardiomyopathy indicators (Ankrd/Carp, Nppb, Myh7 and Myh6). Expression fold change relative to vehicle were represented for DOX 3 mg/kg/week at 2, 4 and 6 weeks time point (n= 6) for (A) Ankrd/Carp, (B) Nppb, (C) Myh7 and (D) Myh6. For each time point and each probe set, vehicle values were averaged and normalized to 1. The same correction was applied to the DOX treated values. Affymetrix probe-set number is indicated in brackets. Error bars represent standard deviation. T-test was performed for vehicle- vs. DOX-treated at each time point. *P<0.05, **P<0.01, ***P<0.005, NS=Non-Significant. (No t-test for #, as n=2).

A significant increase of Ankyrin repeat domain 1 (Ankrd1/Carp), natriuretic peptide precursor type B (Nppb) and myosin heavy chain 7 (Myh7/β) mRNA and a significant decrease of myosin heavy chain 6 (Myh6/α) mRNA was observed in the groups treated with DOX at 3 mg/kg/week. Together, these genomic cardiomyopathy indicators were significantly altered between 2 and 4 weeks of treatment and remained altered after 6 weeks of treatment.

Furthermore, they were not affected by EPS and DZR alone (data not shown). It is important to note that only two out of six animals survived 6 weeks dosing of DOX 3 mg/kg/week.

2.4.2 Perturbation of rat heart microRNAs during chronic DOX treatment

Expression profiles of 518 rodent microRNAs were generated by using a low density array qPCR platform (TLDAs). Approximately 370 microRNAs gave a measurable signal below the arbitrary cut-off set at 35 qPCR cycle times (Ct), with no major distinction between controls and treated samples. The administration of 4 weekly doses of DOX 3 mg/kg/week led to increased levels of 17 microRNAs and decrease levels of 8 microRNAs (p-value below 0.05) (Table 2-1). Notably, a subset of those was consistently modulated at the earlier 2 weeks time point (fold change > 1.5 are italicized in Table 2-1).

Table 2-1: Chronic DOX treatment (3 mg/kg/ week) alters levels of 25 microRNAs from week 2 onwards.

DOX 3mg/kg/week	2 weeks		4 weeks	
	FC	P value	FC	P value
mmu-miR-367 [#]	4.58	1.97E-01	74.69	1.76E-06
mmu-miR-215 [#]	1.11	9.41E-01	51.42	7.45E-03
mmu-miR-216b [#]	42.58	4.54E-03	24.17	2.92E-03
mmu-miR-383 [#]	2.22	5.69E-01	21.50	5.97E-04
mmu-miR-692 [#]	1.79	5.16E-01	17.71	9.47E-03

mmu-miR-135b	1.74	1.07E-01	**12.69**	**4.04E-02**
mmu-miR-667	1.49	3.08E-01	**7.19**	**1.45E-02**
mmu-miR-298	-1.44	1.83E-01	**3.67**	**1.69E-02**
*mmu-miR-145**	1.60	8.87E-02	**3.42**	**1.09E-02**
mmu-miR-208b [#]	1.09	7.94E-01	**3.23**	**3.05E-03**
mmu-miR-21*	1.16	7.36E-01	**3.21**	**4.26E-02**
mmu-miR-877	1.55	1.79E-01	**2.74**	**2.33E-02**
mmu-miR-709 [#]	1.04	8.38E-01	**2.51**	**7.28E-03**
mmu-miR-708 [#]	1.11	7.08E-01	**2.34**	**1.57E-02**
mmu-miR-31	-1.02	9.68E-01	**2.32**	**3.95E-02**
mmu-miR-34c [#]	1.02	9.55E-01	**2.25**	**3.06E-02**
rno-miR-29b-2*	-1.10	6.84E-01	**2.01**	**3.41E-02**
mmu-miR-218-2* [#]	1.03	9.79E-01	**-8.61**	**3.45E-03**
mmu-miR-434-5p	-3.21	3.29E-01	**-6.14**	**4.81E-02**
rno-let-7e*	1.49	6.00E-01	**-3.95**	**1.84E-02**
mmu-miR-337-5p	-2.86	2.30E-01	**-3.76**	**4.37E-02**
mmu-miR-335-3p	1.43	1.33E-01	**-2.52**	**3.47E-02**
mmu-miR-384-3p	1.07	7.52E-01	**-2.38**	**4.32E-02**
mmu-miR-221	1.37	1.15E-01	**-2.08**	**1.85E-02**
rno-miR-1*	-1.16	8.05E-01	**-2.01**	**3.29E-02**

Variation of cardiac microRNA levels versus vehicle are reported for animals treated with DOX 3 mg/kg/week for 2 and 4 weeks. Values were calculated via the relative quantification ($\Delta\Delta$Ct) method by using the mammalian U6 snRNA as a normalizer. MicroRNAs showing same trend at 2 and 4 weeks are italicized. # indicates microRNAs selected for further analysis. Significant P values (<0.05) are in bold. FC= fold change.

2.4.3 MiR-367, miR-215, miR-216b, miR-208b and miR-34c are specifically dysregulated by chronic DOX treatment

A subset of ten microRNAs whose expression was altered by chronic DOX treatment was selected based on their lowest p-values (Table 2-1) and evaluated their expression profile in additional individual animals from the same DOX treatment groups (originating from the same in vivo study) using single Taqman

assays (Supplementary figure 2-2). Five of these microRNAs (miR-208b, miR-215, miR-216b, miR-34c and miR-367) displayed a consistent dose-dependent response to DOX at 2 and 4 weeks and were thus chosen as candidates for further expression profiling across the larger in vivo study treatment groups including co-treatment of DOX with DZR and treatment with EPS only (Figure 2-3).

Following two weeks treatment with DOX alone, a slight up-regulation of miR-208b, mir-216b and miR-367 was observed in the heart of most animals. Following 4 weeks of treatment with DOX alone, the level of all five microRNA candidates were significantly increased and dose dependency was observed for miR-208b, miR-215, miR-216b and miR-367. Following 6 weeks of treatment with DOX alone, the levels of miR-208b, miR-215, miR-216b and miR-367 were significantly increased. Dose dependency was difficult to assess at the 6 week time point since only 2 animals survived in the high dose group.

Following treatment with DZR alone for 2 and 6 weeks, none of the five candidate microRNAs was affected. After treatment with the combination of DOX at 2 mg/kg/week and DZR at 50 mg/kg/week, the level of miR-208b, miR-216b and miR-367 was either slightly increased or not affected. For all five candidate microRNAs, the level was lower when compared to the group of animals treated for 2 weeks with DOX alone at 2 mg/kg/week. Following 6 weeks of treatment with the same DOX/DZR combination, the level of miR-208b, miR-216b and miR-367 was increased. However, this level was lower for miR-208b and miR-216b when compared to the group of animals treated 6 weeks with DOX alone at 2 mg/kg/week and higher for miR-367.

Following 2 and 6 weeks of treatment with EPS, the expression level of the 5 microRNA candidates was minimally affected and considered as non statistically significant since no dose and time dependency was observed (although these

changes might still be biologically relevant). Together, these data suggested that the variation of these five microRNAs was DOX-specific.
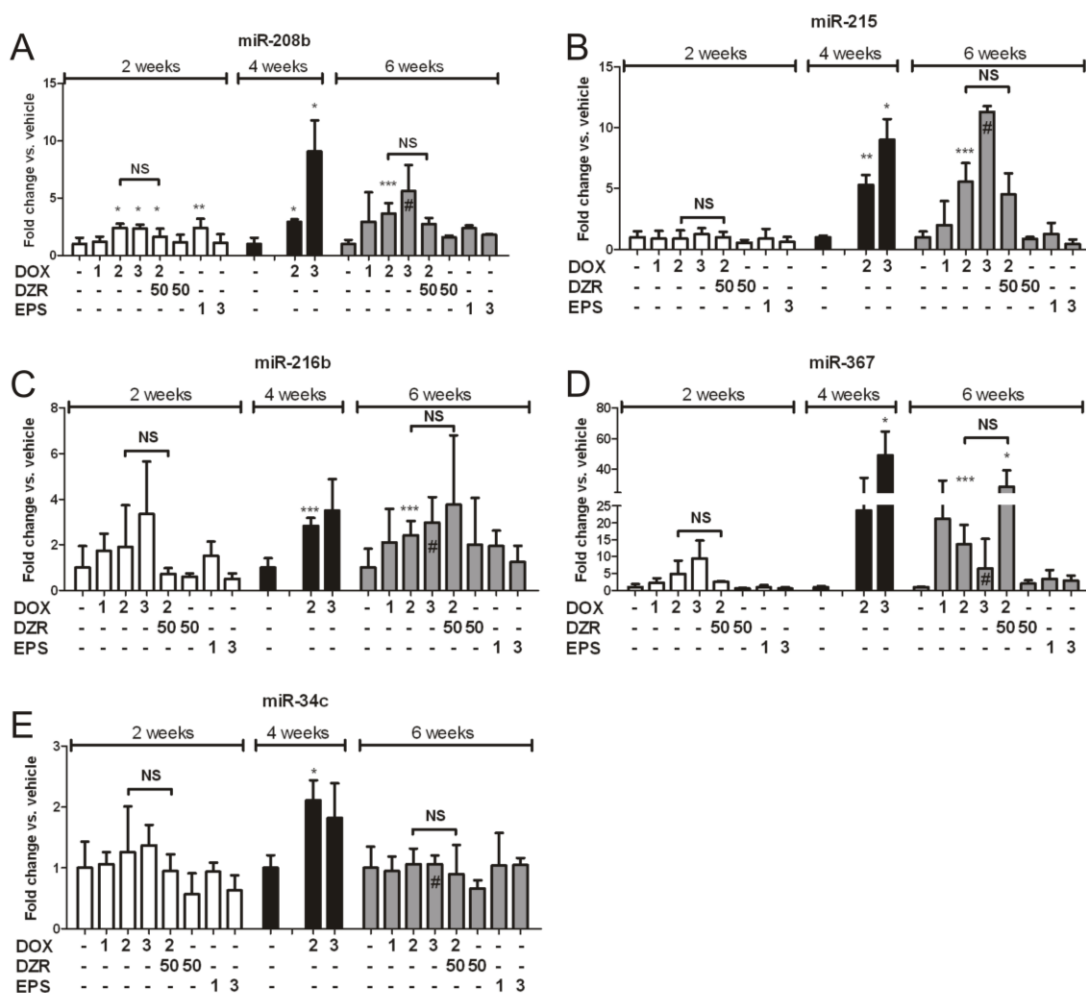
Figure 2-3: Relative quantification of DOX-responder microRNAs in rat heart across all groups. Relative quantification of (A) miR-208b, (B) miR-215, (C) miR-216b, (D) miR-367 and (E) miR-34c in DOX, DOX + DZR, EPS groups, normalized versus vehicle treated animals. Expression levels were measured by single assay qPCR (n= 3, except #, n= 2). DOX: Doxorubicin, DZR: dexrazoxane, EPS: etoposide; numbers indicate the weekly dose of each compound in mg/kg/week. Empty spaces represent non-sampled animals. The vehicle treated is the first column of each time-point. The animals used in this experiment were distinct from the ones represented in Table 2-1. Error bars represent SD. T-test results are indicated by asterisks for significant DOX-treated groups vs. their own vehicle-treated, unless otherwise specified by horizontal range bars; *P<0.05, **P<0.01, ***P<0.005, NS=Non-Significant).

In summary, chronic treatment with DOX at 3 mg/kg/week for 2 weeks led to increases in miR-208b, miR-216b and miR-367, with fold change intensities comparable to those shown by genomic cardiomyopathy indicators (Ankrd/Carp,

Nppb, Myh7 and Myh6) (Figure 2-2 and Table 2-1), suggesting that microRNAs may enhance the predictivity of established genomic indicators of drug-induced cardiomyopathy (e.g. myosin genes, troponins, natriuretic peptides) when assessing the potential for drug-induced cardiotoxicity.

2.4.4 DOX induced up-regulation of miR-216b and miR-367 earlier than tissue lesions as observed by histopathology

We then compared the variation of DOX-specific microRNA perturbations with the severity of cardiac tissue lesions induced by drug (cytoplasm vacuolation) in order to evaluate their sensitivity. Increasing doses of DOX led to higher cumulative vacuolation grading with time (Supplementary table 2-2), as well as progressively larger fold changes for microRNAs miR-208b, miR-216b and miR-367 versus vehicle. Notably, treatment with DOX 1 mg/kg/week during 2 weeks did not cause any detectable vacuolation, but showed a statistically significant increase of miR-216b (Figure 2-4). Treatment with DOX at 2 and 3 mg/kg/week for 2 weeks showed a statistically increase of miR-216b and miR-367 (Figure 2-4). Interestingly, we observed that miR-208b profile across treatment groups had the best statistically significant correlation score in comparison to the severity of vacuolation (Spearman R squared = 0.76, p= 4.45E-04) (Supplementary table 3). Therefore miR-208b may be implicated in the pathology mechanism of the vacuolation. These data suggest that DOX-induced perturbations of cardiac microRNA expression can precede overt tissue histopathology and that specific microRNAs may be useful as genomic indicators of drug-induced cardiac toxicity.

Treatment	Path grading	FC miR-216b	SEM miR-216b	P value miR-216b	FC miR-367	SEM miR-367	P value miR-367
Control	0	1.00	0.19		1.00	0.23	
DOX 1 mg/kg/week	0	2.77	0.47	0.02	1.35	0.11	0.17
DOX 2 mg/kg/week	2	3.82	0.85	0.03	2.45	0.31	0.01
DOX 3 mg/kg/week	5	2.80	0.69	0.04	2.07	0.16	0.01
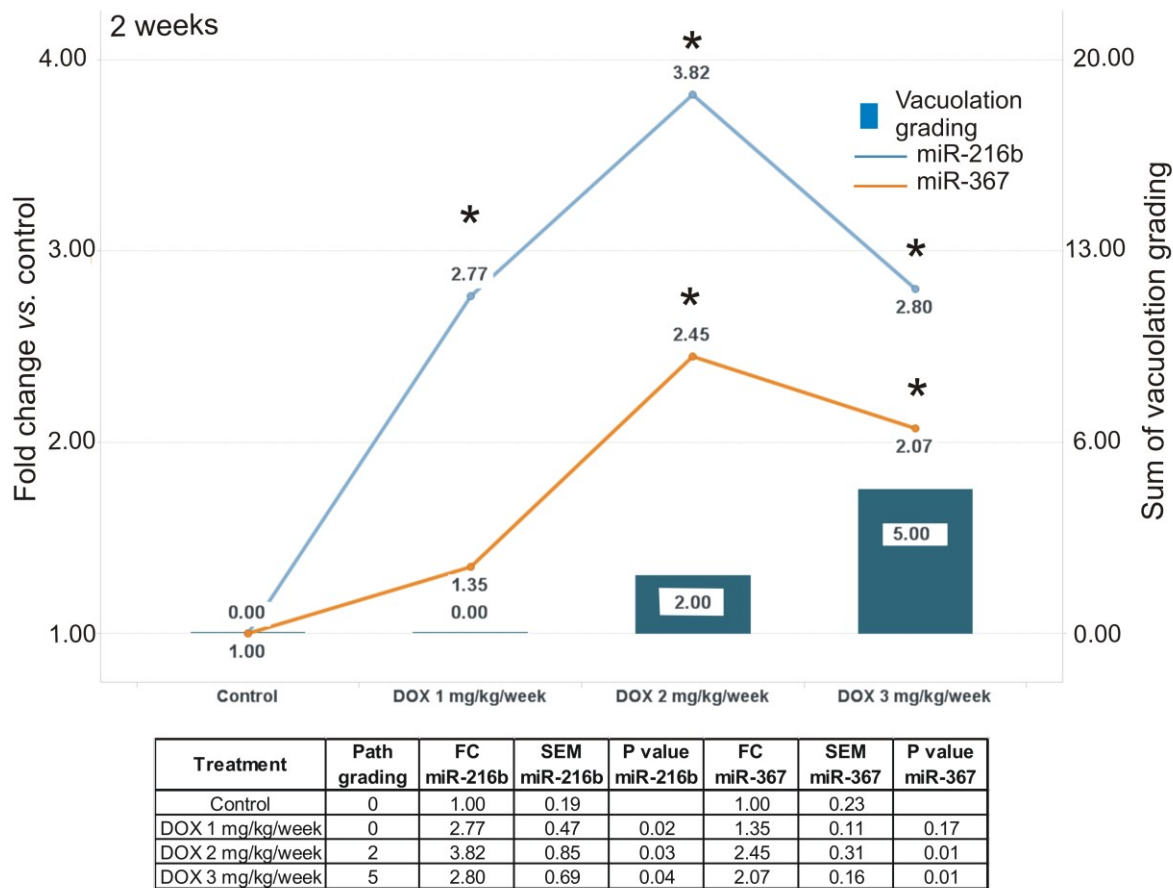
Figure 2-4: DOX-induced upregulation of miR-216 and miR-367 expression precedes the detection of overt histopathological lesions in cardiac tissue. Blue bars show cumulative vacuolation grade. X axis shows the DOX regimen in mg/kg/week received by the animals at 2 weeks. Y axes report cumulative histopathological scores and microRNA fold change *vs.* untreated cardiac tissues (normalized at value 1). Path grading = cumulative vacuolation score. FC= fold change. SEM= standard error on the mean.

2.4.5 MiR-34c regulates Sipa1 mRNA at the post-transcriptional level and is involved in autophagy process

Out of the 7804 mRNA targets for DOX-inducible microRNAs predicted by Microcosm, 132 (1.7%) mRNAs showed the expected profile of anti-correlated expression relative to its putative targeting microRNA (>1.5 fold change at DOX 3mg/kg/week for 4 weeks *vs.* vehicle) (Supplementary table 2-3). Since this low prediction rate could have been obtained by statistical chance, the putative microRNA-mRNA interaction of miR-34c/Sipa1was selected for further

experimental evaluation. Treatment with DOX at 1, 2 and 3 mg/kg/week showed a dose-dependent decrease of signal-induced proliferation-associated 1 (Sipa1) and a dose-dependent increase of miR-34c (Figure 2-5A). Similarly, the treatment of H9C2 rat myoblasts with DOX at 0.1 and 1µM for 24h showed a decrease of Sipa1 mRNA and an increase of endogenous miR-34c (Figure 2-5B). The transfection of miR-34c mimics in H9c2 showed a statistically significant decrease of Sipa1 mRNA, as the transfection of miR-34c hairpin inhibitor (HI) showed a statistically significant increase of Sipa1 mRNA (Figure 5C). Notably miR-34c over-expression exacerbated Sipa1 down-regulation induced by DOX, while the transfection with mir-34c hairpin inhibitor significantly rescued the expression (Figure 2-5C), suggesting that miR-34c is mediating the DOX-induced regulation of Sipa1. Finally, to confirm whether the regulatory effect of miR-34c on the levels of Sipa1 mRNA was direct, 2 luciferase-based reporter expression vectors (pmiR-GLO-Sipa1 WT and mutated) were generated by cloning a 200 nucleotide region surrounding the predicted microRNA seed from each of their 3'-UTR regions downstream of a firefly luciferase reporter gene. The co-transfection of the Sipa1 reporter with miR-34c in HEK 293 cells showed a significant decrease in the luciferase signal. This decrease was observed to a lower extent with other members of the miR-34 family (Figure 2-5D) suggesting that miR-34c interacted directly and specifically with the 3'-UTR of Sipa1.

We have also used the rat myoblast H9c2 cell line to assess the DOX-induced autophagy process through the regulation of Ambra1 mRNA. Transfection of miR-34c mimics showed a statistically significant increase of Ambra1 mRNA. Transfection of miR-34c hairpin inhibitor showed a statistically significant decrease of Ambra1 mRNA. Treatment with DOX at 0.1 µM for 16h in the presence of miR-34c mimics showed a statistically significant increase of Ambra1 mRNA. Treatment with DOX at 0.1 µM for 16h in presence of miR-34c hairpin

inhibitor showed a statistically significant decrease of Ambra1 mRNA when compared to the presence of miR-34c mimics.
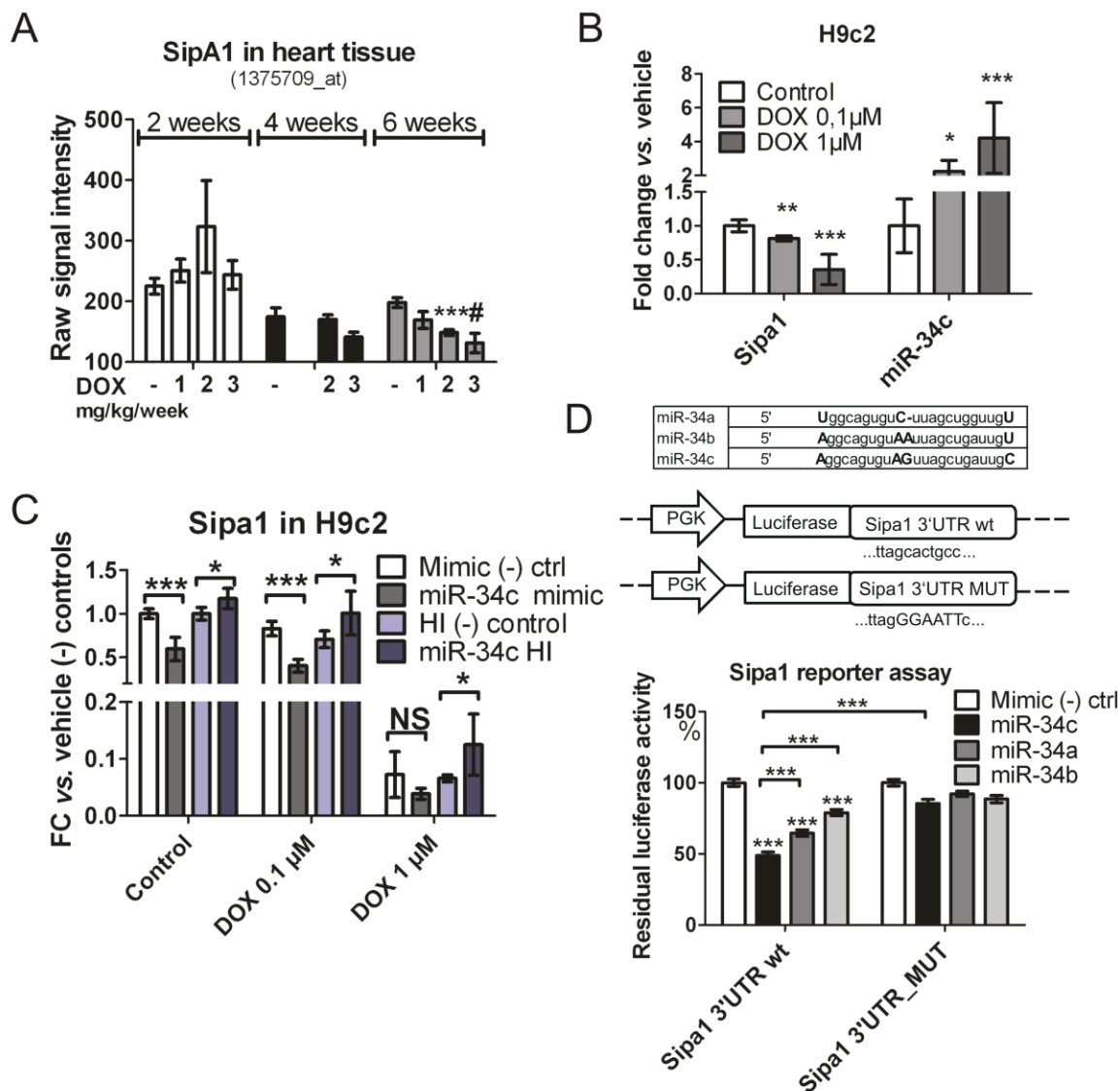
Figure 2-5: miR-34c directly controls DOX-induced Sipa1 mRNA decrease. (A) Sipa1 mRNA raw expression values decreased in the heart of rats treated with DOX. #, n= 2. (B) DOX treatment for 24h caused a decrease of Spa1 mRNA and an increase of miR-34c in cardiac myoblast cells (H9c2). (C) H9c2 endogenous Sipa1 mRNA was decreased by transfection of miR-34c mimic, and increased using a miR-34c hairpin inhibitor (HI). Transfection with miR-34c mimic and inhibitor respectively exacerbated and rescued Sipa1 mRNA levels in H9c2 treated with DOX 0.1 and 1µM overnight in comparison to negative controls. (D) Alignment of mammalian miR-34 family. Capital letters indicate mismatch in the sequence. Sipa1 3'-UTR wt and MUT construct: 12 nt surrounding the predicted seed are shown. HEK 293 cells were co-transfected with pmiR-GLO-Sipa1 and the indicated miRNA mimic or a *C. elegans* negative control mimic. Averaged and normalized Renilla luciferase signal was obtained from 3 independent experiments, each

The extent of Ambra1 mRNA regulation is slight but comparable between the *in vivo* systems and the H9c2. Treatment with DOX in H9c2 didn't affect the level of miR-208b ruling out a potential implication of miR-208b with autophagy in this cellular model. Therefore miR-34c is directly implicated in the DOX-induced modulation of Sipa1 mRNA regulation.
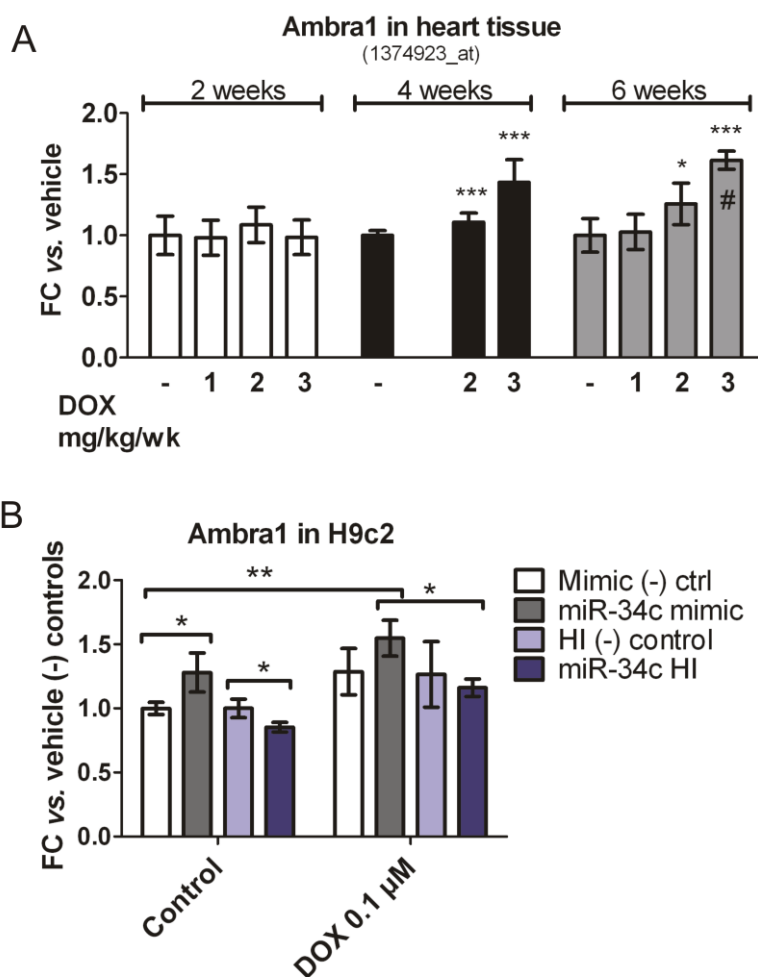
Figure 2-6: Ambra1 expression was induced by DOX treatment *in vivo* and miR-34c could control its endogenous expression levels in H9c2 cardiac myoblasts. (A) Fold change of Ambra1 probe set in rat heart tissue treated with DOX. Fold change and statistical significance were assessed *vs.* each vehicle group. n= 4 to 6 (except #, n= 2) (B)

Endogenous levels of Ambra1 were measured after miR-34c over-expression (miR-34c mimic) or inhibition (miR-34c HI) in absence of presence of DOX 0.1. Fold change value were normalized *vs.* the respective negative transfection controls in the untreated condition (n=3). *P<0.05, **P<0.01, ***P<0.005. FC= fold change.

2.5 Discussion

In the present study 25 microRNAs were found to be dysregulated by pharmacological intervention with DOX in the rat heart. A subset of microRNA candidates were further investigated and associated to their putative targets.

The chronic weekly intravenous administration of DOX was designed to trigger cardiomyopathy symptoms within a 6 week time-frame allowing us to identify mRNAs and microRNAs that are altered in the early stages of the drug-induced cardiac remodeling. Cardiac toxicity at a cumulative dose of 12 mg/kg (3 mg/kg/week for 4 weeks) was confirmed by histopathological lesions and altered expression of genomic indicators associated to contraction performance (Myh6, Myh7) and heart failure (Ankrd1/CARP, Nppb) (Figure 2) (Caulfield and Wolkowicz, 1990; de Bold *et al.*, 1996; Dorn *et al.*, 1994; Mikhailov and Torrado, 2008; Suzuki *et al.*, 1998). In most of the cases the dysregulation of these genes reached a significantly higher level between 2 and 4 weeks of the DOX 3 mg/kg/week dosing regimen. Additionally, 2 weeks treatment with DOX at 2 mg/kg/week onward caused cytoplasm vacuolation to occur with increasing severity.

We profiled hundreds of microRNAs via TLDAs in the heart of rats that showed clear signs of toxicity (DOX 3mg/kg for 4 weeks), and found that 25 microRNAs were dysregulated with p-values below 0.05. Seven out of 25 microRNAs (miR-367, miR-216b, miR-383, miR-692, miR-135b, miR-145*, miR-877, miR-434-5p and miR-337-5p), were already modulated after 2 weeks of DOX at 3 mg/kg/week (Table 2-1). Comparable changes for many of these

microRNAs were confirmed in additional animals, and a subset of five microRNAs (miR-208b, miR-215, miR-216b, miR-367 and miR-34c) and were largely unaffected by the direct Top2 inhibitor etoposide (EPS) (Figure 2-3). However DOX-induced up regulation of these microRNAs was not lessened by co-treatment with DZR, suggesting that these were not involved in the molecular pathways triggered by DZR.

It is noteworthy that miR-21 and miR-146a, previously linked to DOX-induced apoptosis in cardiomyocytes *in vitro* (Horie *et al.*, 2010; Wang and Li, 2010a) or by single administration of a high dose of DOX *in vivo* (Horie *et al.*, 2010), were not among the microRNAs regulated by our chronic DOX dosing regime in Sprague Dawley rats. Similarly, miR-1 and miR-133, known to have alternative effects on oxidative stress-induced apoptosis in myocytes (Xu *et al.*, 2007), the former being pro-apoptotic and the latter anti-apoptotic, were not altered in the present study. These discrepancies may derive from several key differences in the model systems and study designs used. Importantly, primary cells or cell lines treated *in vitro* are not comparable to the whole cardiac tissue when exposed to metabolized systemic DOX and the work of Horie *et al.* explores the acute effect of a single high dose of DOX, which contrasts to the chronic regimen administered here. Our present study describes global changes in microRNAs during chronic drug-induced cardiac toxicity, in a way that closely resembles the safety signals observed in a clinical setting.

In our study miR-215, part of the miR-192/miR-215 cluster, and miR-34c, involved in DNA damage mediated proliferation arrest (Braun *et al.*, 2008; Cannell *et al.*, 2010; Georges *et al.*, 2008; He *et al.*, 2007), were up-regulated, consistent with the pharmacological effect of DOX. However miR-215 and miR-34c were not significantly up-regulated by EPS administration, suggesting that this compound may not trigger a p53 response under these experimental conditions.

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Interestingly miR-215, up regulated by DOX in this study, was also linked to p53 mediated cell cycle arrest [37,38], consistent with the pharmacology of DOX and partially replicating expression changes of miR-34c upon treatment, thus strengthening the biological relevance of our findings and consistency with previously published mechanisms (Cannell *et al.*, 2010).

MicroRNA-208b, encoded from the intron 28 of rat Myh7, is associated to maintenance of myocardial performance together with 2 other myomirs ,i.e. miR-208a/miR-499, which play a pivotal role in the myosin balance (van Rooij *et al.*, 2009). Consistent with previous work, the DOX-induced expression of miR-208b parallels the modulation of expression of its host gene Myh7 (Supplementary figure 2-1B) and occurs in parallel to the appearance of vacuolation (Supplementary table 3). The concomitant decrease of miR-208a and Myh6 levels upon DOX treatment (Supplementary figure 2-1A) further support the importance of the regulatory role of myomiRs-myosin interactions in the myosin switch, which is associated with progressive structural changes and pathological cardiac remodeling (van Rooij *et al.*, 2009).

Of particular interest is the predictive potential of microRNAs as early tissue indicators of drug-induced cardiac lesions. The lowest dose of DOX tested in our study (1 mg/kg/week for 2 weeks) did not cause detectable tissue vacuolation (Supplementary table 2-2). However, miR-216b was already up-regulated (>1.5 fold) under these conditions, and miR-367 concomitantly increased at the earliest time point (2 weeks) in the different treatment groups. Treatment with DOX at all doses and time points was associated with sustained up-regulation of these microRNAs, together with miR-208b and miR-215 at later time points. Further studies will be needed to investigate the potential of circulating microRNAs as indicators of DOX- induced cardiac injury.

In order to gain further insight into the cellular processes that may be modulated upon DOX-induced cardiac toxicity, high confidence target mRNAs of

DOX-dysregulated microRNAs were identified by using the Microcosm prediction tool (Supplementary table 2-3 and Supplementary table 2-3). In particular, only the genes that had an anti-correlated change of expression at the mRNA level *vs.* the cognate microRNA were retained. The screening for putative targets of microRNAs in DOX treated animals, revealed that a number of DOX-responsive microRNAs may regulate mRNAs involved in cardiac tissue remodeling (data not shown), but we also speculated that the Sipa1 mRNA transcripts could be targeted by miR-34c directly. Sipa1 gene product is an activator of Rap GTPase and can influence proliferation rate and metastatic potential in various types of malignancies (Crawford *et al.*, 2006; Minato and Hattori, 2009; Shimizu *et al.*, 2011). Moreover cAMP-mediated Rap signaling, and its modulators, played a role in significant aspects of cardiovascular physiology, including cardiomyocytes contraction and hypertrophy (Jeyaraj *et al.*, 2011). Importantly, Sipa1 mRNA showed an anti-correlated pattern with miR-34c in H9c2 cells treated with DOX 1 μM during 24h (Figure 2-5B). Reporter assay experiments further support that Sipa1 mRNA is a target of the miR-34 family, in particular miR-34c (Figure 2-5D). In addition, our observations in H9c2 myoblast cell line suggest that miR-34c could be involved in the stimulation of the positive autophagy regulator Ambra1, also in presence of DOX. In fact, miR-34c over expression increased the endogenous levels of Ambra1 mRNA in H9c2 cells (both with and without DOX). Vice versa transfection of a miR-34c-directed hairpin inhibitor showed a statistically significant decrease of Ambra1 mRNA level. However, the functional relevance of this finding in autophagy regulation and the exact mechanism of this regulation will have to be elucidated. Further studies, such as gain- or loss-of-function *in vivo* investigations, will be required to elucidate the functional significance of altered Sipa1 expression for cardiac responses to DOX. Immunochemistry staining of autophagy markers like Ambra1 in heart tissues will also be required to elucidate the functional involvement of

miR-215, miR-216b, miR-367, miR-208b and miR-34c in the DOX-induced autophagy process since they seem to be specifically associated to vacuoles appearance.

In conclusion, 25 microRNAs implicated in DOX-induced cardiac toxicity *in vivo* were identified. Among them, miR-216b, which was significantly regulated before overt toxicity, has the potential of a genomic indicator of cardiac toxicity. Our study also provides the basis for understanding the global role of microRNAs in DOX-induced cardiac remodeling and toxicity, and provides evidence the direct miR-34c/Sipa1 functional interaction for the first time.

2.6 Supplementary Material and Methods

Supplementary table 2-1: Doxorubicin study design in rats.

Group		No. of animals Male	Doxorubicin (mg/kg/week)	Dexrazoxane (mg/kg/week)	Etoposide (mg/kg/week)	Dose duration (weeks)
1	Control	6	0	0	0	2
2	DOX Low	6	1	0	0	2
3	DOX Mid	6	2	0	0	2
4	DOX High	6	3	0	0	2
5	EPS Low	6	0	0	1	2
6	EPS High	6	0	0	3	2
7	DZR	6	0	50	0	2
8	DZR + DOX Mid	6	2	50	0	2
9	Control	6	0	0	0	4
10	DOX Mid	6	2	0	0	4
11	DOX High	6	3	0	0	4
12	Control	6	0	0	0	6
13	DOX Low	6	1	0	0	6
14	DOX Mid	6	2	0	0	6
15	DOX High	6	3	0	0	6
16	EPS Low	6	0	0	1	6
17	EPS High	6	0	0	3	6
18	DZR	6	0	50	0	6
19	DZR + DOX Mid	6	2	50	0	6

2.6.1 Histopathology investigations of the heart

At necropsy, the heart was oriented with the left ventricle uppermost (i.e. aorta stem pointing towards operator) and then cut in half longitudinally. Each heart was longitudinally bisected from base to apex along the middle of the ventricular free walls to reveal right and left ventricles; right and left atria; both atrioventricular valves and the root of the aorta. One half of the bisected heart was placed in 4% formaldehyde/1% glutaraldehyde while the other half was flash-frozen for other studies. The fixed hemi-sections of heart were processed for methylmethacrylate plastic embedment, sectioned at 1-2 μm, stained with toluidine blue and examined histologically. Figure 2-1 shows representative histologic cardiac sections with evidence of cardiomyocellular injury, more specifically microvesicular vacuolation typical of doxorubicin cardiomyopathy (Bertinchant *et al.*, 2003; Herman *et al.*, 1985) (Figure 2-1B and C). Vacuolar changes in ventricular and atrial cardiomyocytes were recorded separately and semi-quantitatively graded using a simple cardiomyopathy scoring method based on the frequency and severity of observed changes on a four step arbitrary scale from minimal to marked (1=Minimal, 2=Mild, 3=Moderate and 4=Marked).

2.6.2 Murine Sipa1 3'-UTR region cloned for reporter assay

Wild type:

5'-

cgttccaatctgaatctggacctgcttggaactgcctggcccctcagagcaactgggtcatactagtgcccttcctcag gacttcttccctgcgctgaggcgcgtcttagcactgcccctcttcccagcccatttggtggctaatgcctgtccctgttt gtaaatatcctgtaaagaaaaggagacatcagagtttaaaaaaaagaaacaacaagaagaagcaaacaactct -3'

MiR-34 seed mutant:

5'-

cgttccaatctgaatctggacctgcttggaactgcctggcccctcagagcaactgggtcatactagtgcccttcctcag

89

gacttcttccctgcgctgaggcgcgtcttagGGAATTcccctcttcccagcccatttggtggctaatgcctgtccc

tgtttgtaaatatcctgtaaagaaaaggagacatcagagtttaaaaaaaagaaacaacaagaagaagcaaacaactct

2.6.3 Murine Tnni3k 3'-UTR region cloned for reporter assay

Wild type:

5'-

ccaggtctgacatacacctaaggggagttttcccatcagactgacagcagtgattttatccatggcaggctcacttcca

attataatgctctgccctctgaggtctcttcaagtcgtcttgcttattctgcgctcacgtaattcccttctacagggtaggct

ttcaattgctcagagcatgaagtaccaaggagcagatatagaccgtgcatgaggaactgttcttagtttgatatttaaag

tccttaattgcctggggggtggggttcaagtctgtgtagat

2.7 Supplementary Results

2.7.1 Body and organ weight

Treatment with DOX caused dose-dependent decreases in mean body weight and weight gain in animals dosed with 2 or 3 mg/kg/week. Treatment with combination of DOX and DZR similarly induced a significant decrease in mean body weight gain. The changes in mean body weight and body weight gain appear to directly correlate with decreases in mean food consumption. A similar decrease was not noted in animals that received DZR or EPS alone, indicating that the decrease in mean body weight gain was attributable to DOX. Of the animals that survived to scheduled euthanasia and necropsy, the overall, significantly decreased heart weight parameters indicated a progressive, irreversible DOX-related toxic effect at dose levels of 2 or 3 mg/kg/week. Decrements in heart weight parameters were mitigated when DZR was given in conjunction with DOX illustrating protective properties of DZR (data not shown). More details will be given in a forthcoming publication by Gerrish *et al.*.

2.7.2 Hematology and clinical chemistry

Dose and time dependent alterations in hematology and serum chemistry were caused by DOX once weekly for 2, 4, or 6 weeks. There were minimal alterations in the clinical pathology from rats given DZR or EPS only (data not shown). In general, many of the alterations in rats given DOX in combination with DZR were of much smaller magnitude suggesting that it alleviates many of the side effects of DOX. More details will be given in a forthcoming publication by Gerrish *et al.*

2.7.3 Histopathology scores of cytoplasmic vacuolation in the heart

DOX-induced cardiomyopathy is characterized by sarcoplasmic micro- and macro-vacuolisation of the heart atrium and ventricle. These are generally categorized as minimal, mild, moderate and marked. In order to visualize the histopathological diagnoses we combined the pathology scores from the individual animals in each treatment group.

Supplementary table 2-2: Atrial and ventricular vacuolation incidence increases with doses of DOX. Microscopic histopathological findings. Severity of lesion is graded by numbers. Low= 1mg/kg/week regimen; Mid= 2 mg/kg/week regimen; High= 3 mg/kg/week regimen.

	Treatment/Dose/Duration	Control 2 wks	Dox Low 2 wks	Dox Mid 2 wks	Dox High 2 wks
	Number of animals	6	6	5	6
Atrial vacuolation	minimal				
	mild				2
	moderate				
	marked				
Ventricular vacuolation	minimal			2	1
	mild				
	moderate				
	marked				
	Treatment/Dose/Duration	Control 4 wks		Dox Mid 4 wks	Dox High 4 wks
	Number of animals	6		6	6
Atrial vacuolation	minimal				1
	mild				1

		Control 6 wks	Dox Low 6 wks	Dox Mid 6 wks	Dox High 6 wks
	moderate				
	marked				
Ventricular vacuolation	minimal				1
	mild			2	1
	moderate				
	marked				
Treatment/Dose/Duration		**Control 6 wks**	**Dox Low 6 wks**	**Dox Mid 6 wks**	**Dox High 6 wks**
Number of animals		**6**	**6**	**6**	**6**
Atrial vacuolation	minimal		2	2	1
	mild		1	1	2
	moderate		1	2	1
	marked				
Ventricular vacuolation	minimal		5	2	4
	mild		1	3	1
	moderate			1	
	marked				

There is a progressive increase in the cumulative pathology score in the hearts from DOX-treated animals (Supplementary table 2-2).
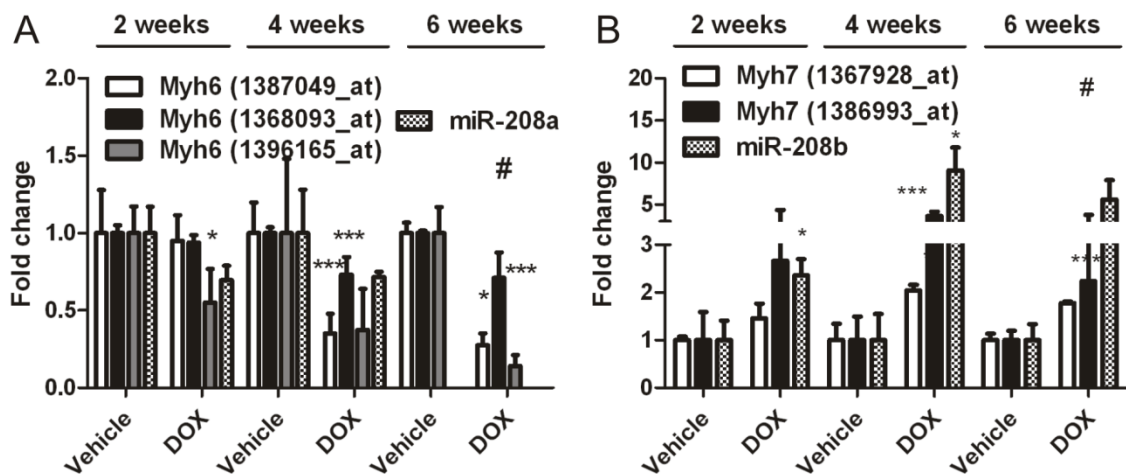
Supplementary figure 2-1: MicroRNA-208a and 208b are regulated similarly as their hosting transcripts upon DOX treatment (Myh6 and Myh7 respectively) (n= 3). (A) Myh6 and miR.208a and (B) Myh7 and miR-208b fold changes vs. control in DOX 3mg/kg/week treated animals.
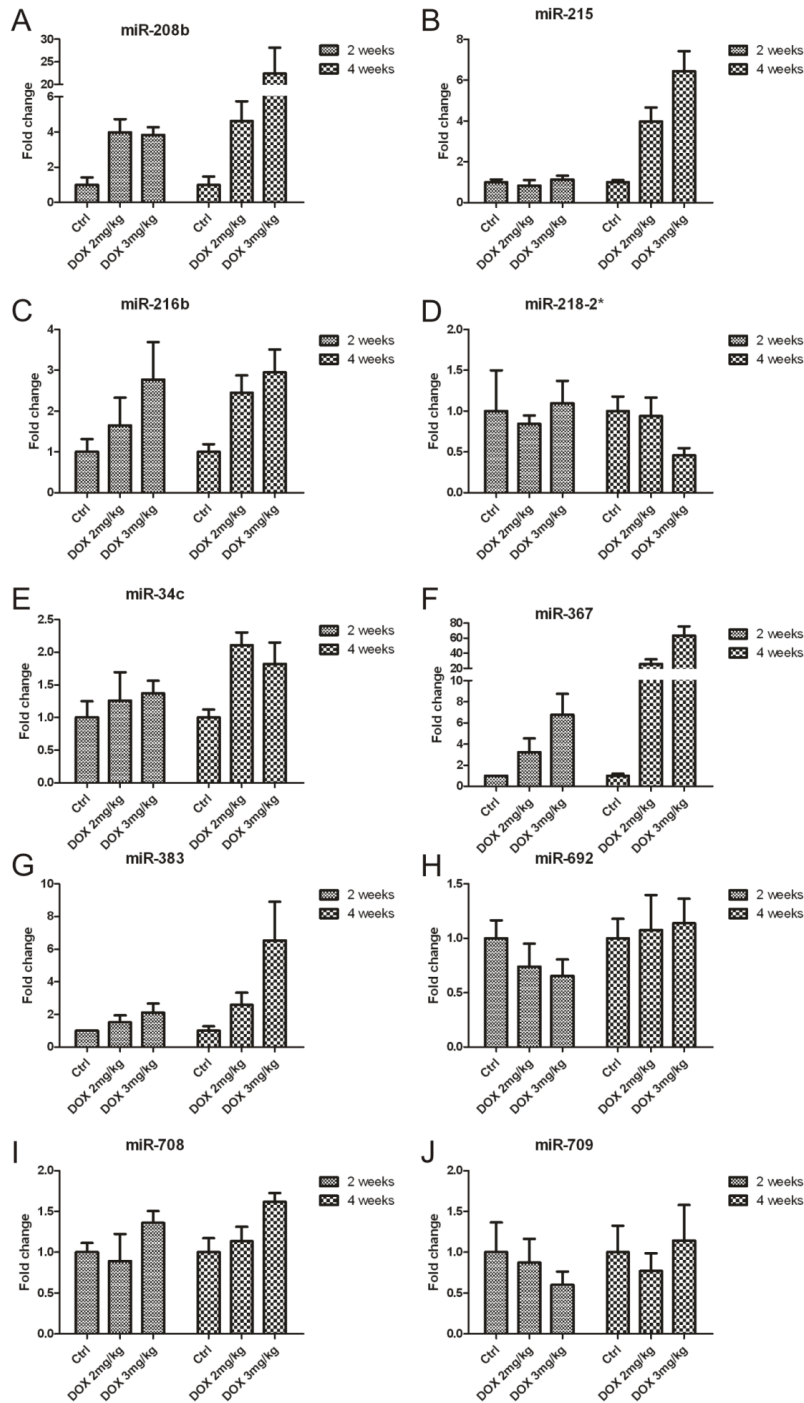
Supplementary figure 2-2. Single assay validation of TLDA data. Ten microRNAs among the DOX 3 mg/kg/week 4 weeks dysregulated microRNAs were assayed in the remaining 3 animal tissues at the same dose and in 3 animals for each indicated dose and timepoint (2 and 3 mg/kg for 2 and 4 weeks). All except miR-709 and miR-692 confirmed the trends observed with the LDA-qPCR technique. Fold changes for the given doses are indicated *vs.* untreated animals. (A) miR-208b. (B) miR-215. (C) miR-216b. (D) miR-218-2*. (E) miR-34c. (F) miR-367. (G) miR-383. (H) miR-692. (I) miR-708. (J) miR-709.

2.7.4 Putative targets of microRNAs responding to high dose DOX at 4 weeks

Supplementary table 2-3: Number of Microcosm predicted targets of 16 out of 25 microRNAs, responding to DOX 3 mg/kg/week after 4 weeks.

Up-regulated	Predicted	Raw>80	cutof f FC 1.3	cutof f FC 1.5	Gene symbols
mmu-miR-367	762	411	31	12	Alb, Igfbp5, Serpinf1, Cryl1, Itga6, Pros1, Sdc2, Dclk1, Man2a1, Marcks, Itgae Igtp
rno-miR-215	429	226	18	6	Dpp4, Bst2, Pdp2, Pcbd1, Pc, Man2a1
hsa-miR-216b	707	336	21	9	Gbp2, Cryl1, Map2k6, Afap1l1, Hrasls, Tmod4, Pxmp2, Pqlc1, Hfe2
mmu-miR-383	443	226	25	12	Cdh11, Slc25a25, Pdia4, Cryl1, Pllp, Fn1, Dtx3l,

					Abhd14b, Ccl5, Cd302, Ros1 Igtp
rno-miR-135b	345	172	16	5	Sord, Casp12, Angpt1, Ugdh, Actr3b
mmu-miR-667	651	337	20	8	Gimap4, Sctr, Dgat2, Comt, Adi1, Pik3ap1, Igtp, Ethe1
rno-miR-298	410	204	11	8	Rbp1, Gamt, Pgcp, Gimap4, Prelp, Gpld1, Ggcx, Marcks
mmu-miR-208b	572	399	11	5	Nr4a1, Marcksl1, Cd53, Ethe1, Adhfe1
mmu-miR-877	325	163	15	4	Cfh, Prelp, Rev3l, Nr1d1
mu-miR-708	333	158	18	10	Cfh, Fxyd3, Srprb /// Tf, Gamt,

					Slc25a25, Cryl1, Pfkfb1, Cp, Wfdc1, Ttc7
rno-miR-31	485	233	27	12	Col3a1, Serpinf1, Clcn4-2, Pdia4, Mt1a, Fkbp4, Ttr, Prelp, Faim3, Wfdc1, Adfp, Pc
rno-miR-34c	532	276	24	9	Fxyd3, Stard10, Cryl1, Itga6, Ppm1j, Nr1d1, Mecr, Marcks, Nid2
Down-reg					
rno-miR-337	395	193	15	5	Srpk3, Eef2k, Lrrfip1, Ecm1, Cdc25b
rno-miR-335	444	238	20	10	Lrrc48, Max, Ppm1b, Lrrfip1, LOC497978,

					Sln, Ctgf, LOC500700, Baiap2, Fhl1
hsa-miR-384	584	303	24	13	Eya2 Pkp4 Speg Plcl1, Nt5c3l, Itga5, Zyx, Nt5e, Ttc27, Ptgds, Mllt11, Mdk, Myl1
rno-miR-221	387	191	10	4	Pdlim7, Tmbim1, Rbm3, Bex1

Raw gene expression was filtered to be higher than 80 in at least 50% of the conditions at the cumulative dose of 12 mg/kg. The absolute fold change anti-correlated to the cognate microRNA was used to further reduce the lists at 2 different stringencies. Number of genes passing these filters is indicated for each signature-microRNA for which prediction was available.

2.7.5 Bias in indexed NGS microRNAs profiling

In order to obtain information on the whole microRNAs population, we prepared cDNA libraries of small RNA samples isolated from the cardiac tissue of at least 5 animals per treatment condition.

As mentioned, the reduced size of the cellular microRNAs repertoire allows sequencing of multiple samples in the same run (multiplexing). We initially attempted pooling small RNA samples ligated with 5' adapters carrying the indexed sequence (barcode) 3' end, (grey oligo sequence in Supplementary figure

2-3, Supplementary table 2-4). However, the composition of the sequenced libraries was biased by the index used, as showed by a principal component analysis in Supplementary figure 2-4. Samples prepared from the animals belonging to different pharmacological treatment groups clustered by the "barcode" sequence and not by the condition, possibly due to sequence "preferences" of the ligase or polymerase enzymes used in the sample preparation, which resulted in apparently enhanced o depleted read counts of a number of microRNAs. We therefore proceeded to profiling microRNAs using TLDA cards, as described in the coming paragraphs.
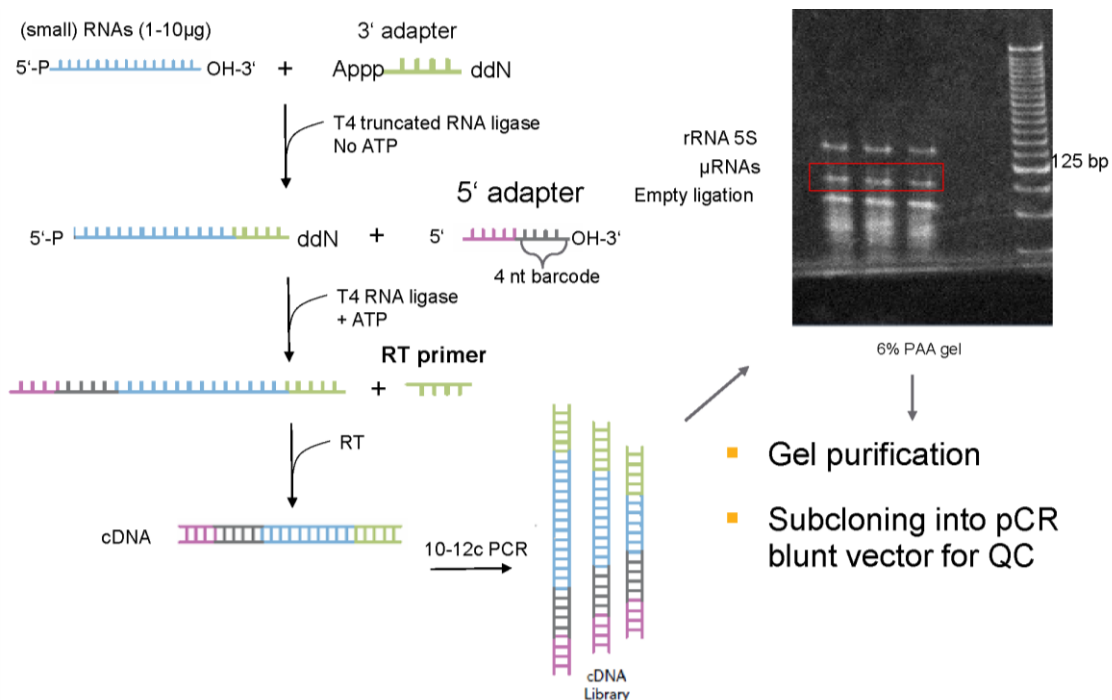
Supplementary figure 2-3: Next generation sequencing library with 5' adapters indexed at their 3' end. Sequential ligation at the 3' (green) and the indexed 5' (pink and grey) adapters to small RNAs (blue) is followed by library reverse transcription and PCR amplification. Only the products running at 123 nt (red box) are excised from polyacrylamide gel in order to avoid primers duplexes and unspecific PCR products.

More recently validated protocols for multiplexing were released by sequencing suppliers, thanks to the increased read length and improved chemistry that allowed paired-end runs for microRNAs applications. In this case the

"barcode" sequence is at the 5' of the adapter and is read during a second round of sequencing in the opposite direction. This strategy did not lead to any anomaly in the clustering of samples (data not shown).

Supplementary table 2-4: Barcoded adapters used in preliminary sequencing experiment.

Name	Sequence : (5' to 3')
Barcode 1	5'GUUCAGAGUUCUACAGUCCGACGAUC**AAAC**
Barcode 2	5'GUUCAGAGUUCUACAGUCCGACGAUC**ACCC**
Barcode 3	5'GUUCAGAGUUCUACAGUCCGACGAUC**AGGC**
Barcode 4	5'GUUCAGAGUUCUACAGUCCGACGAUC**AUUC**
Barcode 5	5'GUUCAGAGUUCUACAGUCCGACGAUC**CACC**
Barcode 6	5'GUUCAGAGUUCUACAGUCCGACGAUC**CCGC**

Libraries of small RNA samples were prepared using 6 different adapters. The 3 sub-terminal nucleotides (bold) were read within a multiplexed sequencing experiment and used to assign each read to the samples of origin.
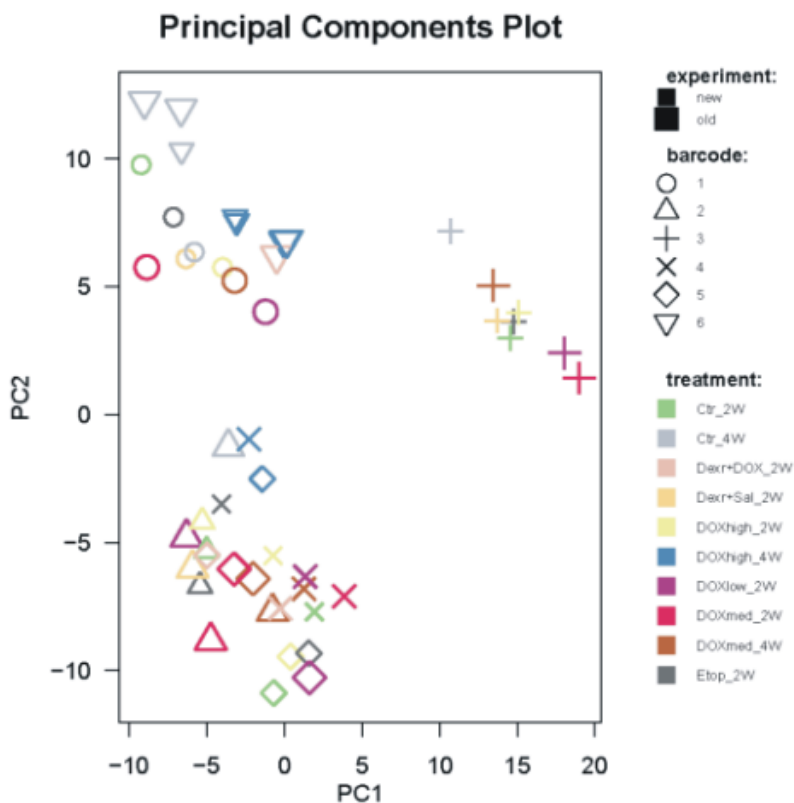
Supplementary figure 2-4: Sequenced libraries cluster according to the index sequence (symbols) and not by condition (colors) when adapter indexing is proximal to ligation site.

2.7.6 Effect of DOX 2mg/kg/week and its combination with antidote DZR on microRNAs related to cardiac diseases after 6 weeks

MicroRNAs involved in cardiac diseases include miR-1, miR-15 family, the myomiRs (miR-208a/b and miR-499), miR-21 and miR-29. Of particular interest miR-21 and miR-29b as fibrotic tissue remodeling is one of the most characterized outcomes of cardiac stress induced by anthracyclines (Jones *et al.*, 2006; Migrino *et al.*, 2008). Members of the miR-15/16 family were implicated in cardiac remodeling, including angiogenesis and post-ischemic injuries (Hullinger *et al.*, 2012).

Treatment with DOX 2 mg/kg/week (medium dose) significantly altered the levels of miR-208b, miR-21 and miR-1. However only for miR-208b this change was conspicuous (>2 folds vs. vehicle) and relieved by co-treatment with the antidote DZR. DOX treatment did not lead to significant alteration of miR-499 and miR-15a/miR-16, while a small increase was observed in the co-treatment group, but not in the DZR alone group. Neither treatment significantly altered miR-208a. No DOX-related effect was detected in the case of miR-29b, but a small decrease was caused by the treatment with DZR alone (Supplementary figure 2-5).

In conclusion, among the microRNAs tested, the variation of miR-208b measured in the cardiac tissue was a conspicuous and specific change induced by DOX, and also reflected the protective effect of DZR.
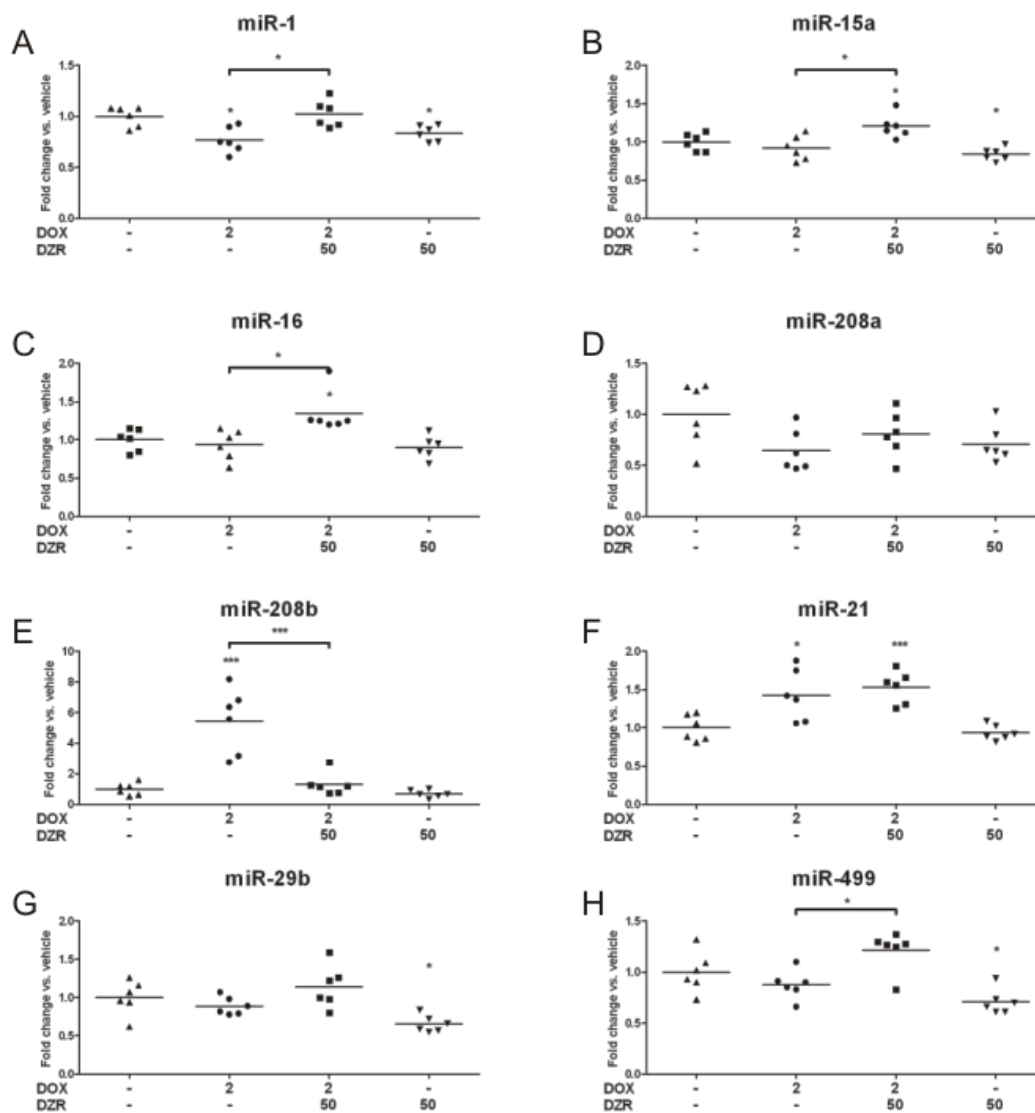
Supplementary figure 2-5: Variation of levels of microRNAs implicated in cardiac diseases in the heart tissue of rats after a 6 weeks treatment with DOX 2 mg/kg/week alone, or in combination with DZR. Fold change vs. vehicle is indicated for individual animals (n=6). Stars indicate statistical significance vs. vehicle, or otherwise indicated. *P<0.05, **P<0.01, ***P<0.005 (n=6).

2.7.7 Early changes in gene expression suggest both structural and functional damage are induced by DOX

Changes in expression level of genes implicated in glycolysis/gluconeogenesis, Nrf2-mediated oxidative stress, mitochondrial activity, ion homeostasis, carbohydrate metabolism and proliferation were among the early changes induced by DOX in SH rats by Thompson and colleagues in 2009 (Thompson *et al.*, 2009). In our study, DOX 3mg/kg/week caused decrease of Ryr2, Gpd2 and Igfbp5 transcripts, and an increase of Hmox1, Pfkp and Scn1a from week 2 onwards (Supplementary figure 2-6). Ryanodin receptor 2 (Ryr2) plays a major role in calcium homeostasis, and intracellular ion disturbances are linked to heart failure and arrhythmias (Lehnart *et al.*, 2009; Yano *et al.*, 2005a; Yano *et al.*, 2005b). The decrease of mRNA levels of glycerol-3-phosphate dehydrogenase 2 (Gpd2), part of the glycerol phosphate shuttle in mitochondria, and of insulin-like growth factor binding protein 5 (Igfbp5), associated with cell proliferation and cancer (Akkiprik *et al.*, 2008), might suggest a loss of myofibrillar functionality and regeneration potential.

Also, higher levels of phosphofructokinase (Pfkp) level contribute to the impairment of carbohydrate metabolism efficiency caused by chemically-induced oxidative stress (Janero *et al.*, 1994), after 4 doses of DOX 3mg/kg.

Sodium channel, voltage-gated, type I, alpha (Scn1a) is a neuronal sodium channel isoform and its expression level was previously characterized by Xi *et al.* in a model of failing heart in rodents, and found increased upon pressure overload (Xi *et al.*, 2009). Scn1a transcript levels are increased by DOX in the present study, which implies a disruption of the physiological action potential that resembles the failing heart.

In our study DOX 3 mg/kg/week induced changes in expression level of genes implicated in these processes from week 2 onwards (Supplementary figure 2-6).
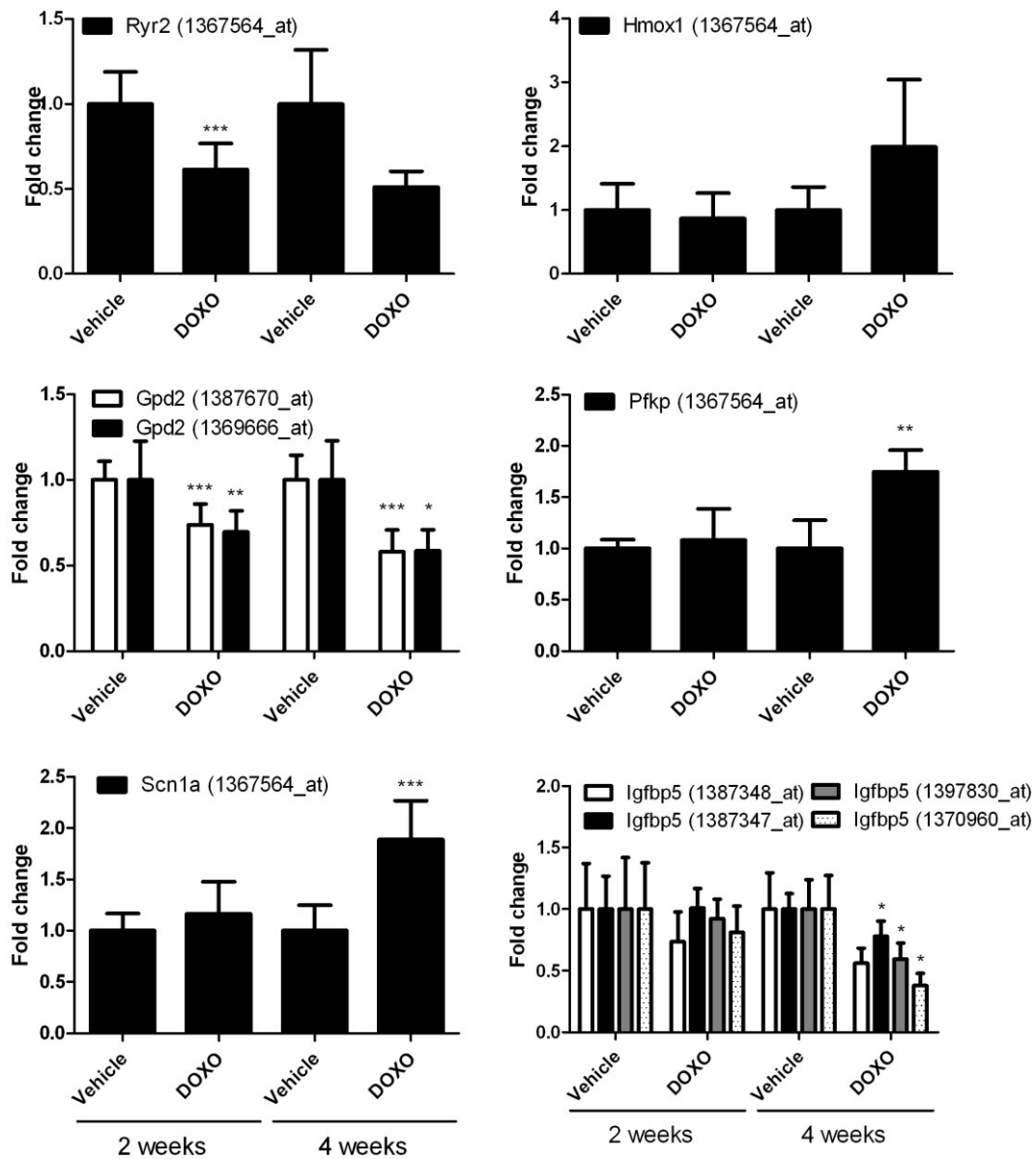
Supplementary figure 2-6: Treatment with DOX alters the expression of genes involved in several cellular processes, critical to cardiac function. Fold change is represented by the ratio between averaged DOX vs. vehicle-treated signal intensity values for each probe-set, obtained by microarray genomic profiles. Error bars represent standard deviation. *P<0.05, **P<0.01, ***P<0.005 (n=6).

The behavior of such a gene-signature suggests that both metabolic and structural functions are deeply compromised by the DOX treatment, as described by previous works.

2.7.8 DOX induces expression of anti-tumoral microRNAs

Consistently with the pharmacological action of DOX, and according to the characterization of some microRNAs based on previous works, there is a general signature of block of proliferation as suggested by the 4 anti-tumoral microRNAs up-regulated (miR-215, miR-216b, miR-31 and miR-708), the 2 p53 transcriptional responders also up-regulated (miR-34c and miR-215) and 1 downregulated onco-miR (miR-221) as shown in Supplementary table 2-5.

Supplementary table 2-5: Literature available on DOX dysregulated microRNAs. When information was available only for the other arm of the microRNAs the mature miRNA name is in bold

Early regulated at both 2 and 4 weeks		
Up-regulated	**Function**	**Selected publications**
mmu-miR-367	Expressed in embryo, inhibits Ryr3 gene	(Suh *et al.*, 2004; Zhang *et al.*, 2011)
mmu-miR-216b	Tumor suppressor, targets κ-Ras	(Deng *et al.*, 2011)
mmu-miR-383	Role in spermatogenesis	(Lian *et al.*, 2009)
mmu-miR-145*	80 papers on **miR-145** and inhibition of cells growth, in smooth muscle cells	(Rangrez *et al.*, 2011)
mmu-miR-877	-	-
mmu-miR-135b	Placental μRNA, detectable in plasma, involved in prostate cancer, miR-135a downreg in DMD, osteogenic differentiation	(Chim *et al.*, 2008; Greco *et al.*, 2009; Schaap-Oziemlak *et al.*, 2010; Wang *et al.*, 2008)
mmu-miR-692	-	-
Down-regulated		
mmu-miR-434-5p	-	-
mmu-miR-337-5p	-	-
Late regulated at 4 weeks only		
Up-regulated		
mmu-miR-208b	Transcribed from fetal gene Myh7 (upreg in GEP). Involved in hypetrophic remodeling	(Callis *et al.*, 2009; van Rooij *et al.*, 2009)

mmu-miR-21*	**MiR-21** induces apoptosis in DOXO treated cardiomyocytes, involved in fibrosis	(Han *et al.*, 2011; Roy *et al.*, 2009; Si *et al.*, 2007; Wang and Li, 2010b)
mmu-miR-298	Possible circulating biomarker for brain injury, indirectly regulates CYP3A4	(Liu *et al.*, 2009a; Pan *et al.*, 2009)
mmu-miR-31	28 publications on miR-31 function, tumor suppressor and antimetastatic function in varisous contexts, p53 regulated, dystrophin modulator	(Valastyan *et al.*, 2009)
mmu-miR-34c	65 publications, tumor suppressor, inhibits replication after DNA damage	(Hermeking, 2010)
mmu-miR-708	Tumor suppressor in renal cancer cells	(Saini *et al.*, 2011)
mmu-miR-709	Inhibits post-transcriptional maturation of pro-apoptotic microRNAs in the nucleus	(Tang *et al.*, 2011)
rno-miR-29b-2*	98 publications on miR-29b targets collagen genes, involved in fibrosis and cancer	(Milewicz, 2012)
mmu-miR-215	Together with miR-192, responds to p53 (genotoxic stress) and induces cell cycle arrest	(Braun *et al.*, 2008; Georges *et al.*, 2008)
mmu-miR-667	-	-
Down-regulated		
mmu-miR-218-2*	**MiR-218-2** is tumor suppressor, enriched in the brain	(Uesugi *et al.*, 2011)
mmu-miR-221	105 publications, oncomiR, targets p27	(Galardi *et al.*, 2007; Pineau *et al.*, 2010)
mmu-miR-335-3p	16 publications **Mir-335-5p** is a tumor suppressor, implicated in various cancer types. Also associated with obesity and lipid metabolism dysfunctions	(Nakanishi *et al.*, 2009; Ronchetti *et al.*, 2008)
mmu-miR-384-3p	-	-
rno-let-7e*	Let-7 family is associated with cancer and organism development	(Ambros, 2011; Osada and Takahashi, 2011)
rno-miR-1*	**MiR-1** is the most abundant miR in heart, involved in differentiation, regulation of proliferation and apoptosis, myocardial infarction	(Bostjancic *et al.*, 2010; Sluijter *et al.*, 2010; Tang *et al.*, 2009; Xu *et al.*, 2007)

Chapter 3: Heart structure-specific gene expression (transcriptomic) profiling reveals conserved microRNA-mRNA interactions relevant to cardiac physiology and function

3.1 Abstract

MicroRNAs are short non-coding RNAs that regulate gene expression at the post-transcriptional level and play key roles in heart development and cardiovascular disease. Here, we have characterized the expression and distribution of microRNAs across 8 cardiac structures (left and right ventricles, apex, papillary muscle, septum, left and right atrium and valves) in rat, canine and Cynomolgus monkey using miRNA-seq. Conserved microRNA signatures enriched in specific heart structures across these species were identified for cardiac valve (miR-let-7c, miR-125b, miR-127, miR-199a-3p, miR-204, miR-320, miR-99b, miR-328 and miR-744) and myocardium (miR-1, miR-133b, miR-133a, miR-208b, miR-30e, miR-499-5p, miR-30e*). The relative abundance of myocardium- (miR-1) and valve-enriched (miR-125b-5p and miR-204) microRNAs was confirmed using in situ hybridization. Potential microRNA-mRNA interactions were explored using anti correlation expression analysis in matched samples and microRNA target prediction algorithms. These included miR-1/Timp3, miR-125b/Rbm24, miR-204/Tgfbr2 and miR-208b/Csnk2a2, for which respective post-transcriptional inhibition was confirmed by over-expression in human pulmonary smooth muscle cells and in a luciferase reporter assay. In conclusion, we have generated a high-resolution heart structure-specific mRNA/microRNA expression atlas for three mammalian species relevant to biomedical research and uncovered novel microRNA regulatory circuits that provide novel insight into cardiac physiopathology.

3.2 Introduction

MicroRNAs are ~22 nucleotides long non-coding RNAs, genomically encoded and conserved throughout evolution in all superior organisms. MicroRNAs are regulators of gene expression, acting mainly at the post-transcriptional level, by directing the RISC complex to target mRNAs via imperfect complementarity (Fabian *et al.*, 2010) which mediates inhibition of translation and mRNA decay. The expression levels of many genes can be influenced by microRNAs (Lim *et al.*, 2005). However, the lack of perfect complementarity between microRNAs and their target mRNAs jeopardizes the prediction of the targets, and *in silico* prediction tools need further optimization (Thomas *et al.*, 2010).

During development, cellular microRNA pools are highly dynamic, tuned by temporal and spatial cues (Martinez *et al.*, 2008; Mendell, 2008; Shkumatava *et al.*, 2009; Stark *et al.*, 2005) and accumulating evidences implicate microRNAs in numerous physiological and pathological processes, as well as responses to xenobiotics, including drug-induced cardiotoxicity (Fukushima *et al.*, 2007a; Izzotti *et al.*, 2010; Lema and Cunningham, 2010; Marsit *et al.*, 2006a; Pogribny *et al.*, 2007; Sathyan *et al.*, 2007; Schembri *et al.*, 2009; Yokoi and Nakajima, 2011; Zhang and Pan, 2009). In particular, several microRNAs preferentially expressed in different types of muscles (miR-1, miR-133, and the myomiRs miR-208, miR-208b and miR-499, among others) play a pivotal role in maintenance of cardiac function (Catalucci *et al.*, 2009; Small and Olson, 2011), and the ablation of microRNAs-RISC machinery can have dramatic effects on cardiac development (Chen *et al.*, 2008b; da Costa Martins *et al.*, 2008; Hauck *et al.*, 2008a).

Notably, drug-induced cardiac toxicity, which is often irreversible, ranks among the most frequent reasons for compound attrition due to safety liabilities during pharmaceutical development. Histopathological read-outs, the golden

standard in safety assessment, often do not provide mechanistic explanations. These can be addressed by detection of drug-induced toxic events at earlier stages by genomic profiling, which also helps generating mechanistic hypotheses. In principle, given the central role played by microRNAs in post-transcriptional regulation, a change in the level of a specific microRNA may be prodromal to expression change of a target gene, suggesting that integrated mRNAs/microRNAs expression profiling can improve the understanding of drug-induced events at the molecular levels and translatable to the clinical context.

Although the sequences conservation of microRNAs across species has been thoroughly studied, systematic data on the degree of similarity of microRNAs distribution within complex organs and/or tissues across mammalian species are still lacking. Availability of an expression atlas can help to address the role of microRNAs pool in gene expression homeostasis in specific cell types and tissue structures at the steady state, revealing novel relevant regulatory circuits, and also be a potential source for circulating lesion markers.

In this work, we generated *ad hoc* data sets of matching microRNA and mRNA profiles from 8 cardiac structures, including apex, left and right ventricular walls, papillary muscle, septum, left and right atrial walls and cardiac valves, from 3 preclinical mammalian species relevant to human biology (*Rattus norvegicus, Canis familiaris, and Macaca fascicularis*). We identified highly expressed microRNAs that can cluster apart the different cardiac structures and found that a number of microRNAs showed a specific pattern of expression conserved in the 3 analyzed species, with particular emphasis on the cardiac valves. By comparing mRNAs with microRNAs expression across the cardiac structures, we could identify genes that had highly anti-correlated expression pattern with their predicted targeting microRNA. Among these, we confirmed that miR-1, miR-208b, miR-204 and miR-125b could directly modulate genes relevant for

cardiac/muscle physiopathology (i.e. Timp3, Csnk2a2, Tgfbr2, Akap2 and Rbm24).

Our data provide a large, comprehensive and homogenous amount of cross-species information for hypothesis-generation on the role of microRNAs, and can be further used to 1) identify microRNAs signatures characteristics of cardiac structures, 2) further elucidate microRNAs/mRNAs interactions relevant for cardiac physiology and 3) propose novel tissue injury specific indicators.

3.3 Material and methods

3.3.1 Experimental strategy

Different cardiac structures were dissected by hand from at least 3 Wistar rats, Beagle dogs and Cynomolgus monkeys (Cyno), as indicated in Figure 3-1. Small (<200 nt) and long RNA was isolated from the very same samples. Messenger RNAs transcriptome was generated via Affymetrix Gene Chip technology from all available samples (Figure 3-1). Based on the mRNAs profiles, we sub-selected the 3 samples from each structure that had the lowest cross-contamination with neighboring regions, and proceeded with microRNAs next-generation sequencing (miRNA seq) (Figure 3-1). Sequencing reads were mapped to miRBase, version 17, containing 1424 human, 720 murine and 383 canine entries. In parallel, rat microRNAs were also profiled using a TLDA qPCR approach. On average, a Spearman correlation of 0.835 ± 0.016 was observed when comparing the TLDA with the miRNA seq data set, confirming the reliability of the miRNA seq approach (Supplementary figure 3-4).
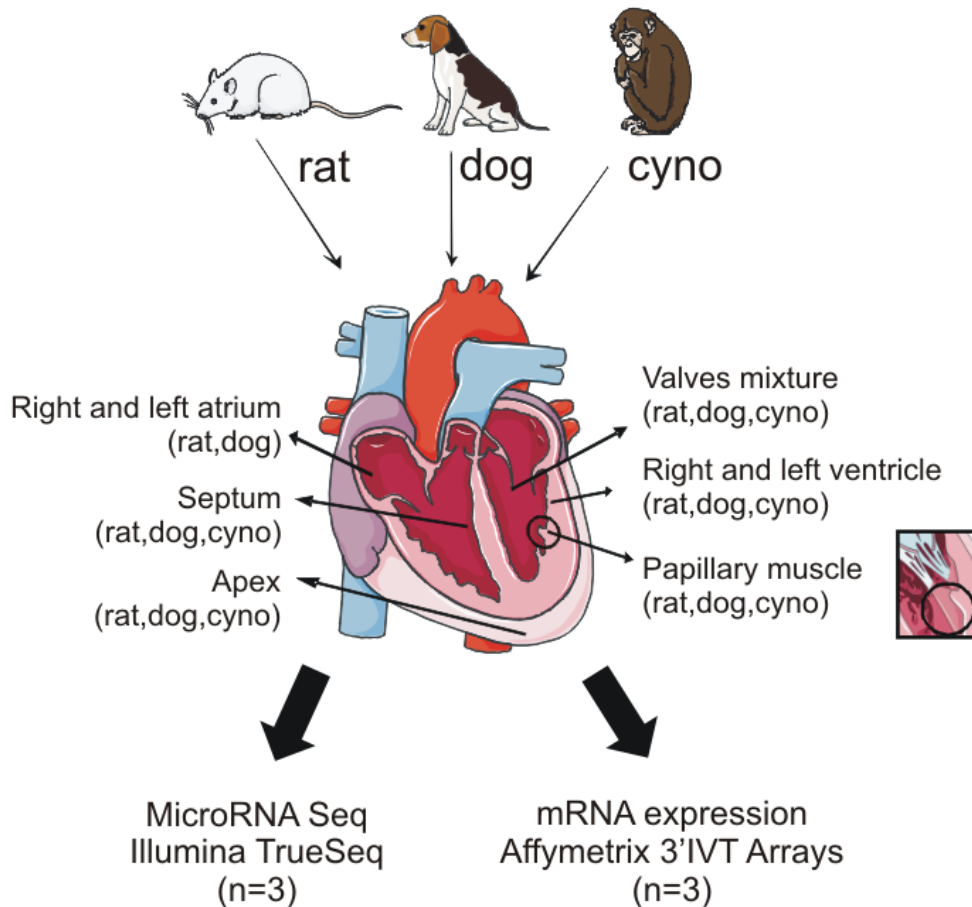
Figure 3-1: Cross-species microRNA/mRNA cardiac atlas. Data sets obtained for rat, dog and/or Cynomolgus monkey are indicated. Inset: a magnified depiction of a papillary muscle.

3.3.2 Heart tissue dissection

Hearts were obtained from naïve adult male Wistar rats, during necropsy. Following brief infusion with saline, a first cut was made parallel to the auricles, adjacent to the level of the atria; both portions were again rinsed with saline, in order to remove most of the blood. Cardiac valves (mitral and tricuspid) were dissected at first, followed by atria, septum, ventricles and apex. All tissues were snap frozen in liquid nitrogen and stored at -80°C until RNA isolation. Similar procedure was carried out for male adult Beagle dogs and female adult Cynomolgus monkeys (*Macaca fascicularis*).

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3.3.3 RNA isolation

Cardiac frozen tissue, sectioned and snap frozen as described in the previous section, was homogenized in Qiazol reagent according to manufacturer instructions. Organic extraction of RNA was achieved by adding chlorophorm and high speed centrifugation at 4°C. RNA-containing upper aqueous phase was mixed with 1 volume of 70% EtOH and loaded into RNeasy minispin columns and briefly spinned at room temperature according to miRNeasy mini kit instructions (Qiagen). The content of this filter-column was then processed to obtain total RNA, while the first flow through, enriched in <200 nt RNA (small RNA fraction) was then processed through an RNeasy Clean-up column, according to manufacturer instructions (Qiagen, MD). Small RNA was eluted in 15 µl of nuclease free water, quantified via 260nm absorbance and quality checked using the Bioanalyzer small RNA chips.

3.3.4 Gene expression profiling

Processing of total RNA and Gene Chip experiments were conducted as recommended by the manufacturer (Affymetrix, Santa Clara, CA). Messenger RNAs isolated from rat, canine and Cynomolgus were hybridized on 3'IVT arrays Rat230_2, Canine_2 and HG-U133_Plus_2 respectively.

HPASMC cells endogenous genes expression was obtained using Taqman assays for the corresponding human transcript, according to manufacturer instructions. Briefly 300 ng of RNA were reverse transcribed to cDNA using the High Capacity Reverse Transcription kit. Two µl of cDNA were used in a 20 µl qPCR reaction in 7900HT machines (all Applied Biosystems, Life Technologies, Carlsbad, CA), 18S was used as reference housekeeping gene.

3.3.5 MicroRNA Next Generation Sequencing

Indexed miRNA sequencing libraries were prepared from 100 ng small RNA using TruSeq Small RNA Sample Preparation Kits (Illumina). After PCR

amplification multiplexed libraries were generated by equimolary pooling of individual libraries. The multiplexed libraries were size selected on 6% TBE-PAGE gels (Invitrogen). The libraries were loaded on HiSeq Single Read Flow Cells v1.5 using TruSeq Single-Read Cluster Generation Kits (v2). The sequencing runs were done on HiSeq 2000 instruments.

3.3.6 TLDA qPCR

Three-hundreds and eighty rodent microRNAs were profiled using the Taqman Low Densitiy Array (TLDA) technology (Applied Biosystems, Life Technologies, Carlsbad, CA) according to the manufacturer instructions (Applied Biosystems, Life Technologies, Carlsbad, CA). Mammalian snRNA U6 was used as housekeeping gene.

3.3.7 Localization of microRNAs via ISH

Detection of microRNA by ISH was performed using double-digoxigenin labeled miRCURY LNA™ probe provided by Exiqon A/S (Vedbaek, Denmark).

ISH on rat heart tissue section was done using the fully automated instrument Ventana Discovery Ultra® (Roche Diagnostics, Rotkreuz, Switzerland). All chemicals were also provided by Roche Diagnostics except the "microRNA ISH optimization kit" provided by Exiqon A/S. Briefly, formalin fixed paraffin embedded sections were de-paraffinized and rehydrated under solvent-free conditions (EZprep solution) and pretreated by enzymatic digestion (Proteinase K at 12 μg/ml for 16 minutes at 37°C). Hybridization was performed adding to each slide 50 nM of DIG-LNA probe diluted in the Exiqon hybridization buffer and incubated for 3 hrs. After hybridization, sections were washed on stringency conditions (see Supplementary table 3-1 for details).

DIG-label LNA probe detection was performed using an Alkaline Phosphatase-conjugated Sheep anti-digoxigenin antibody (Roche Diagnostics) diluted 1/500 in antibody diluent. Antibody incubation was carried out for 30 min at 37°C

followed by chromogenic detection using BlueMap™ Kit with a substrate incubation time of 6 hrs.

Counterstaining using ISH nuclear fast red was performed for 2 min. Sections were mounted in Glycerol-gelatin mounting medium (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) and post-mounted using Pertex™.

3.3.8 Data analysis of gene expression profiles and microRNAs sequencing

The generated ".cel" files were loaded in Genespring 11.5.1 (Agilent Technologies, Santa Clara, CA) and further processed according to the standard RMA workflow. Expression data were analyzed at the gene level. Preferentially enriched gene lists were generated by retaining the top 10th percentile most expressed genes in all replicates of at least one structure. Genes that were 5 fold more expressed in one cardiac structure *vs.* the median of all the remaining were retained.

Illumina NGS reads were aligned to the respective miRBase v.17 (Griffiths-Jones *et al.*, 2008) reference sequences for the different species and to the in-silico computed sequences of their precursor molecules using the Bowtie short readaligner (Langmead *et al.*, 2009). The miRNA abundance was quantified using an in-house NGS analysis pipeline, normalized to robust estimates of the individual sequencing library sizes. MicroRNAs hierarchical Euclidean centroid-linkage clustering and distance matrix were plotted using MeV package (Saeed *et al.*, 2006). The normalized read counts were averaged per structure and the top 10th percentile most expressed microRNAs in at least one structure were compared across the other data sets of the same species in order to determine those preferentially expressed in each structure (average expression fold change >2, top 10th percentile expressed). All mRNA expression values were computed using the standard Bioconductor RMA normalization and quantification algorithm (Gautier *et al.*, 2004). The mouse miRNA target predictions were extracted from TargetScan (Version 5.2) (Grimson *et al.*, 2007) and translated to the rat and dog

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genomes by homology mapping based on the ENSEMBL gene orthology predictions. These were systematically plotted against the corresponding microRNA across cardiac samples in order to generate a Spearman correlation scores from -1 to +1. Genes expressed at a lower level in samples enriched with their targeting microRNAs, and vice versa, obtained a more negative score and will be referred to as anti-correlated.

The degree of correlation between qPCR and miRNAseq profiles was obtained by computing Pearson and Spearman correlation factors (r and r_s respectively) for each sample pair and for the microRNAs detected by both technologies.

Gene set enrichment analysis (GSEA) was performed using Ingenuity Pathways Analysis 8.8 (Ingenuity® Systems).

3.3.9 Cell culture, transfection and reporter assay

Human pulmonary artery smooth muscle cells (HPASMC, Lonza CC-2581 lot# 7F33558) were cultured according to supplier instructions. The day of transfection, cells were trypsinized, rinsed with HEPES, and resuspended in 500 µl Nucleofector solution (Lonza, CH) supplemented with 50 nM of microRNA-mimics (Dharmacon, Lafayette, CO) according to Amaxa transfection protocol A-033. After 24 h, cells were lysed in Qiazol to retrieve RNA.

Regions of interest of mRNAs 3'UTR (Supplementary figure 3-7 and Supplementary Material and Mehtods) were cloned by synthesis in pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega, Madison, WI). Target seeds were mutated using QuikChange II Site-Directed Mutagenesis Kits (Agilent Technologies, Santa Clara, CA). Primers were designed according to manufacturer instructions.

Hek293 cells were cultured according to supplier instructions. Cells ($2\text{x }10^5$ per well, in 24-wells plates) were co-transfected with 5 ng of pmiR-GLO reporter construct of interest and synthetic microRNA mimics (40 nM) (Dharmacon, Lafayette, CO) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). A C.

elegans mimic with no affinity for mammalian targets was used as negative control. Dual-GLO Luciferase Assay System (Promega, Madison, WI) was used to obtain the luciferase/Renilla relative luminescence, according to manufacturer instructions. Data were averaged for n=4 in 3 independent experiments.

3.4 Results

3.4.1 MicroRNA profiles separate the different structures of the heart as efficiently as mRNAs

Between 260 and 340 unique mature microRNAs were found expressed (normalized counts >10) in each cardiac structure in the different species. A hierarchical Euclidean linkage clustering showed that microRNAs profile can group the samples in at least 3 distinct clusters, comprising the two ventricles, apex, septum and papillary muscles (myocardium), the 2 atria (atria) and the cardiac valves (Figure 3-2A) with a threshold of > 7.5. Similarly, principal component analysis of microRNAs and mRNAs data sets showed that individual samples from these 3 main structures grouped together (Figure 3-2B and C). In fact valvular profiles were clearly separated from atrial and ventricular profiles. Conversely, expression profiles from ventricles, septum, papillary muscles and apex were virtually indistinguishable from each other and therefore pooled together for further analysis under the name of "myocardium". This was consistent with the cell type composing these structures, where cardiomyocytes are predominant. Similarly, left and right atria profiles were evaluated together in further analysis based on the similarity of their profiles.
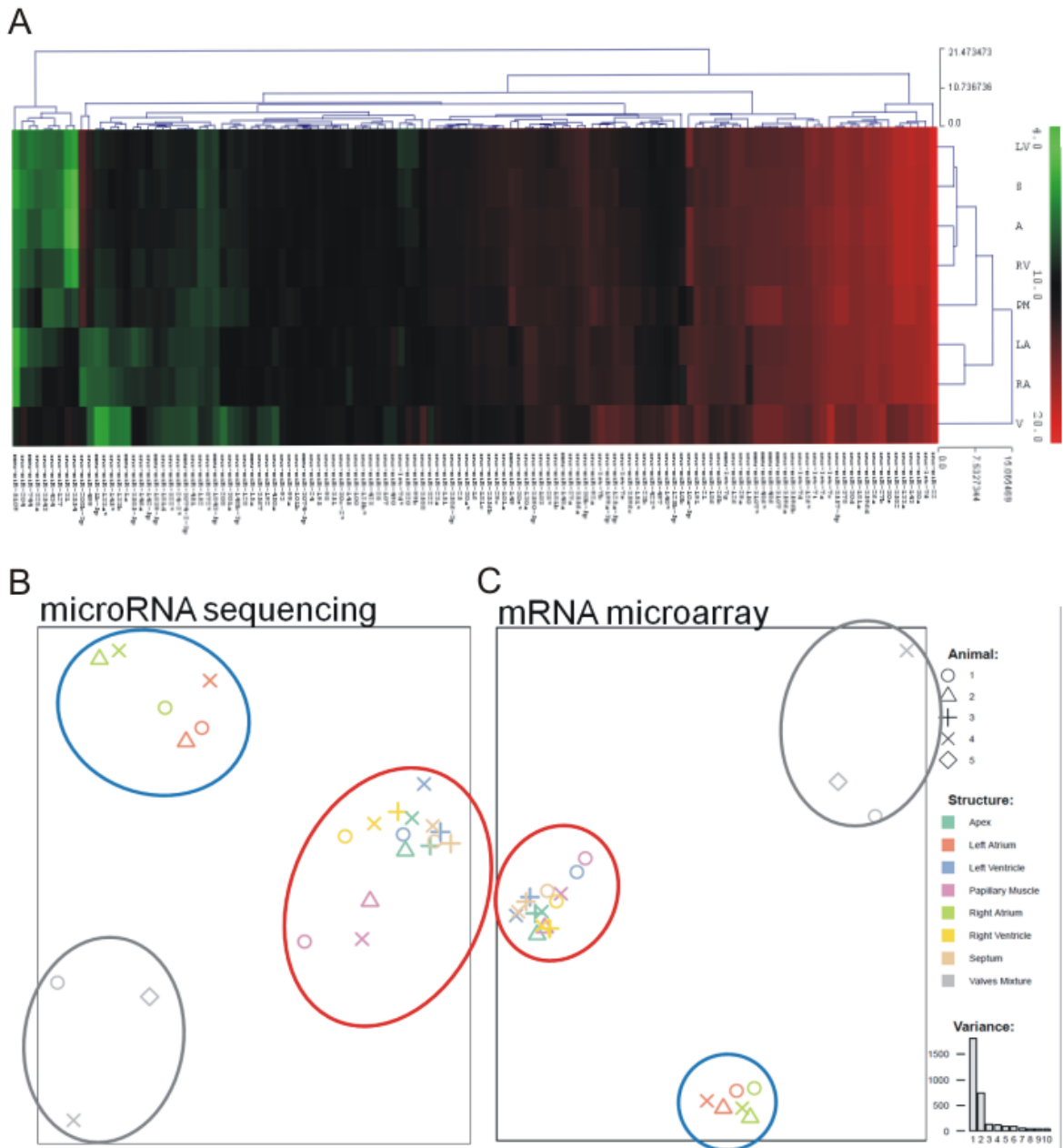
Figure 3-2: Distribution of microRNAs in rat cardiac structures. (A) Cardiac samples are grouped according to the structure using microRNAs signatures (top 10%) in a hierarchical Euclidean-linkage clustering. (B-C) cardiac structures are separated by microRNAs and mRNA distribution in a principal component analysis. Red circles: myocardial tissue (apex, left and right ventricle, septum, papillary muscle). Blue circles: left and right atrium. Grey circles: cardiac valves. A, apex. LA, left atrium. LV, left ventricle. PM, papillary muscle. RA, right atrium. RV, right ventricle. S, septum. V, valves.

The clusters obtained were reproducible and consistent across several individuals, suggesting that the structure-specific signatures were robust enough and were not affected by inter-individual differences (Supplementary figure 3-1).

3.4.2 Conserved structure-enriched microRNAs and mRNAs across species

For each species, we identified microRNAs that were preferentially expressed in each of the 3 described major cardiac structures (valves, myocardium or atria), by a two step procedure. At first we retained only the microRNAs in top 10th percentile most expressed for each structural cluster. Secondly, we further compared the expression of each highly expressed microRNAs across structures, retaining those that were preferentially enriched in one structure only (see Data Analysis section in Material and Methods for details). In this way, we obtained lists of microRNAs likely to play a role in gene expression homeostasis in cardiac valves, myocardium or atria. These lists are available as supplementary information (Supplementary table 3-2, Supplementary table 3-3, Supplementary table 3-4). The 3 cardiac structures could still be discriminated in the 3 species by a hierarchical clustering when using these limited microRNAs signatures (data not shown). The commonly called heart-specific microRNAs were indeed amongst the most expressed microRNAs in the myocardium, but were present to a much lower extent in valves and atria. Applying a similar analytical process, structure-enriched messenger RNAs were also identified (see Data Analysis section in Material and Methods for details).

In an attempt to determine the degree of conservation of cross-species structure-specific distribution of microRNAs, we found that 9 unique microRNAs were highly enriched in the cardiac valves of rat, canine and Cynomolgus monkey (miR-let7c, mIR-125b, miR-127, mir-199a-3p, miR204, miR-320, miR-99b, miR-328 and miR-744) (Figure 3-3A), 7 in the myocardium (miR-1, mir-133a, miR-133b, miR-208b, miR-30e, miR-499-5p, miR-30e*)(Figure 3-3A). At the messenger mRNA level, 4 (S100a4, Inhba, Mfap4 and Cdh11) and 1 (Myl2)

mRNAs were found to be expressed consistently in cardiac valves or myocardium respectively of rat, canine and monkey (Figure 3-3B).
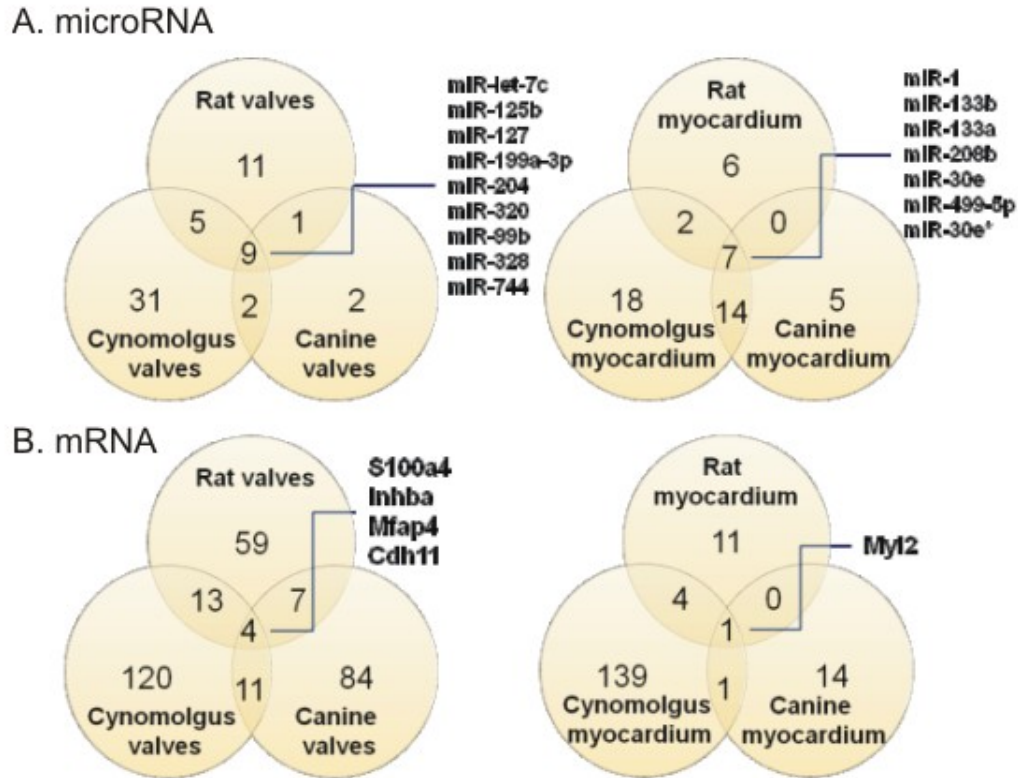
Figure 3-3: Number of microRNAs (A) and mRNAs (B) preferentially expressed in cardiac valves or myocardium across species. Myocardium includes apex, ventricles, septum and papillary muscles. Fold change thresholds were >= 2 for microRNAs in (A), and >=5 from mRNAs in (B).

A few microRNAs were selected for further analysis and their expression relative to apex is shown in Figure 3-4. Mir-1 is highly expressed in almost all structures, but to a much lower extent in the valves, in all species. MiR-208b is more specific of the myocardium structures, and is almost absent in the atria of rat and canine as well as in the valves of all species. MiR-125b-5p and miR-204 are enriched in the valves of all 3 species while less abundant in all other structures. Notably, mir-125b-5p expression is not restricted to the valves in dog, as for rat and Cynomolgus monkey.
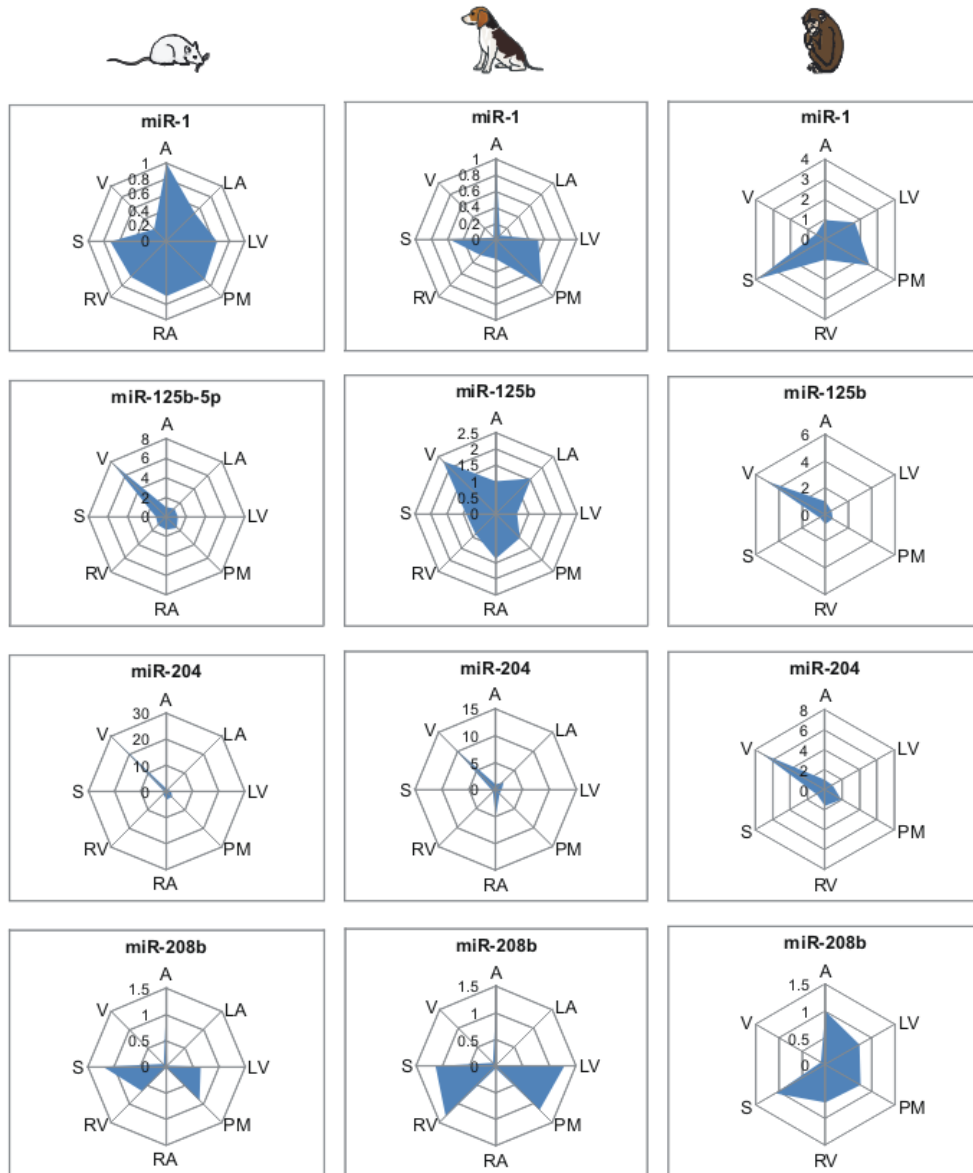
Figure 3-4: Distribution of miR-1, miR-125b-5p, miR-204 and miR-208b in the cardiac structures. Axes represent fold change vs. apex. A, apex; LA, left atrium; LV, left ventricle; PM, papillary muscle; RA, right atrium; RV, right ventricle; S, septum; V, valve.

3.4.3 Confirmation of microRNAs distribution by in situ hybridization

Relative expression obtained by ISH for miR-1, miR-125b-5p and miR-204 were consistent with the distribution patterns observed by miRNAs seq and qPCR.

MiR-1, miR-204 and miR-125b were detected in rat cardiac tissue by *in situ* hybridization (ISH). Representative micrographs are shown in Figure 3-5. Coherently with our miRNAs seq data signal for miR-1 was more intense in the myocardium than in the valves (Figure 3-5G, H and I), and staining for miR-204 and 125b-5p was more intense in the valves than in the rest of the heart (Figure 3-5A to F).

MiR-1 showed an intense and uniform staining in the ventricle, while was weakly detectable in cardiac valves (Figure 3-5 G, H and I and Supplementary table 3-5). The signal for miR-204 appeared more intense at the level of endothelial cells in the valves (Figure 3-5 A and B). However, specificity of miR-204 for endothelial cells will have to be investigated further via ISH in other endothelial cells-rich tissues. Mir-204 was weakly expressed in myocardial tissue, and the staining was almost undetectable (Figure 3-5 C). Similarly to miR-204, also miR-125b-5p showed a more intense signal in the cardiac valves than in the myocardium (Figure 3-5 D, E and F). MiR-125b-5p was strongly expressed in the myocardium but was more preponderant in the cardiac valves according to expression data (Supplementary table 3-5). While signals for miR-1 and miR-125b-5p were strong, miR-204 was at the limit of detection, consistently with its relative abundance as observed by miRNAseq and qPCR (Supplementary table 3-5). Several attempts of staining for miR-208b were unsuccessful, possibly due to the low expression level of miR-208b (Ct value ~27 in rat myocardium).

In conclusion, the results of the *in situ* hybridization correlate well with the relative distribution of microRNAs observed using the miRNA seq/TLDA approach and suggest that the precise dissection of the rat heart successfully allowed the isolation of expression profiles from individual structures.
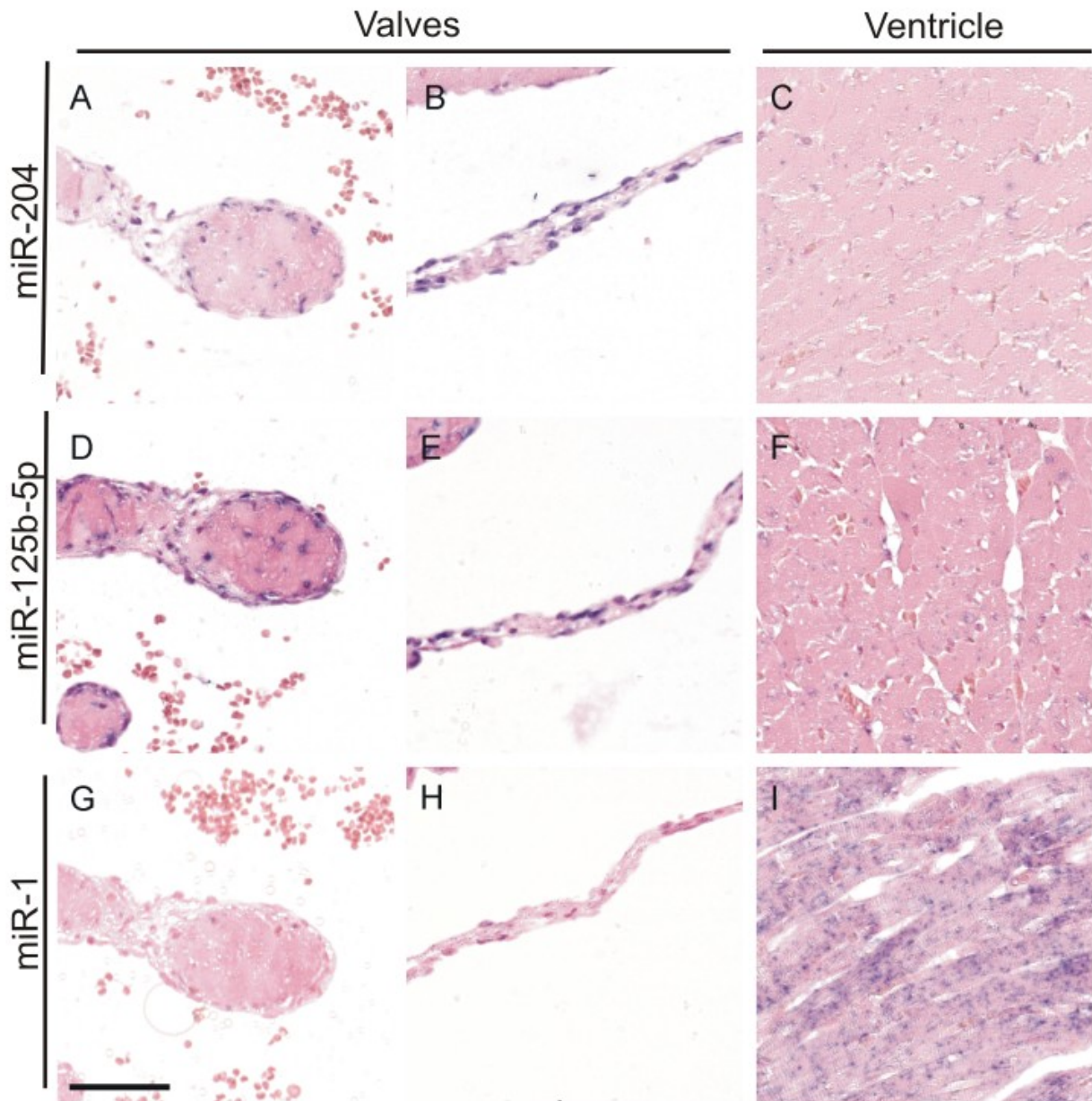
Figure 3-5: Localization of miR-204, miR-125b-5p and miR-1 in rat heart. MiR-204 in valves (A-B) and myocardium (C). MiR-125b-5p in valves (D-E) and myocardium (F). MiR-1 in valves (G-H) and myocardium (I). Bar = 50 μm.

3.4.4 Integration of gene expression and microRNA profiles at the steady state

MicroRNAs targets were obtained from Targetscan (human database was used for Cyno microRNAs). Spearman correlation scores for the distribution of each

microRNA and their predicted mRNAs targets were calculated across all samples, in order to identify avoidant expression profiles.
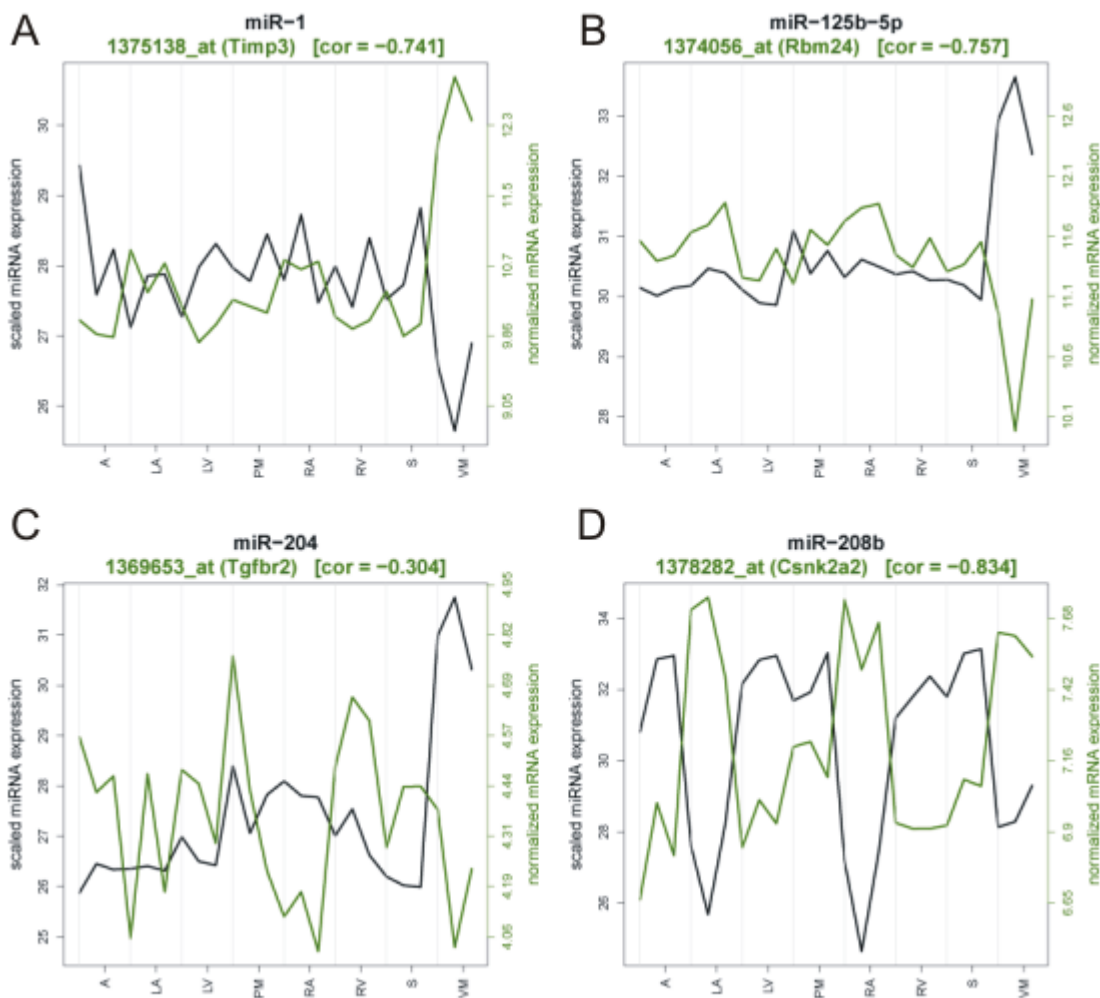
Figure 3-6: Rat cardiac disease relevant genes are negatively correlated with putative targeting microRNA. Timp3 and miR-1 (A), Rbm24 and miR-125b-5p (B), Tgfbr2 and miR-204 (C), Csnk2a2 and miR-208b (D). Green curve represents log2 normalized intensity for the indicated probe set. Black curve represents log2 scaled and normalized microRNA read counts. Three replicates are plotted for each structure.

Expression profiles of four microRNAs interesting for their distribution pattern (miR-1, miR-125b, miR-204 and miR-208b) and 4 respective predicted target mRNAs that show anti-correlated distribution in rat are shown in Figure 3-6. Interestingly Timp3, Rbm24, Tgfbr2 and Csnk2a2 were already reported to be

implicated in cardiac physiology and/or muscular differentiation (Bujak and Frangogiannis, 2007; Hauck *et al.*, 2008b; Jin *et al.*, 2010; Kandalam *et al.*, 2010; Limana *et al.*, 2011; Litchfield, 2003; Miyamoto *et al.*, 2009). The sequence of the microRNAs predicted binding site is conserved across species, reinforcing the hypothesis that the interactions could be relevant for heart biology (Supplementary figure 3-5). Expression profiles of miR-1/Timp3 and miR-125b-5p/Rbm24 were also anti-correlated with microRNAs expression in the canine heart (Supplementary figure 3-2) and Cynomolgus monkey (Supplementary figure 3-3). Expression profiles of miR-204/Tgfbr2 in canine and Cynomolgus monkey as well as miR-208b/Csnk2a2 in Cynomolgus monkey were instead positively correlated. Human Timp3 was found down-regulated upon miR-1 over expression in HeLa cells by Lim and colleagues (Lim *et al.*, 2005), although it was not investigated whether the effect was directly mediated by the microRNAs at the post-transcriptional level by a direct reporter assay. Interestingly, Tgfbr2 was shown to be directly targeted by miR-204 in human epithelial cells (Wang *et al.* 2010a), providing us with a suitable positive control for further confirmatory experiments.

Transfection of miR-1 in HPASMC caused a decrease of about 50% of both the endogenous Timp3 mRNAs level (Figure 3-7A) and of the corresponding rodent Timp3 3'UTR reporter assay in HEK 293 (Figure 3-7E). Interestingly, we demonstrated that only one out of 2 predicted binding sites was active: seed mutation of site 1 (pmiR-GLO-Timp3-S1-MUT), but not site 2 (pmiR-GLO-Timp3-S2-MUT), could rescue expression of reporter gene luciferase, suggesting that site 2 is not involved in the regulation of this gene by miR-1. Notably, Limana *et al.* previously reported that the site 2, but not site 1, was targeted by miR-206, which has identical 5' seed to miR-1, whilst site 1 did not respond to miR-206 over expression. Additional interactions mediated by the 3' end of the microRNA were crucial for alternate miR-1/206 regulation of Timp3, beyond the

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canonical 5' seed, suggesting an alternative effect of highly related microRNAs in different cell types or tissues.
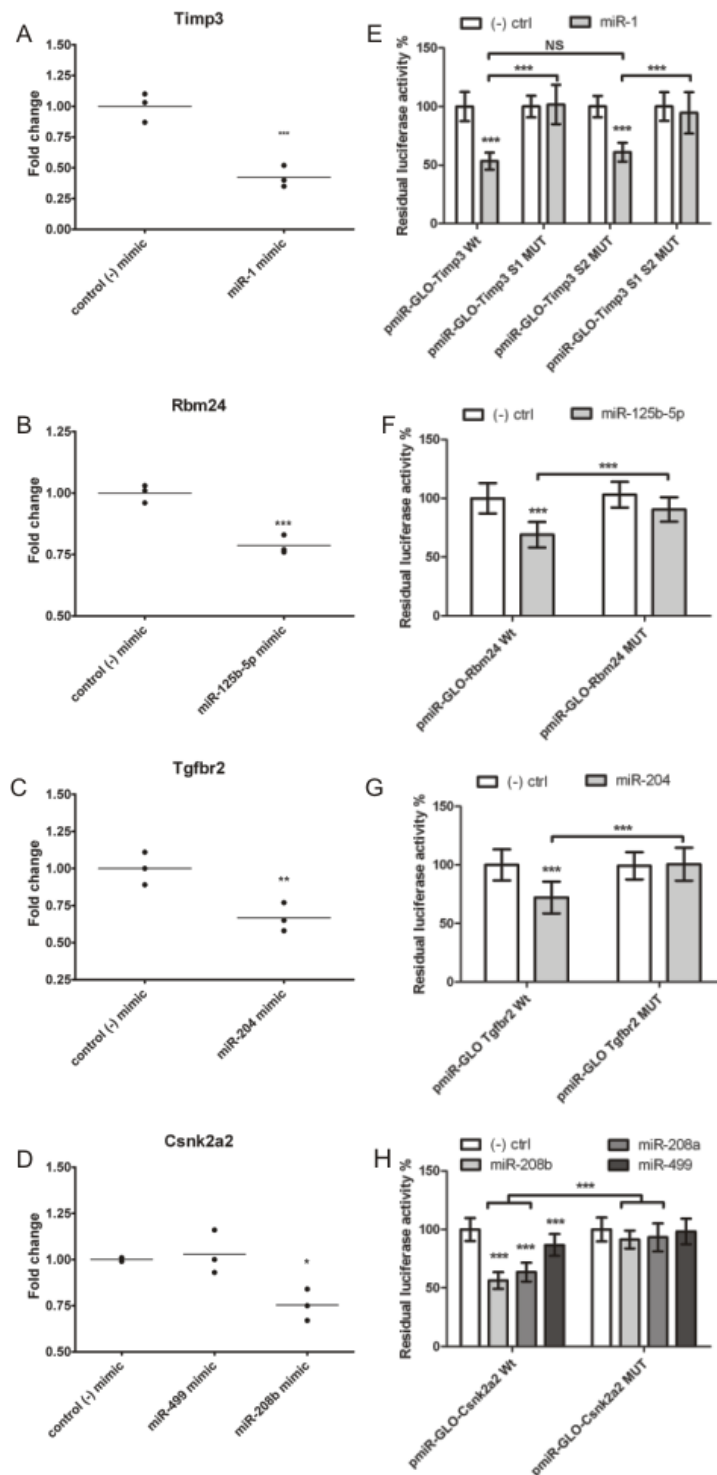
Figure 3-7: Anti-correlated microRNA targets are directly inhibited by microRNA over expression. (A-D) Endogenous expression of Timp3, Rbm24, Tgfbr2 and Csnk2a2 genes is

Notably, miR-206 was not detected in any of the cardiac structures of any of the 3 species investigated in this study, in accordance to what reported by Rao *et al.* (Rao *et al.*, 2009), while it is expressed in the skeletal muscles (data not shown).

Transfection of miR-125b-5p in HPASMC significantly reduced Rbm24 levels by 25% (Figure 3-7B), consistently with the effect observed with the 3'UTR reporter assay in HEK 293 (Figure 3-7F). Mutation of the predicted target site rescues luciferase gene activity. The function of Rbm24 and Rbm38 stabilize the mRNAs of myogenin and p21, respectively, in skeletal muscle (Jin *et al.*, 2010; Miyamoto *et al.*, 2009). The levels of their mRNAs increase during myoblastic differentiation, while at the same time miR-125b-5p expression drop (Ge *et al.*, 2011). Interestingly, Rbm38 is also anti-correlated to miR-125b in our cardiac samples, and has a predicted miR-125b targeting site in its 3'UTR. However only Rbm24 is directly affected by miR-125b co-transfection in the luciferase assay (Figure 3-7F), while luc-Rbm38 signal was unaffected (data not shown).

Transfection of mir-204 significantly decreased the level of Tgfbr2 mRNA by 20% in both HPASMC (Figure 3-7C) and in co-transfection with the corresponding Tgfbr2 3' UTR luciferase reporter assay (Figure 3-7G).

MiR-208b over expression lowered Csnk2a2 mRNA level in HPASMC by 25% (Figure 3-7D), and co-transfection with the reporter assay caused a 50% inhibition of luciferase activity in HEK cells (Figure 3-7H). Although the 3 myomiRs miR-208a/miR-208b/miR-499 are almost identical at the seed sequence, miR-499 was much less potent at inhibiting luc-Csnk2a2 than miR-

208a/miR-208b, suggesting a functional role of 3' compensatory interaction between the microRNA and the target in this case (Figure 3-7D and H).

In summary, we have verified that 4 genes important for the cardiac/muscular physiology are post-transcriptionally regulated by miR-1, miR-125b-5p, miR-204 and miR-208b, and the respective anti-correlated profiles suggest that these microRNAs are responsible for the homeostasis of these transcripts across tissues and species.

3.5 Discussion

The tissue-specific deletion of Dicer in murine myocardial lineage causes lethality, demonstrating the pivotal role played by microRNAs in cardiovascular development (Chen *et al.*, 2008b). In particular, miR-1 and miR-133, which are abundant microRNAs in the heart, are implicated in cardiovascular development and myocardial lineage differentiation, as they tightly control expression of muscle genes and repress "unwanted" gene levels, through a network of target transcription factors (Ivey *et al.*, 2008; Liu *et al.*, 2008; Schlesinger *et al.*, 2011; Zhao *et al.*, 2007). Noteworthy, miR-1, miR-133, mir-30, miR-208a, miR-208b, mir-499, miR-23a, miR-9 and miR-199a were shown to be functionally involved in cardiovascular diseases such as heart failure and hypertrophy (Latronico and Condorelli, 2011; Thum *et al.*, 2007; van Rooij *et al.*, 2009; van Rooij *et al.*, 2006; van Rooij *et al.*, 2007), and have been proposed as therapeutics or disease related drug targets (Montgomery *et al.*, 2011; van Rooij and Olson, 2007). However one of the issues of studying the function of specific genes/microRNAs in the cardiac organ is the heterogeneity of cell type and morphological composition. In order to have a better understanding of the significance of different microRNA in the physiology of distinct heart structures we have generated a comprehensive heart structure-specific transcriptomic resource for

three mammalian species that should facilitate the investigation of microRNA/mRNA regulatory interactions in cardiac physiopathology mechanisms and may also enhance the identification of cardiac tissue injury biomarkers.

Conserved microRNA signatures were identified in valves (miR-let-7c, miR-125b, miR-127, miR-199a-3p, miR-204, miR-320, miR-99b, miR-328 and miR-744) and in ventricular-specific regions of the myocardium (miR-1, miR-133b, miR-133a, miR-208b, miR-30e, miR-499-5p, miR-30e*) of Wistar rat, Beagle dog and Cynomolgus monkey. The putative targets of conserved valvular and myocardial microRNAs found in this study are significantly enriched in genes implicated in Gene Expression Regulation, Cellular and Organism Development according to gene ontology terms enrichment (Supplementary figure 3-6), suggesting that they may play important roles in defining the molecular identity of heart-specific structures through direct post-transcriptional regulation of mRNA targets and their downstream effects on gene regulatory proteins. Conversely, microRNAs that exhibit a dissimilar heart-structure specific distribution across species are likely to be of high interest for translational science applications including the drug cardiosafety assessment.

Here we have focused on the characterization of four microRNAs, including myocardial specific miR-1 and miR-208b and valve enriched mir-204 and miR-125b-5p, based on their distinct heart-structure-specific distribution patterns and known roles in cardiac physiology, disease and pathological remodeling. Numerous putative microRNA targets were found to have an anti-correlated distribution with these 4 microRNAs across cardiac tissues supporting the biological relevance of predicted microRNA-mRNA interactions. We demonstrated that miR-1 could directly control Timp3 expression at the post-transcriptional level. Timp3 is a regulator of adult myogenesis implicated in the fibrotic pathological remodeling of infarcted heart, and atrial fibrillation (Barth *et*

al., 2005; Kandalam *et al.*, 2010; Limana *et al.*, 2011; Liu *et al.*, 2010). Interestingly, Timp3 has 2 predicted sites for miR-1/206 family and despite miR-1 and miR-206 (an already known regulator of Timp3) having identical seed sequence, miR-206 targets specifically the second site (Limana *et al.*, 2011), while miR-1 specifically targets the first site (Figure 3-7E). These observations suggest that duplication of targeting sites may be a strategy for redundant regulation in cardiac cell types having different microRNA repertoires. RNA binding motif proteins 24 and 38 (Rbm24 and Rbm38) play a role in muscle differentiation (Jin *et al.*, 2010; Miyamoto *et al.*, 2009) and carry a miR-125b-5p targeting site. Interestingly, the function of miR-125b in controlling proliferation/differentiation switches was characterized in a number of cell types, such as immune system and muscular cells (Ge *et al.*, 2011; Le *et al.*, 2011; Lee *et al.*, 2005; Murphy *et al.*, 2010; Rossi *et al.*, 2011). We found that miR-125b-5p over-expression could lower Rbm24, but not Rbm38, levels in HPASM cells, and that the down-regulation was directly mediated by the microRNAs as verified *via* luciferase reporter assay. The direct relationship between the RNA regulatory protein Rbm24 and miR-125b-5p reinforces the key responsibilities of this microRNA in cell fate switches. TGF-β receptor 2 (Tgfbr2) has been functionally connected to cardiovascular diseases (Barth *et al.*, 2005; Bujak and Frangogiannis, 2007; Kato *et al.*, 2009; Wang *et al.*, 2010a) and miR-204 was observed to directly inhibit Tgfbr2 mRNA in a direct manner both in HPASM cells and in a luciferase assay, thus extending the potential roles for miR-204 in cardiac pathogenesis. Casein kinase 2a2 (Csnk2a2) and miR-208ab have both previously been implicated in cardiovascular pathologies (Eom *et al.*, 2011; Hauck *et al.*, 2008b; van Rooij *et al.*, 2007) and we have shown here for the first time that they are inter-related based on the striking anti-correlation between their expression profiles in cardiac tissues. Additional predicted targeting sites for miR-221/222, implicated in muscular development (Cardinali *et al.*, 2009; Davis *et al.*,

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2009; Liu *et al.*, 2009b), were also found in Timp3 and Rbm24 3' UTRs (Supplementary figure 3-7), however the corresponding luciferase reporter assays were unaffected when miR-221 mimic was co-transfected in Hek 293 cells (data not shown) suggesting that miR-221/222 could not directly regulate these genes at the post-transcriptional level.

MicroRNAs can easily be quantified in body fluids using assays that are fully translatable across species based on the sequence conservation (Wang *et al.*, 2009) and numerous publications support the use of tissue-specific microRNAs in the peripheral circulation as marker of tissue injury (Starkey Lewis *et al.*, 2011; Wang *et al.*, 2010b; Wang *et al.*, 2009; Wang *et al.*, 2011b). Furthermore, heart ventricular microRNAs (miR-1, miR-133 and miR-499) were found to be increased in the plasma of patients with myocardial infarction, and might represent an alternative to the classical cTnI cardiac injury biomarkers (Cheng *et al.*, 2010; Corsten *et al.*, 2010; D'Alessandra *et al.*, 2010a; Wang *et al.*, 2010b; Wang *et al.*, 2011b). MicroRNAs involved in drug-induced cardiac injury may also provide earlier safety endpoints as their expression change could be prodromal to the corresponding target gene/protein variations. Here, for the first time, we describe conserved valve-enriched microRNAs that may help to monitor or predict valvular disease and drug-induced valvular injury, for which no translational circulating biomarkers currently exist (Bhattacharyya *et al.*, 2009).

The identification of microRNAs that are highly specific for a cell type or morphological structure is challenging, as most of them are ubiquitous, but this may be overcome by measuring a signature of several microRNAs. This strategy can help to discriminate between closely related tissues (such as skeletal *vs.* cardiac muscle), or isolate the signal of an under-represented structure in global genomics analyses.

In conclusion, data presented here provided a valuable resource to investigate potential roles of specific microRNAs as conserved regulators of cell homeostasis

and tissue pathological remodeling. Our data sets can be further analyzed to answer different open questions, such as cell type specific genes/microRNAs distribution, involvement of microRNAs in genome plasticity, disease and cellular homeostasis and co-regulation between intronic microRNAs and their hosting transcripts. One important future direction is to obtain suitable clinical samples for the extension of this molecular atlas to human heart-specific structures. A similar transcriptomic atlas strategy can also be extended to other heterogeneous organs in order to characterize potential tissue structure-specific specific mechanisms and biomarkers.

3.6 Supplementary Material and Methods

3.6.1 In situ hybridization

Supplementary table 3-1: LNA probe hybridization conditions

Probe Name	Reference number	Hybridization temperature	Stringency washes
hsa-miR-204	18098-15	55°C	0.5x SSC at 37°C (3x times)
hsa-miR-125b	18022-15	57°C	0.5x SSC at 37°C (3x times)
rno-miR-1	41336-15	47°C	2.0x SSC at 37°C (3x times)

3.6.2 Cloned regions of Timp3, Rbm24, Rbm38, Akap2, Tgfbr2 and Csnk2a2

Relative position of cloned stretches is shown in Supplementary figure 3-7. Mutations are in indicated in bold.

Timp3 WT 5'-3' (Murine NM_011595.2):

AGTTTAATCTTTTTTTCTTTTGTTGTTTGATGGGAAATTGTGACATTC
CAAGTTGACTTTTTTTTATTATCTGAACAGATTATGGGTATTTATCCTC
TATTAGTATCATATCGTTTTCATTCCACTTTAGAAACACAGTTACCAGT
TGAGTATTTCACCTGTTCACTTACTGTATAATTTAAAATCATTTATGTA

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GCTGAGACACTTTGATACTTCAATTATATCGAGAAAACCCCCTCTAGA
AGGAATGTATTTGTTGCTAAATTTTGTAGCACTGTTTACAGTTTTCCTC
CATGTTA

Timp3 S1-S2 MUT 5'-3' (Murine NM_011595.2):

AGTTTAATCTTTTTTTCTTTTGTTGTTTGATGGGAAATTGTG**TGTAAG
G**AAGTTGACTTTTTTTTATTATCTGAACAGATTATGGGTATTTATCCTC
TATTAGTATCATATCGTTTT**GTAAGGT**CTTTAGAAACACAGTTACCAG
TTGAGTATTTCACCTGTTCACTTACTGTATAATTTAAAATCATTTATGT
AGCTGAGACACTTTGATACTTCAATTATATCGAGAAAACCCCCTGAAG
AAGGAATGTATTTGTTGCTAAATTTTGTAGCACTGTTTACAGTTTTCCT
CCATGTTA

Rbm24 WT 5'-3' (Rat NM_001191100):

AGGACACGATGGCAGGGGTACCCATGGGACTTCACTTTTGTATGGG
GATTTTTATTTTTTTTGCTCTTTTTTATAGTATCAGGGAAGCAAACTGC
CTTTTCCAAGTTAGAAAACGCTACGTGAATCTAGCTGAACCAGGGAAT
ACGGAGTCTCTAAAGGAAGGAACTTTAGAAGTGACACTGTAAAATTAT
GTATTCATCTCATGGCATAAGTTATTCAGTAGGTCTAGATGTAGCATAT
TAAATATTAACCTATTCAACTAAAGATGTTGGCTTTGGATTTATTTAAA
TTCTTATGTGC

Rbm24 MUT 5'-3' (Rat NM_001191100):

GAGAGGACACGATGGCAGGGGTACCCATGGGACTTCACTTTTGTAT
GGGGATTTTTATTTTTTTTGCTCTTTTTTATAGTA**AGTCCCT**AGCAAAC
TGCCTTTTCCAAGTTAGAAAACGCTACGTGAATCTAGCTGAACCAGGG
AATACGGAGTCTCTAAAGGAAGGAACTTTAGAAGTGACACTGTAAAA
TTATGTATTCATCTCATGGCATAAGTTATTCAGTAGGTCTAGATGTAGC
ATATTAAATATTAACCTATTCAACTAAAGATGTTGGCTTTGGATTTATT
TAAATTCTTATGTGC

Rbm38 WT 5'-3' (Rat NM_001108965.1):

CACCCAGCCCCCCATTGTCACCTGTACCAGTCTGGAGCATGACCTAG
CTGCACAGGGAGAAGGTCCAGCCCACACACGTCTGGGAGGGGAGGAG
TGGGCATCTCTGGCAGCATGGGCTGCCCACAGGCTGGGCAGTGGGTCC
CTCCACACCTACCTCAGGGAGCCAGTGTGTAGGCACTCCACAGCATGC
CTGTTAGTCAAGGCCTGGAGGACAGAGGGAGTAATGTTATAATAATAT
TTTTATTAGAATGTTCTGATTATAAAAAATAAAACTTGTTTTCTTTAAA
G

Akap2 WT 5'-3' (Human NM_001004065):

ATTGTGTGTTTGTAAAATGTGTATGTTCATGAGTAAGGGTGTGTGTG
TGTGTGTATTAAAATTCCAGAGTGACCGTGGCACTTGGGTGTACAGGT
AATTCCTCCAGAGCTGTTTGCTGGCTTCAGGAGTGGAGTGAGAATTTC
TTTTTTATGAAAAGGGATATAAAGGCACCGAGCTGATGCAGTATTTGT
AATATTAAGTTGACCTAACAAGGTATTTGCATGAGTCACAATTACAAA
GTTTTGAGCGGTTTTGTAATTTGACATTTAGGAGAGTCTCCTATTTATT
CTCATACTTTAC

Akap2 MUT 5'-3' (Human NM_001004065):

ATTGTGTGTTTGTAAAATGTGTATGTTCATGAGTAAGGGTGTGTGTG
TGTGTGTATTAAAATTCCAGAGTGACCGTGGCACTTGGGTGTACAGGT
AATTCCTCCAGAGCTGTTTGCTGGCTTCAGGAGTGGAGTGAGAATTTC
TTTTTTATGA**TTTCCCT**TATAAAGGCACCGAGCTGATGCAGTATTTGT
AATATTAAGTTGACCTAACAAGGTATTTGCATGAGTCACAATTACAAA
GTTTTGAGCGGTTTTGTAATTTGACATTTAGGAGAGTCTCCTATTTATT
CTCATACTTTAC

Tgfbr2 WT 5'-3' (Human NM_001024847):

CTCACTTCTGGGTTATCAGCATAAACTGGAATGTAGTGTCAGAGGAT
ACTGTGGCTTGTTTTGTTTATGTTTTTTTTTCTTATTCAAGAAAAAAGA
CCAAGGAATAACATTCTGTAGTTCCTAAAAATACTGACTTTTTTCACTA
CTATACATAAAGGGAAAGTTTTATTCTTTATGGAACACTTCAGCTGTA

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CTCATGTATTAAAATAGGAATGTGAATGCTATATACTCTTTTTATATCA
AAAGTCTCAAGCACTTATTTTTATTCTATGCATTGTTTGTCTTTTACATA
AATAAAA

Tgfbr2 MUT 5'-3' (Human NM_001024847):

CTCACTTCTGGGTTATCAGCATAAACTGGAATGTAGTGTCAGAGGAT
ACTGTGGCTTGTTTTGTTTATGTTTTTTTTTCTTATTCAAGAAAAAAGA
CCAAGGAATAACATTCTGTAGTTCCTAAAAATACTGACTTTTTTCACTA
CTATACAT**TTTCCCT**AAGTTTTATTCTTTTATGGAACACTTCAGCTGTA
CTCATGTATTAAAATAGGAATGTGAATGCTATATACTCTTTTTATATCA
AAAGTCTCAAGCACTTATTTTTATTCTATGCATTGTTTGTCTTTTACATA
AATAAAA

Csnk2a2 WT 5'-3' (Rat NM_001107409):

CTTCTACCCGGTGGTGAAGGAGCAGTCCCAGCCTTGTGCTGAGAAC
ACCGTGCTTTCCAGTGGTCTCACCGCAGCACGATGAAGCCTGGGGAAT
CGACGGTCTGTTGCGGTTCCTCCCACTTTTCCATAAGCAGAACAAGAA
CCAAATCAAAACGTCTTAACGCGTGTAGCGAGATCACGTCCCGAGAG
CAGACACAAAATGGTGGCAGGCTTGGCGAACAGGAACTAGACCACCC
GAAGGGCAGCCCACCACCGTAAATCAGACCTCACTTCCGAATGTAAA
AGGTTCACATGCCTTTG

Csnk2a2 MUT 5'-3' (Rat NM_001107409):

CTTCTACCCGGTGGTGAAGGAGCAGTCCCAGCCTTGTGCTGAGAAC
ACCGTGCTTTCCAGTGGTCTCACCGCAGCACGATGAAGCCTGGGGAAT
CGACGGTCTGTTGCGGTTCCTCCCACTTTTCCATAAGCAGAACAAGAA
CCAAATCAAAA**GCAGAAT**ACGCGTGTAGCGAGATCACGTCCCGAGAG
CAGACACAAAATGGTGGCAGGCTTGGCGAACAGGAACTAGACCACCC
GAAGGGCAGCCCACCACCGTAAATCAGACCTCACTTCCGAATGTAAA
AGGTTCACATGCCTTTG
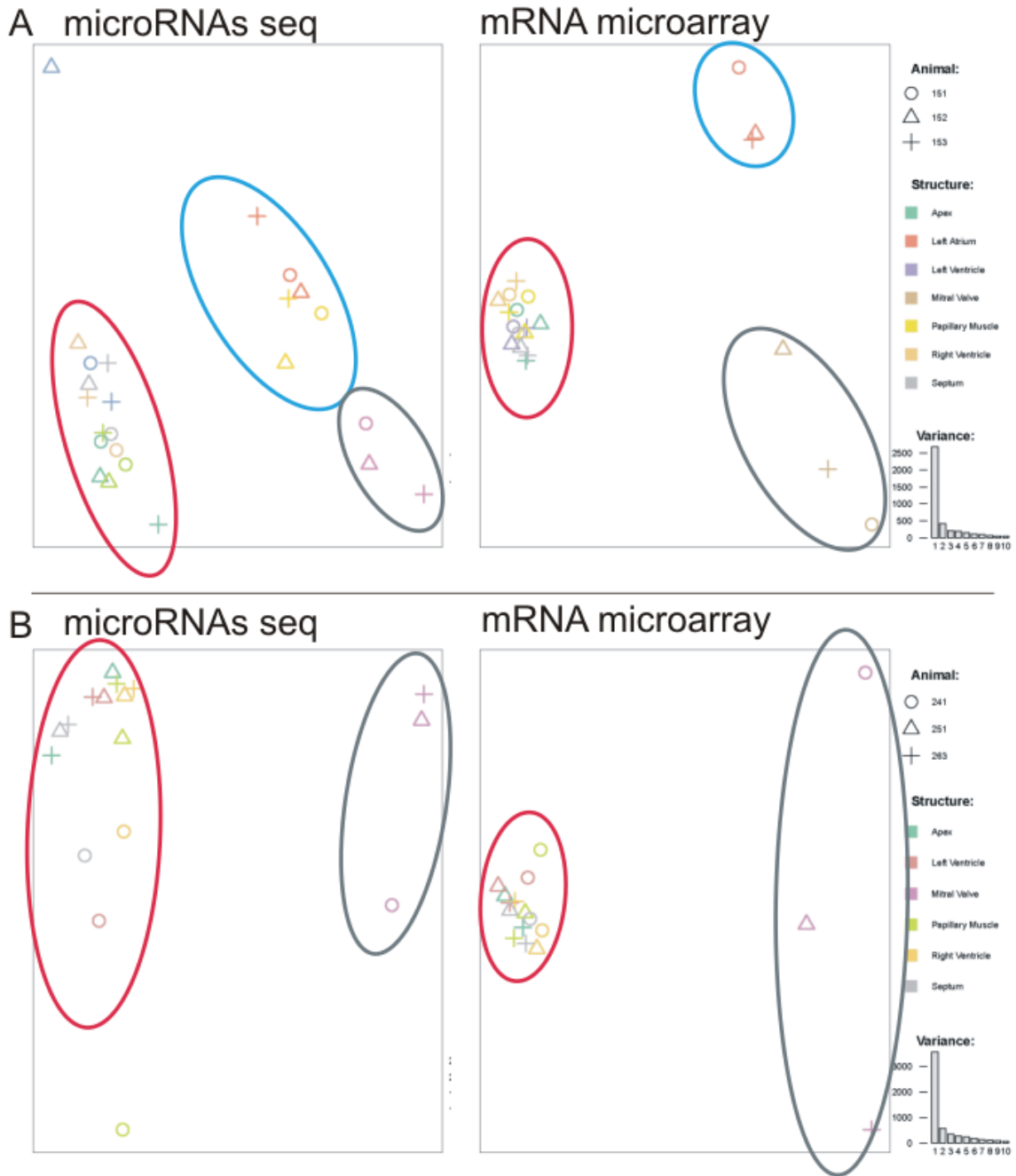
Supplementary figure 3-1: Cardiac structures are similarly clustered by microRNA and mRNA profiles of dog (A) and Cynomolgus (B), accordingly to their histological characteristics. Red circles: myocardial tissue (apex, left and right ventricle, septum, papillary muscle). Blue circles: left and right atrium. Grey circles: cardiac valves. A, apex. LA, left atrium. LV, left ventricle. PM, papillary muscle. RA, right atrium. RV, right ventricle. S, septum. V, valve.

Supplementary table 3-2: Rat cardiac structure enriched microRNAs

Valves			
miRNA	id	Sequence	Fold enrichment
rno-let-7e	mirnaUID2686	TGAGGTAGGAGGTTGTATAGTT	17.94
rno-miR-3554	mirnaUID1040	CTATGCCAGCATCTTGCCT	17.93
rno-miR-204	mirnaUID0307	TTCCCTTTGTCATCCTATGCCT	17.68
rno-miR-10b	mirnaUID2476	TACCCTGTAGAACCGAATTTGTG	7.31
rno-miR-497,mmu-miR-497	mirnaUID0236	CAGCAGCACACTGTGGTTTGTA	6.42
mmu-miR-5109	mirnaUID0403	TGTTGCGGACCAGGGGAATCCGA	6.42
rno-miR-199a-3p,mmu-miR-199a-3p,mmu-miR-199b	mirnaUID2023	ACAGTAGTCTGCACATTGGTTA	5.14
rno-miR-140*,mmu-miR-140*	mirnaUID2487	TACCACAGGGTAGAACCACGG	4.90
rno-miR-434,mmu-miR-434-3p	mirnaUID0606	TTTGAACCATCACTCGACTCCT	4.84
rno-miR-31	mirnaUID1686	AGGCAAGATGCTGGCATAGCTGT	3.71
rno-miR-23b	mirnaUID0633	ATCACATTGCCAGGGATTACC	3.59
rno-let-7b	mirnaUID1072	TGAGGTAGTAGGTTGTGTGGTT	3.21
rno-miR-423*,mmu-miR-423-5p	mirnaUID2095	TGAGGGGCAGAGAGCGAGACTTTT	3.14
rno-miR-320	mirnaUID0965	AAAAGCTGGGTTGAGAGGGCGA	3.07
rno-miR-125a-5p	mirnaUID0109	TCCCTGAGACCCTTTAACCTGTGA	3.01
rno-miR-3596c	mirnaUID1636	ACTATACAACCTCCTACCTCA	2.94
rno-let-7d*	mirnaUID1929	CTATACGACCTGCTGCCTTTCT	2.78
rno-miR-3588	mirnaUID2671	TCACAAGTTAGGGTCTCAGGGA	2.76
rno-let-7c	mirnaUID1210	TGAGGTAGTAGGTTGTATGGTT	2.72
rno-miR-3543	mirnaUID2716	CAGGAGTCGAGTGATGGTTCAAA	2.51
rno-miR-99b	mirnaUID0532	CACCCGTAGAACCGACCTTGCG	2.51
rno-miR-125b-5p	mirnaUID2699	TCCCTGAGACCCTAACTTGTGA	2.29
mmu-miR-744	mirnaUID0939	TGCGGGGCTAGGGCTAACAGCA	2.29
rno-miR-127	mirnaUID0088	TCGGATCCGTCTGAGCTTGGCT	2.29
rno-miR-3585-5p	mirnaUID1381	TTCACAAGAAGGTGTCTTTCAT	2.17

rno-miR-328a,mmu-miR-328	mirnaUID1067	CTGGCCCTCTCTGCCCTTCCGT	2.05

Myocardium			
miRNA	id	Sequence	Fold enrichment
rno-miR-30e	mirnaUID0677	TGTAAACATCCTTGACTGGAAG	30.37
rno-miR-30e*	mirnaUID1498	CTTTCAGTCGGATGTTTACAGC	14.42
rno-miR-133a*,mmu-miR-133a*	mirnaUID2046	AGCTGGTAAAATGGAACCAAAT	3.76
rno-miR-499	mirnaUID1007	TTAAGACTTGCAGTGATGTTT	3.73
rno-miR-133a,mmu-miR-133a	mirnaUID0106	TTTGGTCCCCTTCAACCAGCTG	2.78
rno-miR-3567	mirnaUID1032	TACCAAAAGTAATAATGTGCTG	2.78
mmu-miR-1a	mirnaUID0152	TGGAATGTAAAGAAGTATGTAT	2.70
rno-miR-133b	mirnaUID2652	TTTGGTCCCCTTCAACCAGCTA	2.68
rno-miR-150	mirnaUID1542	TCTCCCAACCCTTGTACCAGTG	2.44
rno-miR-208b-3p,mmu-miR-208b	mirnaUID0997	ATAAGACGAACAAAAGGTTTGT	2.44
rno-miR-450a	mirnaUID2119	TTTTGCGATGTGTTCCTAATGT	2.40
rno-miR-3582	mirnaUID1365	CAGCTGGTTGAAGGGGACCAA	2.35
mmu-miR-149	mirnaUID2194	TCTGGCTCCGTGTCTTCACTCCC	2.07
rno-miR-143,mmu-miR-143	mirnaUID0897	TGAGATGAAGCACTGTAGCTCA	2.04
rno-miR-322,mmu-miR-322	mirnaUID0573	CAGCAGCAATTCATGTTTTGGA	2.03

Atria			
miRNA	id	Sequence	Fold enrichment
rno-miR-31	mirnaUID1686	AGGCAAGATGCTGGCATAGCTGT	7.41
rno-miR-3554	mirnaUID1040	CTATGCCAGCATCTTGCCT	7.41
rno-miR-127	mirnaUID0088	TCGGATCCGTCTGAGCTTGGCT	3.95
rno-miR-490,mmu-miR-490-3p	mirnaUID0503	CAACCTGGAGGACTCCATGCTG	3.02
rno-miR-434,mmu-miR-434-3p	mirnaUID0606	TTTGAACCATCACTCGACTCCT	2.95

Supplementary table 3-3: Monkey cardiac structure enriched microRNAs

Valves			
miRNA	id	Sequence	Fold enrichment
mml-miR-320,hsa-miR-320a,hsa-miR-320d	mirnaUID0965	AAAAGCTGGGTTGAGAGGGCGA	50.76
hsa-miR-320b	mirnaUID0343	AAAAGCTGGGTTGAGAGGGCAA	40.96
mml-miR-214	mirnaUID0512	ACAGCAGGCACAGACAGGCAGT	37.44
hsa-miR-3120-5p	mirnaUID0899	CCTGTCTGTGCCTGCTGTACA	31.12
mml-miR-197	mirnaUID0938	TTCACCACCTTCTCCACCCAGC	22.56
mml-miR-92b	mirnaUID0054	TATTGCACTCGTCCCGGCCTCC	20.74
mml-let-7b	mirnaUID1072	TGAGGTAGTAGGTTGTGTGGTT	11.88
hsa-miR-574-3p	mirnaUID2267	CACGCTCATGCACACACCCACA	9.14
mml-miR-140-3p	mirnaUID2487	TACCACAGGGTAGAACCACGG	9.12
mml-miR-134,hsa-miR-134	mirnaUID1573	TGTGACTGGTTGACCAGAGGGG	8.65
mml-miR-125b	mirnaUID2699	TCCCTGAGACCCTAACTTGTGA	8.44
hsa-miR-1296	mirnaUID0319	TTAGGGCCCTGGCTCCATCTCC	7.86
mml-miR-204	mirnaUID0307	TTCCCTTTGTCATCCTATGCCT	7.83
mml-let-7c	mirnaUID1210	TGAGGTAGTAGGTTGTATGGTT	6.06
hsa-miR-328	mirnaUID1067	CTGGCCCTCTCTGCCCTTCCGT	5.69
hsa-miR-125b-1*	mirnaUID2553	ACGGGTTAGGCTCTTGGGAGCT	5.36
mml-miR-34a	mirnaUID1747	TGGCAGTGTCTTAGCTGGTTGT	5.34
hsa-miR-744	mirnaUID0939	TGCGGGGCTAGGGCTAACAGCA	4.90
mml-miR-127,hsa-miR-127-3p	mirnaUID0088	TCGGATCCGTCTGAGCTTGGCT	4.77
hsa-let-7b*	mirnaUID2339	CTATACAACCTACTGCCTTCCC	4.72
mml-miR-423-5p	mirnaUID2095	TGAGGGGCAGAGAGCGAGACTTTT	4.52
mml-miR-193b	mirnaUID1124	AACTGGCCCTCAAAGTCCCGCT	4.41
mml-miR-100	mirnaUID0973	AACCCGTAGATCCGAACTTGTG	4.07
mml-miR-140-5p	mirnaUID0183	CAGTGGTTTTACCCTATGGTAG	3.99
hsa-let-7d*	mirnaUID1929	CTATACGACCTGCTGCCTTTCT	3.66
mml-miR-409-3p	mirnaUID2437	GAATGTTGCTCGGTGAACCCCT	3.60
mml-miR-99b	mirnaUID0532	CACCCGTAGAACCGACCTTGCG	3.06
hsa-miR-335*	mirnaUID1199	TTTTTCATTATTGCTCCTGACC	2.98
mml-miR-125a-5p	mirnaUID0109	TCCCTGAGACCCTTTAACCTGTGA	2.75
mml-miR-181d	mirnaUID1317	AACATTCATTGTTGTCGGTGGGT	2.66
hsa-miR-106b*	mirnaUID2147	CCGCACTGTGGGTACTTGCTGC	2.60
mml-miR-152	mirnaUID1339	TCAGTGCATGACAGAACTTGG	2.45

miRNA	id	Sequence	
mml-miR-199a-3p,hsa-miR-199b-3p	mirnaUID2023	ACAGTAGTCTGCACATTGGTTA	2.43
mml-miR-423-3p	mirnaUID0925	AGCTCGGTCTGAGGCCCCTCAGT	2.40
hsa-miR-3184	mirnaUID2607	TGAGGGGCCTCAGACCGAGCTTTT	2.40
mml-miR-23a	mirnaUID2468	ATCACATTGCCAGGGATTTCC	2.32
hsa-miR-125b-2*	mirnaUID2235	TCACAAGTCAGGCTCTTGGGAC	2.26
hsa-miR-3120-3p	mirnaUID0801	CACAGCAAGTGTAGACAGGCA	2.24
hsa-miR-214*	mirnaUID1165	TGCCTGTCTACACTTGCTGTGC	2.23
mml-miR-130a	mirnaUID1062	CAGTGCAATGTTAAAAGGGCAT	2.17
mml-miR-92a	mirnaUID0699	TATTGCACTTGTCCCGGCCTGT	2.16
mml-miR-145,hsa-miR-145	mirnaUID1820	GTCCAGTTTTCCCAGGAATCCCTT	2.10
mml-miR-199a,mml-miR-199a-5p	mirnaUID2233	CCCAGTGTTCAGACTACCTGTTC	2.08
mml-miR-28,hsa-miR-28-5p	mirnaUID2058	AAGGAGCTCACAGTCTATTGAG	2.04
mml-miR-181b	mirnaUID0653	AACATTCATTGCTGTCGGTGGGTT	2.03
mml-let-7a,hsa-let-7a	mirnaUID2005	TGAGGTAGTAGGTTGTATAGTT	2.02
mml-miR-191	mirnaUID0921	CAACGGAATCCCAAAAGCAGCTG	2.01
Myocardium			
miRNA	id	Sequence	Fold enrichment
hsa-miR-499a-3p	mirnaUID0074	AACATCACTGCAAGTCTTAACA	12.05
mml-miR-499-5p	mirnaUID1007	TTAAGACTTGCAGTGATGTTT	11.96
mml-miR-208b	mirnaUID0997	ATAAGACGAACAAAAGGTTTGT	10.88
mml-miR-363	mirnaUID2513	AATTGCACGGTATCCATCTGTAA	10.08
hsa-miR-378d	mirnaUID0020	ACTGGACTTGGAGTCAGAAA	6.63
mml-miR-133a	mirnaUID0646	TTGTCCCCTTCAACCAGCTGT	6.04
mml-miR-30e	mirnaUID0677	TGTAAACATCCTTGACTGGAAG	5.87
mml-miR-10b	mirnaUID2476	TACCCTGTAGAACCGAATTTGTG	5.37
mml-miR-133c,hsa-miR-133a	mirnaUID0106	TTTGGTCCCCTTCAACCAGCTG	5.21
hsa-miR-30d*	mirnaUID0175	CTTTCAGTCAGATGTTTGCTGC	4.93
hsa-miR-30e*	mirnaUID1498	CTTTCAGTCGGATGTTTACAGC	4.64
hsa-miR-145*	mirnaUID0432	ATTCCTGGAAATACTGTTCTTG	4.63
mml-miR-133b	mirnaUID2652	TTTGGTCCCCTTCAACCAGCTA	4.55
mml-miR-143	mirnaUID0897	TGAGATGAAGCACTGTAGCTCA	4.33
mml-miR-29b	mirnaUID0341	TAGCACCATTTGAAATCAGTGTT	4.21

mml-miR-29c	mirnaUID2148	TAGCACCATTTGAAATCGGTTA	4.11
mml-miR-22	mirnaUID2497	AAGCTGCCAGTTGAAGAACTGT	4.06
mml-miR-30a-5p,hsa-miR-30a	mirnaUID0712	TGTAAACATCCTCGACTGGAAGC	3.63
mml-miR-30b,mml-miR-30c,hsa-miR-30b	mirnaUID2059	TGTAAACATCCTACACTCTCAGCT	3.50
hsa-miR-30c-1*	mirnaUID0178	CTGGGAGAGGGTTGTTTACTCC	3.36
mml-miR-1,hsa-miR-1	mirnaUID0152	TGGAATGTAAAGAAGTATGTAT	3.23
mml-miR-301a	mirnaUID2381	CAGTGCAATAGTATTGTCAAAGC	3.21
hsa-miR-378i	mirnaUID1256	ACTGGACTAGGAGTCAGAAGG	3.14
mml-miR-338-3p	mirnaUID1371	TCCAGCATCAGTGATTTTGTTGA	3.08
hsa-miR-3065-5p	mirnaUID1514	TCAACAAAATCACTGATGCTGGA	3.08
mml-miR-378,hsa-miR-378c	mirnaUID0205	ACTGGACTTGGAGTCAGAAGAGTGG	2.97
mml-miR-142-5p	mirnaUID0690	CATAAAGTAGAAAGCACTACT	2.93
mml-miR-27b	mirnaUID2565	TTCACAGTGGCTAAGTTCTGC	2.91
mml-miR-340	mirnaUID0680	TTATAAAGCAATGAGACTGATT	2.72
mml-miR-451	mirnaUID2416	AAACCGTTACCATTACTGAGTT	2.53
hsa-miR-126*	mirnaUID1796	CATTATTACTTTTGGTACGCG	2.45
mml-miR-107	mirnaUID1208	AGCAGCATTGTACAGGGCTATCA	2.43
mml-miR-150	mirnaUID1542	TCTCCCAACCCTTGTACCAGTG	2.43
mml-miR-221	mirnaUID2188	AGCTACATTGTCTGCTGGGTTTC	2.41
mml-miR-30d	mirnaUID0941	TGTAAACATCCCCGACTGGAAGCT	2.32
mml-miR-101	mirnaUID1884	TACAGTACTGTGATAACTGAAG	2.22
mml-let-7f	mirnaUID1332	TGAGGTAGTAGATTGTATAGTT	2.21
mml-miR-126,hsa-miR-126	mirnaUID0048	TCGTACCGTGAGTAATAATGCG	2.20
mml-miR-148b	mirnaUID1084	TCAGTGCATCACAGAACTTTGT	2.14
mml-miR-30a-3p,hsa-miR-30a*	mirnaUID1016	CTTTCAGTCGGATGTTTGCAGC	2.12
mml-miR-222	mirnaUID1145	AGCTACATCTGGCTACTGGGT	2.03

Supplementary table 3-4: Canine cardiac structure enriched microRNAs

Valves			
miRNA	id	Sequence	Fold enrichment
cfa-miR-204,hsa-miR-204	mirnaUID0307	TTCCCTTTGTCATCCTATGCCT	8.17
cfa-miR-127,hsa-miR-127-3p	mirnaUID0088	TCGGATCCGTCTGAGCTTGGCT	4.29
cfa-miR-328,hsa-miR-328	mirnaUID1067	CTGGCCCTCTCTGCCCTTCCGT	3.52
hsa-miR-744	mirnaUID0939	TGCGGGGCTAGGGCTAACAGCA	3.32
cfa-miR-199,hsa-miR-199a-3p,hsa-miR-199b-3p	mirnaUID2023	ACAGTAGTCTGCACATTGGTTA	3.15
cfa-miR-320,hsa-miR-320a,hsa-miR-320d	mirnaUID0965	AAAAGCTGGGTTGAGAGGGCGA	3.07
cfa-let-7c,hsa-let-7c	mirnaUID1210	TGAGGTAGTAGGTTGTATGGTT	2.71
cfa-miR-99b,hsa-miR-99b	mirnaUID0532	CACCCGTAGAACCGACCTTGCG	2.40
cfa-let-7e,hsa-let-7e	mirnaUID2686	TGAGGTAGGAGGTTGTATAGTT	2.27
hsa-miR-151-3p	mirnaUID1826	CTAGACTGAAGCTCCTTGAGG	2.25
cfa-miR-149,hsa-miR-149	mirnaUID2194	TCTGGCTCCGTGTCTTCACTCCC	2.21
cfa-miR-181b,hsa-miR-181b	mirnaUID0653	AACATTCATTGCTGTCGGTGGGTT	2.18
cfa-miR-125b,hsa-miR-125b	mirnaUID2699	TCCCTGAGACCCTAACTTGTGA	2.18
cfa-miR-152,hsa-miR-152	mirnaUID1339	TCAGTGCATGACAGAACTTGG	2.07

Myocardium			
miRNA	id	Sequence	Fold enrichment
cfa-miR-208b	mirnaUID0997	ATAAGACGAACAAAAGGTTTGT	70.0304
cfa-miR-31,hsa-miR-31	mirnaUID1686	AGGCAAGATGCTGGCATAGCTGT	17.620426
cfa-miR-499,hsa-miR-499-5p	mirnaUID1007	TTAAGACTTGCAGTGATGTTT	17.01442

hsa-miR-499a-3p	mirnaUID0074	AACATCACTGCAAGTCTTAACA	16.83687
cfa-miR-1,hsa-miR-1	mirnaUID0152	TGGAATGTAAAGAAGTATGTAT	9.188781
hsa-miR-378i	mirnaUID1256	ACTGGACTAGGAGTCAGAAGG	5.7802687
hsa-miR-378d	mirnaUID0020	ACTGGACTTGGAGTCAGAAA	4.8989153
cfa-miR-378,hsa-miR-378,hsa-miR-378c	mirnaUID0205	ACTGGACTTGGAGTCAGAAGAGTGG	4.191974
hsa-miR-133a	mirnaUID0106	TTTGGTCCCCTTCAACCAGCTG	4.075601
cfa-miR-133a,cfa-miR-133c	mirnaUID0013	TTGGTCCCCTTCAACCAGCTGT	4.07559
cfa-miR-30b,cfa-miR-30c,hsa-miR-30b,hsa-miR-30c	mirnaUID2059	TGTAAACATCCTACACTCTCAGCT	3.9003046
cfa-miR-126,hsa-miR-126*	mirnaUID1796	CATTATTACTTTTGGTACGCG	3.619509
hsa-miR-126	mirnaUID0048	TCGTACCGTGAGTAATAATGCG	3.5997996
hsa-miR-486-5p	mirnaUID1023	TCCTGTACTGAGCTGCCCCGAG	3.3498151
cfa-miR-363,hsa-miR-363	mirnaUID2513	AATTGCACGGTATCCATCTGTAA	3.2826152
hsa-miR-486-3p	mirnaUID2078	CGGGGCAGCTCAGTACAGGAT	3.1971457
cfa-miR-133b	mirnaUID2652	TTTGGTCCCCTTCAACCAGCTA	3.1729188
hsa-miR-30d*	mirnaUID0175	CTTTCAGTCAGATGTTTGCTGC	3.1622963
cfa-miR-30d,hsa-miR-30d	mirnaUID0941	TGTAAACATCCCCGACTGGAAGCT	2.9301157
cfa-miR-10b,hsa-miR-10b	mirnaUID2476	TACCCTGTAGAACCGAATTTGTG	2.885671
cfa-miR-30e,hsa-miR-30e*	mirnaUID1498	CTTTCAGTCGGATGTTTACAGC	2.8295717
hsa-miR-30e	mirnaUID0677	TGTAAACATCCTTGACTGGAAG	2.8032448
cfa-miR-30a,hsa-miR-30a	mirnaUID0712	TGTAAACATCCTCGACTGGAAGC	2.5368807
cfa-miR-144,hsa-miR-144	mirnaUID1312	TACAGTATAGATGATGTACTAG	2.46647

miRNA	id	Sequence	Fold enrichment
cfa-miR-22,hsa-miR-22	mirnaUID2497	AAGCTGCCAGTTGAAGAACTGT	2.426751
cfa-miR-29c,hsa-miR-29c	mirnaUID2148	TAGCACCATTTGAAATCGGTTA	2.0725322
Atria			
miRNA	id	Sequence	Fold enrichment
cfa-miR-184,hsa-miR-184	mirnaUID1958	TGGACGGAGAACTGATAAGGGT	41.79
cfa-miR-208a	mirnaUID2674	ATAAGACGAGCAAAAAGCTTGT	31.20
cfa-miR-490,hsa-miR-490-3p	mirnaUID0503	CAACCTGGAGGACTCCATGCTG	4.09
cfa-miR-221,hsa-miR-221	mirnaUID2188	AGCTACATTGTCTGCTGGGTTTC	2.47
cfa-miR-222,hsa-miR-222	mirnaUID1145	AGCTACATCTGGCTACTGGGT	2.38
cfa-miR-204,hsa-miR-204	mirnaUID0307	TTCCCTTTGTCATCCTATGCCT	2.32

Supplementary table 3-5: Normalized miRNA seq reads for mir-1, miR-125b-5p, miR-204 and miR-208b in rat myocardium or valves.

Rat miRNA	Average normalized miRNA seq reads		Average Ct	
	Valves	Myocardium	Valves	Myocardium
mmu-miR-1a	141	1223	15	11
miR-125b-5p	17256	2506	17	15
miR-204	4482	244	18	20
miR-208b	814	11250	30	25
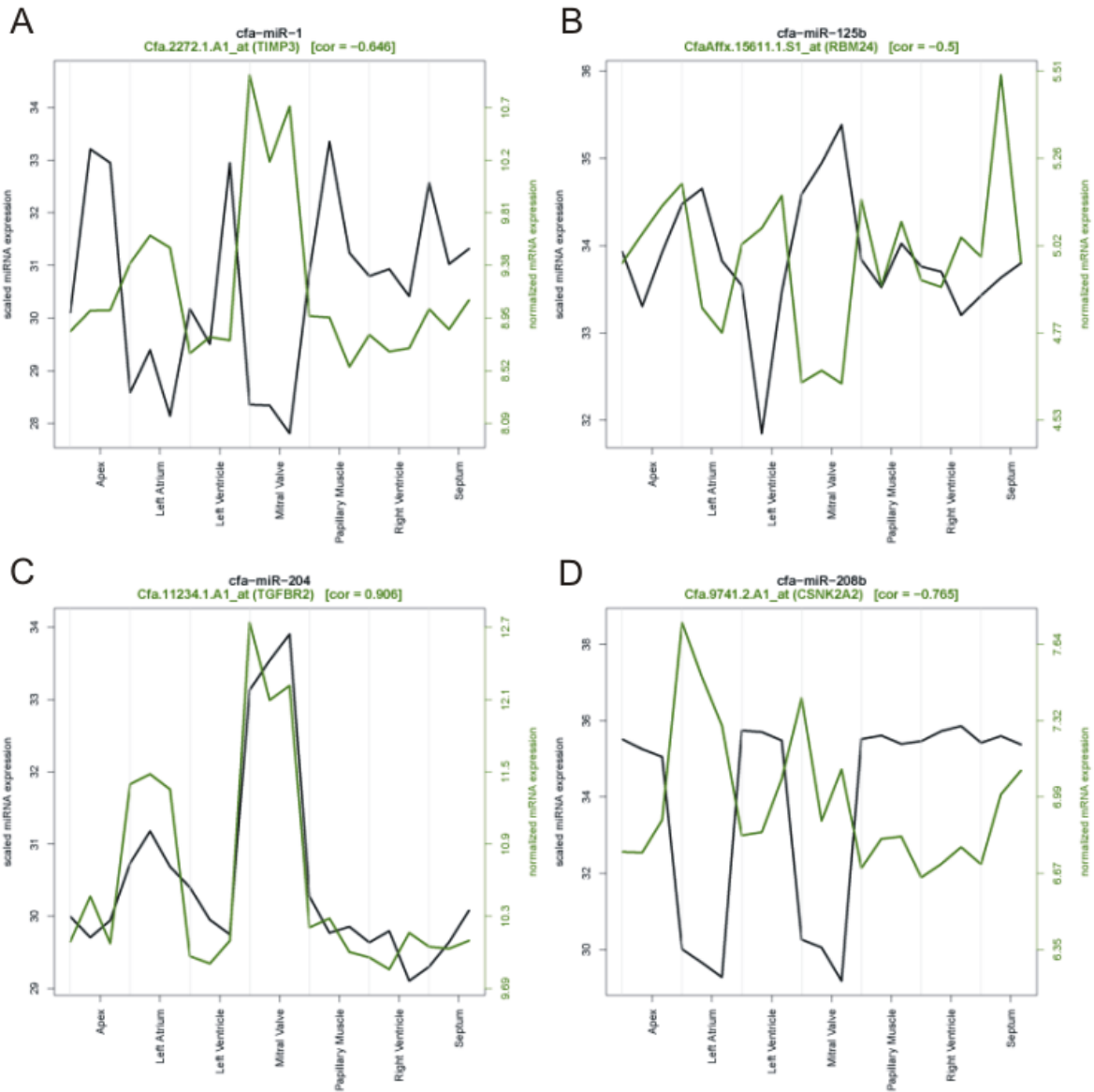
Supplementary figure 3-2: Correlation of 4 cardiac disease relevant genes with putative targeting microRNA in canine. Timp3 and miR-1 (A), Rbm24 and miR-125b-5p (B), Tgfbr2 and miR-204 (C), Csnk2a2 and miR-208b (D). Green curve represents log2 normalized intensity for the indicated probe set. Black curve represents log2 scaled and normalized microRNA read counts. Three replicates are plotted for each structure.
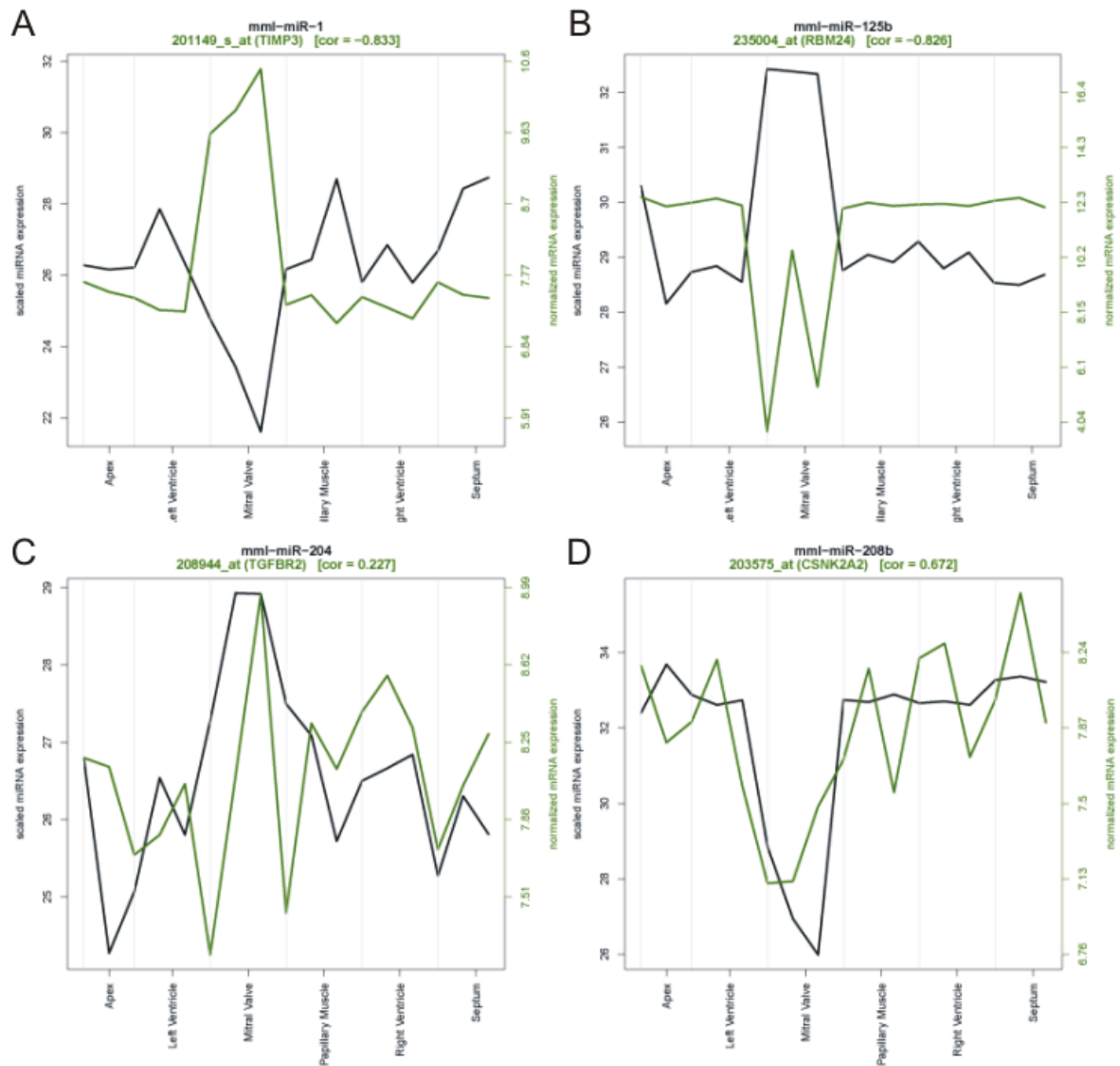
Supplementary figure 3-3: Correlation 4 cardiac disease relevant genes with putative targeting microRNA in Cyno monkey. Timp3 and miR-1 (A), Rbm24 and miR-125b-5p (B), Tgfbr2 and miR-204 (C), Csnk2a2 and miR-208b (D). Green curve represents log2 normalized intensity for the indicated probe set. Black curve represents log2 scaled and normalized microRNA read counts. Three replicates are plotted for each structure.
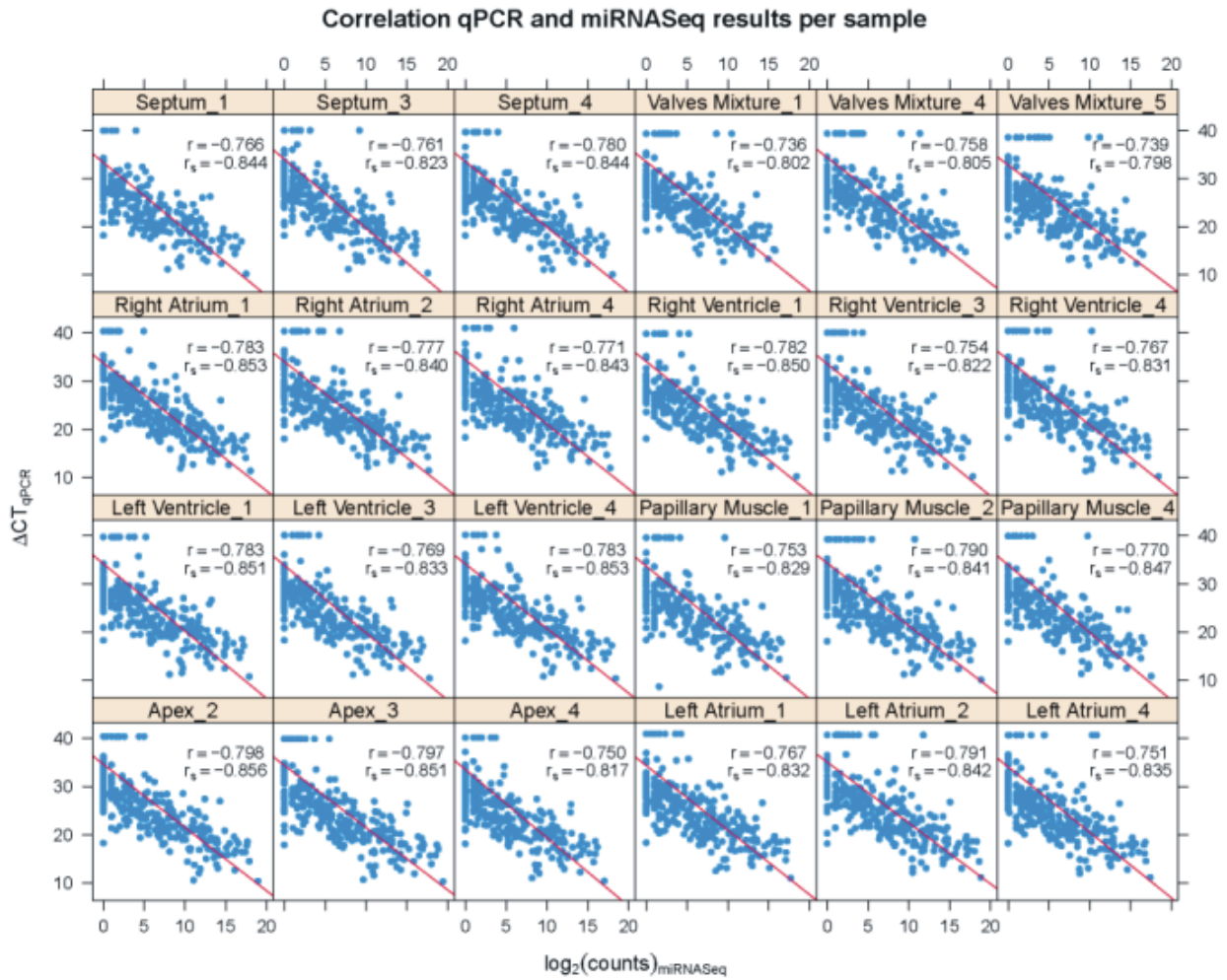
Supplementary figure 3-4: Robustness of microRNA seq and validation with qPCR. Data obtained with miRNAseq or TLDA cards are compared for each rat cardiac sample. r_s = Spearman's rank correlation, r = Pearson's correlation.
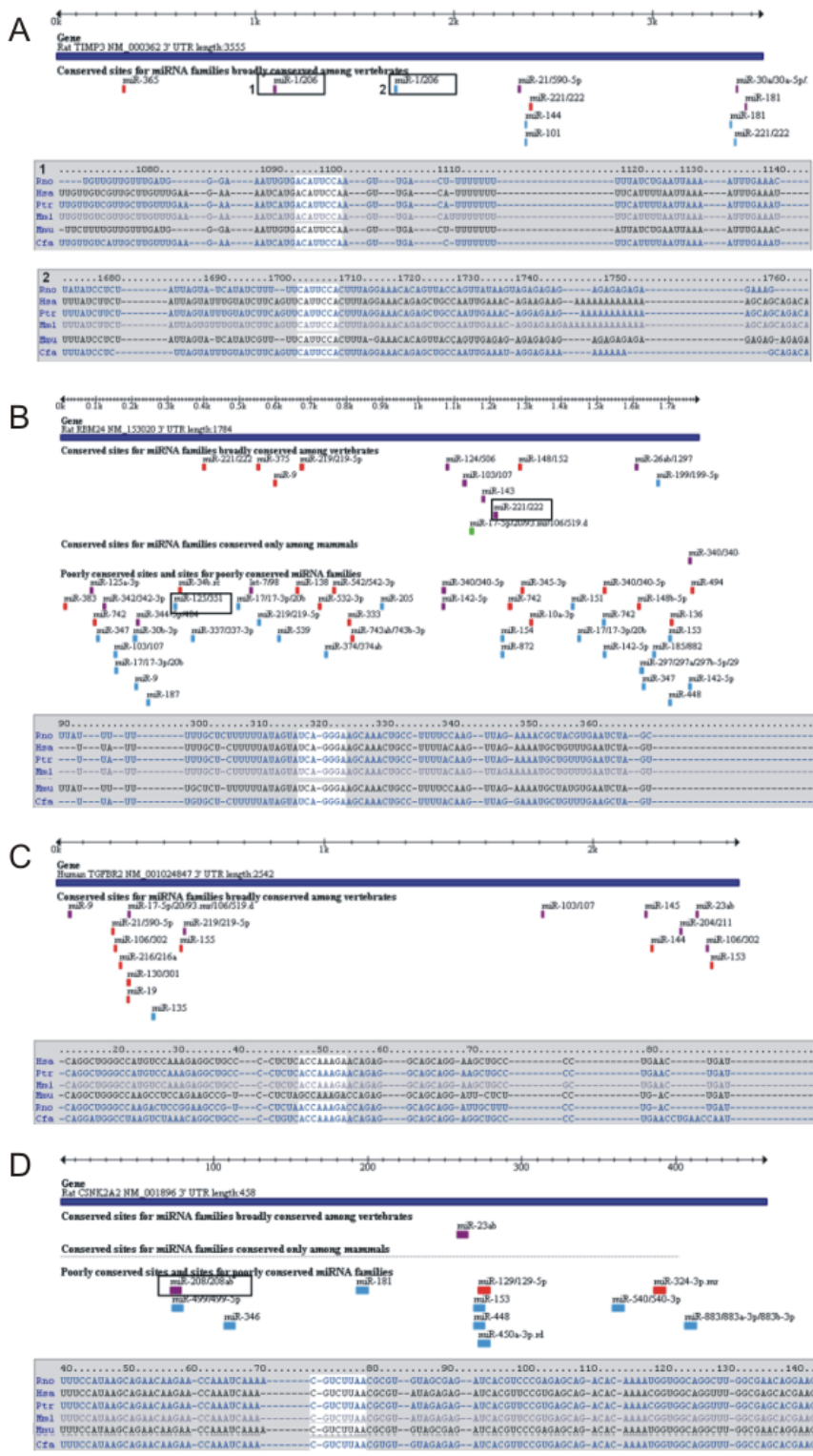
Supplementary figure 3-5: Conservation of (A) Timp3 miR-1/206 targeting seed, (B) Rbm24 miR-125b-5p targeting seed , (C) Tgfbr2 miR-204 targeting seed, (D) Csnk2a2 miR-208b targeting seed. Rno: Rattus norvegicus. Hsa: Homo sapiens. Ptr: Pan trogloditus. Mml: Macaca mulatta. Mmu: Mus musculus. Cfa: Canis familiaris.
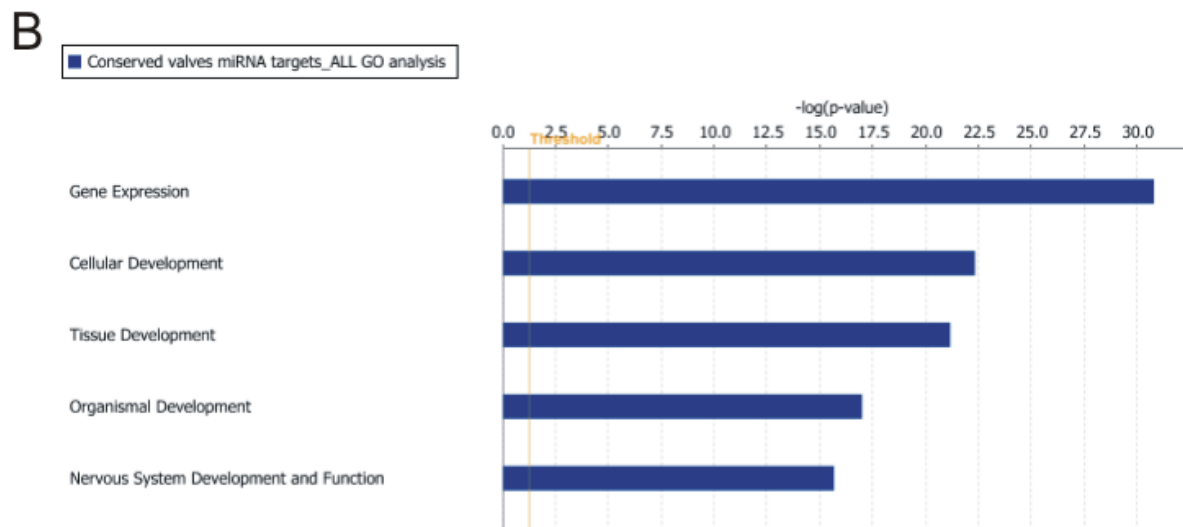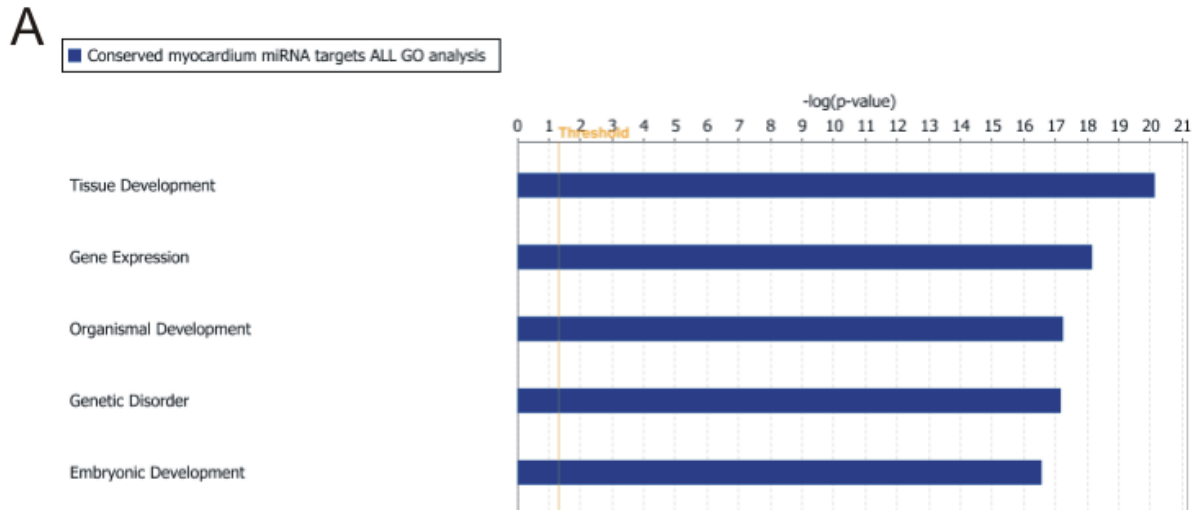
Supplementary figure 3-6: Gene ontology analysis of all Targetscan putative targets of structure-enriched conserved microRNA targets. Targets of conserved myocardial enriched microRNAs (A). Targets of conserved valves enriched microRNAs (B).
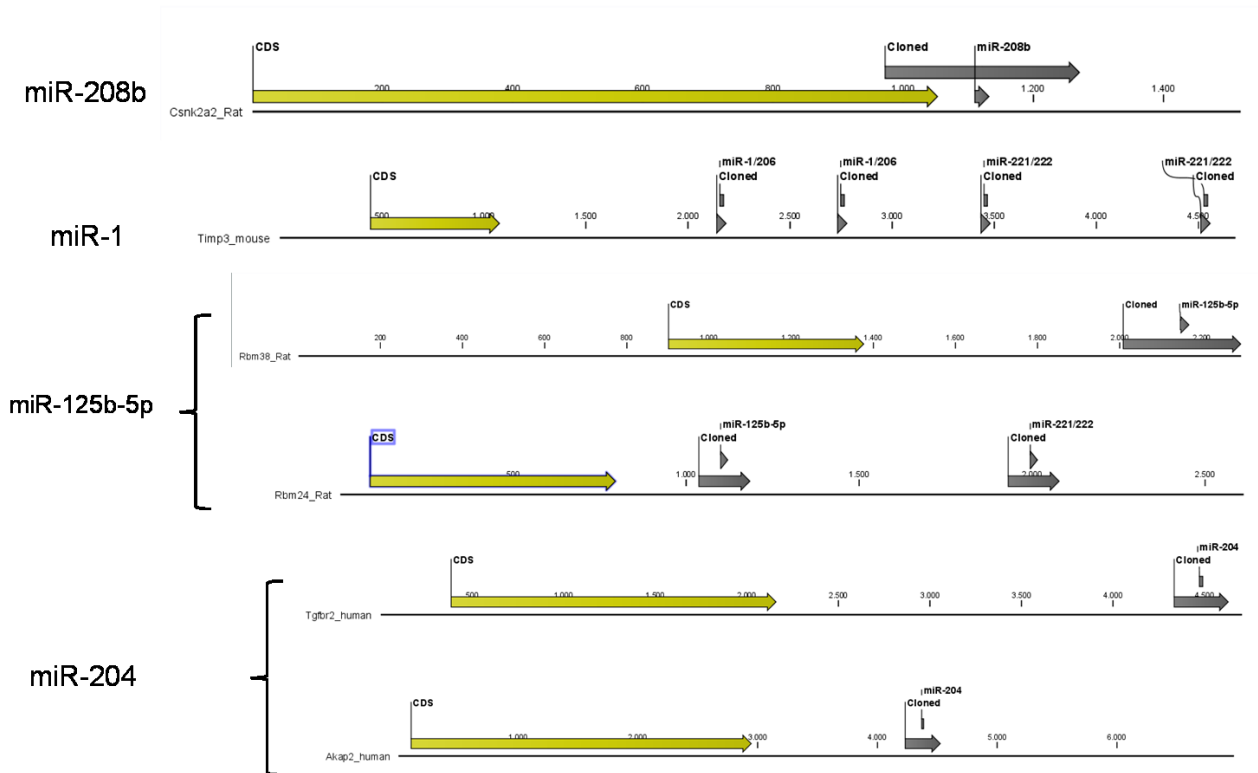
Supplementary figure 3-7: Cloning of 3' UTRs of Timp3, Csnk2a2, Rbm24, Rbm38, Tgfbr2 and Akap2 in pmiR-GLO. Yellow arrows indicate ORFs. Grey arrows identify the region surrounding the microRNA Targetscan targeting site cloned in pmiR-GLO.

Chapter 4:   Concluding discussion

4.1 Brief summary of results

In the context of toxicogenomics, it would be desirable to be able to interpret the results of microRNAs profiling upon xenobiotics administration in terms of "functional signatures", similarly to what is possible today with mRNAs expression. However in order to do this, much more information is needed on the real microRNAs "targetome", and on microRNAs relative distribution across the tissues of the model species.

In this thesis we generated data that can support further elucidation of the molecular mechanisms of cardiac function and drug-induced cardiomyopathy. First, we identified a microRNAs signature altered by an anti-tumoral agent known for its cardiotoxicity (doxorubicin), and proposed a link with concomitant transcriptome variation, and we could identify a novel direct microRNAs-mRNAs interaction (miR-34/Sipa1). Second, we have uncovered the spatial distribution of microRNAs and mRNAs in 8 cardiac structures of rat, dog and Cynomolgus monkey, with a level of detail never achieved before in these model species. A subset of microRNAs with a patterned expression across cardiac structures was further characterized and 3 novel targets relevant for cardiac biology were validated (Timp3, Rbm24, Csnk2a2).

Ideally, our findings will contribute to develop more predictive safety models for drug development, especially for cardiac toxicity.

4.2 MicroRNAs toxicity/pharmacodynamics indicators in the tissue

We identified for the first time the microRNAs dysregulated *in vivo* by DOX in the rat heart. Notably, 2 of them (miR-216b and miR-367) were increased earlier than first signs of cardiotoxicity. Importantly the myomiR miR-208b was

increased concomitantly with its genomic host gene, Myh7, underpinning the occurrence of a dose/time dependent cardiac remodeling. However, since a consistent number of DOX-induced microRNAs were not previously characterized, it was difficult to suggest their implication in pathological events associated to DOX in the heart. In particular, we could not find evidence of a correlation between the DOX-induced microRNAs with autophagy or fibrosis (data not shown and Supplementary figure 2-5), two mechanisms that can contribute to DOX-induced cardiac toxicity (Kobayashi *et al.*, 2010). In first place, there were no clear signs of autophagy induction from the gene expression *in vivo*, and we did not observe any concomitant increase of DOX-induced microRNAs in neonatal rat ventricular myocytes treated with DOX (data not shown). However, it is possible that the signals of the autophagy gene signature would be diluted, as only a few myocytes displayed lesions compatible with autophagy. Importantly, the use of a non-cardiotoxic Top2 inhibitor (etoposide, EPS) suggests that the increase of miR-215, mir-216b, miR-208b, mIR-34c and miR-367 is DOX specific. We could not exclude that these microRNAs could be implicated in other toxic events implicated with DOX cardiotoxicity, such as mitochondrial stress (Thompson *et al.*, 2009).

Nevertheless, consistently with the anti-proliferative effect of DOX, a subset of the dysregulated microRNAs were related to cell proliferation, including the 4 upregulated onco-suppressors (miR-215, miR-216b, miR-31 and miR-708), the 2 p53 transcriptional responders (miR-34c and miR-215) and 1 down-regulated onco-miR (miR-221) (Supplementary table 2-5).

Therefore, although we could not elucidate a critical toxic pathway that directly involved the microRNAs (with the exception of mir-208b over-expression that most likely is a consequence of its host gene up-regulation), those microRNAs that were related to arrest of cell proliferation could be potential pharmacodynamics endpoints for anti-proliferative drugs for example.

4.3 Heterogeneity of heart tissue and cross-species heart atlas

Tissues and organs are heterogeneous, and it is often debatable how tissues should be sampled in order to capture relevant molecular changes. In addition to the well studied ischemic diseases, cardiovascular liabilities relevant to drug safety include dilated cardiomyopathy, arrhythmias, contractile toxicity, valvular and secondary toxicity leading to heart failure (Stummann *et al.*, 2009). Other than ventricle walls, also atrial walls, cardiac valves, papillary muscles and septum can be involved in cardiac pathologies of different etiology. However little is known about the molecular mechanisms implicated in remodeling and diseases affecting these areas. This has particular relevance in the case of drug-induced valvular toxicity, which is often assessed neither during histopathological readouts, nor with toxicogenomics.

We addressed the cardiac heterogeneity issue by combining microRNAs sequencing and gene expression microarrays from 8 distinct cardiac structures and generated a large amount of data highly relevant to the preclinical cardiac safety assessment for rat, dog and Cynomolgus monkey, three species widely used in pharmacological research, but not much as basic research models, and therefore still poorly annotated.

It was evident that each cardiac zone can be differentiated by the pattern of expression of both mRNAs and microRNAs. Structures mainly composed of cardiomyocytes (ventricles, apex, septum and papillary muscles) of course were very similar, while atria and valves were markedly distinct. In a word, when generating the expression profiles of generic heart samples, the cardiomyocytes "fingerprint" is predominant, and we would recommend defining the highly expressed mRNAs or microRNAs as "cardiomyocytes" specific, rather then "heart" specific.
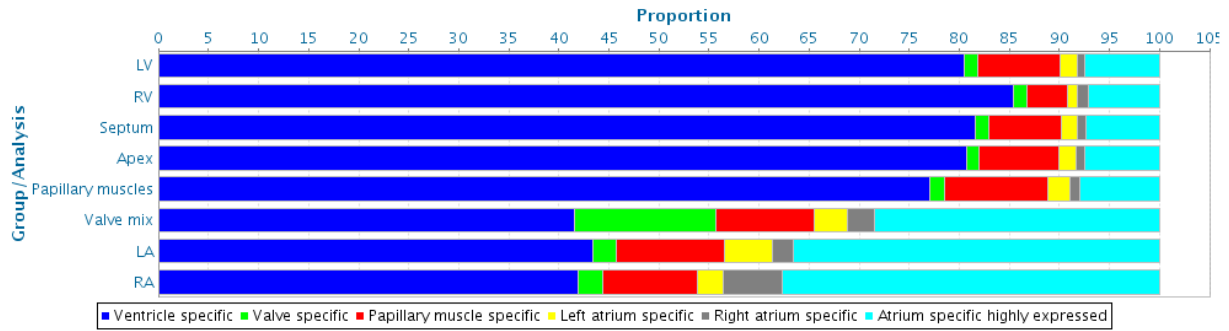
Figure 4-1: Percentual representation of selected gene lists in 8 cardiac structures of the heart. LA=left atrium, RA= right atrium. Six gene lists were manually curated and contained in total 222 Affymetrix probe sets.

In conclusion, our data sets will allow researchers to obtain more accurate cardiac samples and to detect dissection abnormalities by looking at the percentual composition of structure enriched genes or microRNAs, as exemplified in Figure 4-1.

Gene/microRNAs structure signatures can help to understand whether an increase/decrease of a certain transcript in a complex tissue is reflecting a real increase of the average cellular content of the transcripts, or rather an over-/under-representation of a specific cell type/structure due to the applied stimulus.

4.4 Cardiac microRNAs-mRNAs regulatory circuits and pathways

One challenge of microRNAs research is to characterize their function by defining the real targets, among all the predicted ones. Systematic identification of functional microRNAs targets and their co-distribution across tissues/organs/cell types is crucial to elucidate microRNAs-controlled regulatory circuitries. At present, it is not yet possible to associate alteration in specific microRNAs expression to pathways and biological processes (GO analysis) altered upon drug administration, since most the function of the microRNAs is defined by their "targetome". Extensive comparison between microRNAs and

mRNAs expression will open new avenues for the identification of microRNAs implicated in specific pathways.

Typically, the effect of microRNAs on their target genes is observed by introducing a perturbation in the system (*in vitro* or *in vivo*), such as a mutation or pharmacological treatment with an active compound, and we also used pharmacological treatment in the DOX study (see Chapter 2). However we speculated it would be possible to functionalize microRNAs also at the steady state by comparing different tissues. This would identify mainly key microRNAs-mRNAs interactions key to maintain the tissue identity, as discussed in Chapter 3 and briefly below.

4.4.1 Effect of DOX on cardiac remodeling in vivo

We described for the first time that Sipa1 was a direct target of miR-34 family and of m iR-34c in particular. This finding is most likely related to the pharmacological effect of DOX, since Sipa1 has been described as pro-metastatic gene (Park *et al.*, 2005), and miR-34 family as onco-suppressors (Ichimura *et al.*, 2010).

A subset of microRNAs involved in fibrosis was monitored by single assay qPCR across the study conditions, with and without DZR co-administration. However only miR-208b was increased by DOX, and brought down to baseline levels by DZR (Supplementary figure 2-5).

DOX was shown to induce autophagy in cardiomyocytes (Kobayashi *et al.*, 2010) and we found clues of miR-34c activity on autophagic regulator Ambra1 in H9c2 cells treated with DOX (Supplementary figure 2-6B), although the observed effect is most probably indirect.

4.4.2 Anti-correlation of microRNAs with target mRNAs at the steady state

The second strategy we employed to identify and validate microRNAs targets was the comparison of heterogeneous, but still highly similar samples. Matched

mRNAs and microRNAs expression from 8 cardiac structures from 3 mammalian species suggested a number of microRNAs/mRNAs anti-correlations interesting for heart physiology and diseases. Direct inhibition of the transcript mediated by the microRNAs was confirmed for 4 candidate genes by reporter assays.

Unexpectedly, one interesting finding was that each of the 2 targeting sites predicted for miR-1/miR-206 family in Timp3 3'UTR were extremely specific for either miR-1 (as shown in Chapter 3) or miR-206 (as shown by Limana *et al.*). MiR-206 is normally not expressed in the mammalian heart, but was found up-regulated in presence of fibrosis (Limana *et al.*, 2011), while miR-1 is normally down-regulated. The importance of the microRNAs mediated control of this gene was further reinforced by the reportedly high potency of both miR-1 and miR-206 in the Timp3-3' UTR reporter assay, and the different sites of action argues against the belief that members of the same family should share the same targets.

Further functional studies will be needed to elucidate the role in cardiac physiology of the 3 other miRNA/mRNAs couple described in our work (mir-125b-5p/Rbm24, miR-204/Tgfbr2, mir-208b/Csnk2a2).

Chapter 5:   Final remarks and future directions

5.1 MicroRNAs as "early" biomarkers of toxicity in the tissue

In principle, variation of specific microRNAs expression induced by external stimuli (including xenobiotics) can potentially precede downstream molecular alterations of the transcriptome. Therefore microRNAs could be early pharmacodynamics/toxicity biomarkers in the tissue, even earlier than tissue biomarkers (Figure 5-1). This can be exemplified by our finding that miR-1 was able to strongly down-regulate Timp3, a key gene in extra-cellular matrix remodeling and cardiac fibrosis, suggesting that a decrease in miR-1 could be prodromal to an increase in Timp3 associated to a fibrotic process. Furthermore, an important aspect is that microRNAs involved in a specific process (i.e. cell proliferation) may not necessarily be dysregulated by perturbation of that same process (either pharmacological or genetic), as suggested by Jentzsch *et al.* who identified, via a cardiomyocytes hypertrophy screening, microRNAs that were never found altered in hypertrophy models (Jentzsch *et al.*, 2011).

5.2 MicroRNAs as source of circulating biomarkers of tissue lesions

MicroRNAs are increasingly regarded as promising non-invasive damage biomarkers and these data sets are a resource for circulating indicators of cardiac lesions at the preclinical level. Furthermore, since microRNAs sequences and distribution are usually conserved, observations common to the 3 species could be highly relevant to the human. Our cardiac atlas constitutes a repository for selection of translatable circulating microRNAs for cardiac structure lesions in human.

5.3 Non-canonical regulatory circuits and transcriptional regulation of microRNAs

Increasing interest is growing around specific cases in which microRNAs control of expression extends to RNA-RNA interactions, or in which pseudo-genes modulate microRNAs activity (Poliseno *et al.*, 2010; Sumazin *et al.*, 2011). Open questions remain regarding the regulation of microRNAs expression, and our miRNA seq data sets could be a starting ground for elucidating transcriptional regulation mechanisms.
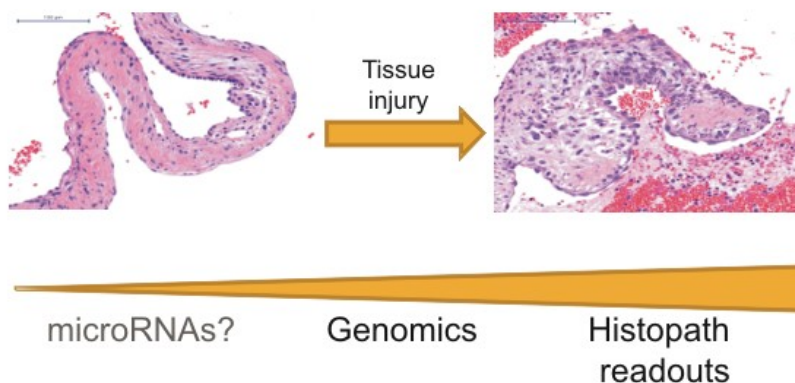
Figure 5-1: microRNAs may be earlier predictors of gene expression in toxicogenomics evaluation of drug-induced tissue injury.

However, the scarce genomic annotation of the pre-clinical species used for pharmaceutical research advocates for better annotation, since many choices regarding the patients are done based on the information extracted from these models.

Reference List

1. Adachi, T., Nakanishi, M., Otsuka, Y., Nishimura, K., Hirokawa, G., Goto, Y., Nonogi, H. and Iwai, N. (2010) **Plasma MicroRNA 499 as a Biomarker of Acute Myocardial Infarction**. *Clinical Chemistry*.

2. Ai, J., Zhang, R., Li, Y., Pu, J., Lu, Y., Jiao, J., Li, K., Yu, B., Li, Z., Wang, R., Wang, L., Li, Q., Wang, N., Shan, H., Li, Z. and Yang, B. (2010) **Circulating microRNA-1 as a potential novel biomarker for acute myocardial infarction**. *Biochem Biophys Res Commun* **391**, 73-77.

3. Akkiprik, M., Feng, Y., Wang, H., Chen, K., Hu, L., Sahin, A., Krishnamurthy, S., Ozer, A., Hao, X. and Zhang, W. (2008) **Multifunctional roles of insulin-like growth factor binding protein 5 in breast cancer**. *Breast Cancer Research* **10**, 212-.

4. Alexiou, P., Maragkakis, M., Papadopoulos, G.L., Reczko, M. and Hatzigeorgiou, A.G. (2009) **Lost in translation: an assessment and perspective for computational microRNA target identification**. *Bioinformatics* **25**, 3049-3055.

5. Alexiou, P., Maragkakis, M., Papadopoulos, G.L., Simmosis, V.A., Zhang, L. and Hatzigeorgiou, A.G. (2010) **The DIANA-mirExTra Web Server: From Gene Expression Data to MicroRNA Function**. *PLoS ONE* **5**.

6. Ambros, V. (2011) **MicroRNAs and developmental timing**. *Curr Opin Genet Dev* **21**, 511-517.

7. Antonov, A.V., Dietmann, S., Wong, P., Lutter, D. and Mewes, H.W. (2009) **GeneSet2miRNA: finding the signature of cooperative miRNA activities in the gene lists**. *Nucleic Acids Research* **37**, W323-W328.

8. Arroyo, J.D., Chevillet, J.R., Kroh, E.M., Ruf, I.K., Pritchard, C.C., Gibson, D.F., Mitchell, P.S., Bennett, C.F., Pogosova-Agadjanyan, E.L., Stirewalt, D.L., Tait, J.F. and Tewari, M. (2011) **Argonaute2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma**. *Proc Natl Acad Sci U S A*.

9. Baek, D., Villén, J., Shin, C., Camargo, F.D., Gygi, S.P. and Bartel, D.P. (2008) **The impact of microRNAs on protein output**. *Nature* **455**, 64-71.

10. Bardi, E., Bobok, I., Olah, V., Kappelmayer, J. and Kiss, C. (2007) **Anthracycline antibiotics induce acute renal tubular toxicity in children with cancer**. *Pathol Oncol Res* **13**, 249-253.

11. Bartel, D.P. (2004) **MicroRNAs: Genomics, Biogenesis, Mechanism, and Function**. *Cell* **116**, 281-297.

12. Barth, A.S., Merk, S., Arnoldi, E., Zwermann, L., Kloos, P., Gebauer, M., Steinmeyer, K., Bleich, M., Kaab, S., Hinterseer, M., Kartmann, H., Kreuzer, E., Dugas, M., Steinbeck, G. and Nabauer, M. (2005) **Reprogramming of the human atrial transcriptome in permanent atrial fibrillation: expression of a ventricular-like genomic signature**. *Circ Res* **96**, 1022-1029.

13. Berezikov, E. (2011) **Evolution of microRNA diversity and regulation in animals**. *NatRevGenet* **12**, 846-860.

14. Bertinchant, J.P., Polge, A., Juan, J.M., Oliva-Lauraire, M.C., Giuliani, I., Marty-Double, C., Burdy, J.Y., Fabbro-Peray, P., Laprade, M., Bali, J.P., Granier, C., de la Coussaye, J.E. and Dauzat, M. (2003) **Evaluation of cardiac troponin I and T levels as markers of myocardial damage in doxorubicin-induced cardiomyopathy rats, and their relationship with echocardiographic and histological findings**. *Clin Chim Acta* **329**, 39-51.

15. Bhattacharyya, S., Schapira, A.H., Mikhailidis, D.P. and Davar, J. (2009) **Drug-induced fibrotic valvular heart disease**. *The Lancet* **374**, 577-585.

16. Biomarkers Definitions Working Group. (2001) **Biomarkers and surrogate endpoints: preferred definitions and conceptual framework**. *Clin Pharmacol Ther* **69**, 89-95.

17. Bostjancic, E., Zidar, N., Stajer, D. and Glavac, D. (2010) **MicroRNAs miR-1 , miR-133a, miR-133b and miR-208 Are Dysregulated in Human Myocardial Infarction**. *Cardiology* **115**, 163-169.

18. Braun, C.J., Zhang, X., Savelyeva, I., Wolff, S., Moll, U.M., Schepeler, T., Orntoft, T.F., Andersen, C.L. and Dobbelstein, M. (2008) **p53-Responsive MicroRNAs 192 and 215 Are Capable of Inducing Cell Cycle Arrest**. *Cancer Research* **68**, 10094-10104.

19. Buck, W.R., Waring, J.F. and Blomme, E.A. (2008) **Use of traditional end points and gene dysregulation to understand mechanisms of toxicity: toxicogenomics in mechanistic toxicology**. *Methods MolBiol* **460:23-44.**, 23-44.

20. Budnitz, D.S., Pollock, D.A., Weidenbach, K.N., Mendelsohn, A.B., Schroeder, T.J. and Annest, J.L. (2006) **National surveillance of emergency department visits for outpatient adverse drug events**. *JAMA* **296**, 1858-1866.

21. Bujak, M. and Frangogiannis, N.G. (2007) **The role of TGF-beta signaling in myocardial infarction and cardiac remodeling**. *Cardiovascular Research* **74**, 184-195.

22. Butte, A. (2002) **The use and analysis of microarray data**. *Nat RevDrug Discov* **1**, 951-960.

23. Callis, T.E., Pandya, K., Seok, H.Y., Tang, R.H., Tatsuguchi, M., Huang, Z.P., Chen, J.F., Deng, Z., Gunn, B., Shumate, J., Willis, M.S., Selzman, C.H. and Wang, D.Z. (2009) **MicroRNA-208a is a regulator of cardiac hypertrophy and conduction in mice**. *The Journal of Clinical Investigation* **119**, 2772-2786.

24.   Cannell, I.G., Kong, Y.W., Johnston, S.J., Chen, M.L., Collins, H.M., Dobbyn, H.C., Elia, A., Kress, T.R., Dickens, M., Clemens, M.J., Heery, D.M., Gaestel, M., Eilers, M., Willis, A.E. and Bushell, M. (2010) **p38 MAPK/MK2-mediated induction of miR-34c following DNA damage prevents Myc-dependent DNA replication**. *Proceedings of the National Academy of Sciences* **107**, 5375-5380.

25.   Car, B. (2006) **Enabling technologies in reducing drug attrition due to safety failures**. *Am Drug Discov* **1**, 53-56.

26.   Cardinali, B., Castellani, L., Fasanaro, P., Basso, A., Alema, S., Martelli, F. and Falcone, G. (2009) **Microrna-221 and microrna-222 modulate differentiation and maturation of skeletal muscle cells**. *PLoSOne* **4**.

27.   Carè, A., Catalucci, D., Felicetti, F., Bonci, D., Addario, A., Gallo, P., Bang, M.L., Segnalini, P., Gu, Y., Dalton, N.D., Elia, L., Latronico, M.V.G., Hoydal, M., Autore, C., Russo, M.A., Dorn, G.W., Ellingsen, O., Ruiz-Lozano, P., Peterson, K.L., Croce, C.M., Peschle, C. and Condorelli, G. (2007) **MicroRNA-133 controls cardiac hypertrophy**. *Nat Med* **13**, 613-618.

28.   Catalucci, D., Gallo, P. and Condorelli, G. (2009) **MicroRNAs in Cardiovascular Biology and Heart Disease**. *Circulation: Cardiovascular Genetics* **2**, 402-408.

29.   Caulfield, J.B. and Wolkowicz, P.E. (1990) **Myocardial connective tissue alterations**. *Toxicol Pathol* **18**, 488-496.

30.   Cesana, M., Cacchiarelli, D., Legnini, I., Santini, T., Sthandier, O., Chinappi, M., Tramontano, A. and Bozzoni, I. (2011) **A Long Noncoding RNA Controls Muscle Differentiation by Functioning as a Competing Endogenous RNA**. *Cell* **147**, 358-369.

31.   Chekulaeva, M., Mathys, H., Zipprich, J.T., Attig, J., Colic, M., Parker, R. and Filipowicz, W. (2011) **miRNA repression involves GW182-mediated recruitment of CCR4-NOT through conserved W-containing motifs**. *NatStruct MolBiol* **18**, 1218-1226.

32.   Chen, B., Peng, X., Pentassuglia, L., Lim, C. and Sawyer, D. (2007) **Molecular and cellular mechanisms of anthracycline cardiotoxicity**. *Cardiovascular Toxicology* **7**, 114-121.

33.   Chen, J., Carney, S.A., Peterson, R.E. and Heideman, W. (2008a) **Comparative genomics identifies genes mediating cardiotoxicity in the embryonic zebrafish heart**. *Physiological Genomics* **33**, 148-158.

34.   Chen, J.F., Murchison, E.P., Tang, R., Callis, T.E., Tatsuguchi, M., Deng, Z., Rojas, M., Hammond, S.M., Schneider, M.D., Selzman, C.H., Meissner, G., Patterson, C., Hannon, G.J. and Wang, D.Z. (2008b) **Targeted deletion of Dicer in the heart leads to dilated cardiomyopathy and heart failure**. *Proc Natl Acad Sci U S A* **105**, 2111-2116.

35.   Cheng, Y., Tan, N., Yang, J., Liu, X., Cao, X., He, P., Dong, X., Qin, S. and Zhang, C. (2010) **A translational study of circulating cell-free microRNA-1 in acute myocardial infarction**. *Clinical Science* **119**, 87-95.

36.   Chi, S.W., Zang, J.B., Mele, A. and Darnell, R.B. (2009) **Argonaute HITS-CLIP decodes microRNA-mRNA interaction maps**. *Nature*, -.

37.   Chim, S.S.C., Shing, T.K.F., Hung, E.C.W., Leung, T.y., Lau, T.k., Chiu, R.W.K. and Dennis Lo, Y.M. (2008) **Detection and Characterization of Placental MicroRNAs in Maternal Plasma**. *Clinical Chemistry* **54**, 482-490.

38.   Collino, F., Deregibus, M.C., Bruno, S., Sterpone, L., Aghemo, G., Viltono, L., Tetta, C. and Camussi, G. (2010) **Microvesicles derived from adult human bone marrow and tissue specific mesenchymal stem cells shuttle selected pattern of miRNAs**. *PLoS One* **5**.

39.   Corsten, M.F., Dennert, R., Jochems, S., Kuznetsova, T., Devaux, Y., Hofstra, L., Wagner, D.R., Staessen, J.A., Heymans, S. and Schroen, B. (2010) **Circulating MicroRNA-208b and MicroRNA-499 reflect myocardial damage in cardiovascular disease**. *Circ Cardiovasc Genet* **3**, 499-506.

40.   Couttet, P., FromontRacine, M., Steel, D., Pictet, R. and Grange, T. (1997) **Messenger RNA deadenylylation precedes decapping in mammalian cells**. *P Natl Acad Sci USA* **94**, 5628-5633.

41.   Crawford, N.P., Ziogas, A., Peel, D.J., Hess, J., Anton-Culver, H. and Hunter, K.W. (2006) **Germline polymorphisms in SIPA1 are associated with metastasis and other indicators of poor prognosis in breast cancer**. *Breast Cancer Res* **8**, R16-.

42.   Croce, C.M. (2009) **Causes and consequences of microRNA dysregulation in cancer**. *Nat Rev Genet* **10**, 704-714.

43.   Cullen, B.R. (2006) **Viruses and microRNAs**. *Nature Genetics* **38**, 525-530.

44.   D'Alessandra, Y., Devanna, P., Limana, F., Straino, S., Di Carlo, A., Brambilla, P.G., Rubino, M., Carena, M.C., Spazzafumo, L., De Simone, M., Micheli, B., Biglioli, P., Achilli, F., Martelli, F., Maggiolini, S., Marenzi, G., Pompilio, G. and Capogrossi, M.C. (2010a) **Circulating microRNAs are new and sensitive biomarkers of myocardial infarction**. *Eur Heart J* **31**, 2765-2773.

45.   D'Alessandra, Y., Devanna, P., Limana, F., Straino, S., Di Carlo, A., Brambilla, P.G., Rubino, M., Carena, M.C., Spazzafumo, L., De Simone, M., Micheli, B., Biglioli, P., Achilli, F., Martelli, F., Maggiolini, S., Marenzi, G., Pompilio, G. and Capogrossi, M.C. (2010b) **Circulating microRNAs are new and sensitive biomarkers of myocardial infarction**. *European Heart Journal*, -.

46.   da Costa Martins, P.A., Bourajjaj, M., Gladka, M., Kortland, M., van Oort, R.J., Pinto, Y.M., Molkentin, J.D. and De Windt, L.J. (2008) **Conditional dicer gene deletion in the postnatal myocardium provokes spontaneous cardiac remodeling**. *Circulation* **118**, 1567-1576.

47.   Davis, B.N., Hilyard, A.C., Nguyen, P.H., Lagna, G. and Hata, A. (2009) **Induction of microRNA-221 by platelet-derived growth factor signaling is critical for modulation of vascular smooth muscle phenotype**. *JBiol Chem* **284**, 3728-3738.

48.   de Bold, A.J., Bruneau, B.G. and Kuroski de Bold, M.L. (1996) **Mechanical and neuroendocrine regulation of the endocrine heart**. *CardiovascRes* **31**, 7-18.

49.   De Rosa, S., Fichtlscherer, S., Lehmann, R., Assmus, B., Dimmeler, S. and Zeiher, A.M. (2011) **Transcoronary Concentration Gradients of Circulating MicroRNAs**. *Circulation* **124**, 1936-1944.

50.   De Windt, L.J., Lim, H.W., Taigen, T., Wencker, D., Condorelli, G., Dorn, G.W., Kitsis, R.N. and Molkentin, J.D. (2000) **Calcineurin-mediated hypertrophy protects cardiomyocytes from apoptosis in vitro and in vivo: An apoptosis-independent model of dilated heart failure**. *Circ Res* **86**, 255-263.

51.   Decristofaro, M.F. and Daniels, K.K. (2008) **Toxicogenomics in biomarker discovery**. *Methods Mol Biol* **460:185-94.**, 185-194.

52.   Deng, M., Tang, H., Zhou, Y., Zhou, M., Xiong, W., Zheng, Y., Ye, Q., Zeng, X., Liao, Q., Guo, X., Li, X., Ma, J. and Li, G. (2011) **miR-216b suppresses tumor growth and invasion by targeting KRAS in nasopharyngeal carcinoma**. *J Cell Sci* **124**, 2997-3005.

53.   Dorn, G.W., Robbins, J., Ball, N. and Walsh, R.A. (1994) **Myosin heavy chain regulation and myocyte contractile depression after LV hypertrophy in aortic-banded mice**. *AmJPhysiol* **267**, H400-H405.

54.   Eiring, A.M., Harb, J.G., Neviani, P., Garton, C., Oaks, J.J., Spizzo, R., Liu, S., Schwind, S., Santhanam, R., Hickey, C.J., Becker, H., Chandler, J.C., Andino, R., Cortes, J., Hokland, P., Huettner, C.S., Bhatia, R., Roy, D.C., Liebhaber, S.A., Caligiuri, M.A., Marcucci, G., Garzon, R., Croce, C.M., Calin, G.A. and Perrotti, D. (2010) **miR-328 functions as an RNA decoy to modulate hnRNP E2 regulation of mRNA translation in leukemic blasts**. *Cell* **140**, 652-665.

55.   Elangbam, C.S. (2010) **Drug-induced valvulopathy: an update**. *ToxicolPathol* **38**, 837-848.

56.   Eom, G.H., Cho, Y.K., Ko, J.H., Shin, S., Choe, N., Kim, Y., Joung, H., Kim, H.S., Nam, K.I., Kee, H.J. and Kook, H. (2011) **Casein kinase-2alpha1 induces hypertrophic response by phosphorylation of histone deacetylase 2 S394 and its activation in the heart**. *Circulation* **123**, 2392-2403.

57.   Fabian, M.R., Sonenberg, N. and Filipowicz, W. (2010) **Regulation of mRNA translation and stability by microRNAs**. *AnnuRevBiochem* **79:351-79.**, 351-379.

58.   Fang, Z. and Rajewsky, N. (2011) **The impact of miRNA target sites in coding sequences and in 3'UTRs**. *PLoSOne* **6**.

59.   Fichtlscherer, S., Zeiher, A.M. and Dimmeler, S. (2011) **Circulating microRNAs: biomarkers or mediators of cardiovascular diseases?** *ArteriosclerThrombVascBiol* **31**, 2383-2390.

60.   Filipowicz, W., Bhattacharyya, S.N. and Sonenberg, N. (2008) **Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight?** *Nat Rev Genet* **9**, 102-114.

61.   Fimia, G.M., Stoykova, A., Romagnoli, A., Giunta, L., Di Bartolomeo, S., Nardacci, R., Corazzari, M., Fuoco, C., Ucar, A., Schwartz, P., Gruss, P., Piacentini, M., Chowdhury, K. and Cecconi, F. (2007) **Ambra1 regulates autophagy and development of the nervous system**. *Nature* **447**, 1121-U1114.

62.   Force, T. and Kolaja, K.L. (2011) **Cardiotoxicity of kinase inhibitors: the prediction and translation of preclinical models to clinical outcomes**. *Nat RevDrug Discov* **10**, 111-126.

63.   Fukushima, T., Hamada, Y., Yamada, H. and Horii, I. (2007a) **Changes of micro-RNA expression in rat liver treated by acetaminophen or carbon tetrachloride--regulating role of micro-RNA for RNA expression**. *J Toxicol Sci* **32**, 401-409.

64.   Fukushima, T., Hamada, Y., Yamada, H. and Horii, I. (2007b) **Changes of micro-RNA expression in rat liver treated by acetaminophen or carbon tetrachloride--regulating role of micro-RNA for RNA expression**. *JToxicolSci* **32**, 401-409.

65.   Gad, S.C. (2008) Current practices in safety pharmacology. John Wiley & Sons, pp. 611-694.

66.   Galardi, S., Mercatelli, N., Giorda, E., Massalini, S., Frajese, G.V., Ciafre, S.A. and Farace, M.G. (2007) **miR-221 and miR-222 Expression Affects the Proliferation Potential of Human Prostate Carcinoma Cell Lines by Targeting p27Kip1**. *J Biol Chem* **282**, 23716-23724.

67.   Gautier, L., Cope, L., Bolstad, B.M. and Irizarry, R.A. (2004) **affy--analysis of Affymetrix GeneChip data at the probe level**. *Bioinformatics* **20**, 307-315.

68.   Ge, Y., Sun, Y. and Chen, J. (2011) **IGF-II is regulated by microRNA-125b in skeletal myogenesis**. *J Cell Biol* **192**, 69-81.

69.   Georges, S.A., Biery, M.C., Kim, S.y., Schelter, J.M., Guo, J., Chang, A.N., Jackson, A.L., Carleton, M.O., Linsley, P.S., Cleary, M.A. and Chau, B.N. (2008) **Coordinated Regulation of Cell Cycle Transcripts by p53-Inducible microRNAs, miR-192 and miR-215**. *Cancer Research* **68**, 10105-10112.

70.   Gianni, L., Herman, E.H., Lipshultz, S.E., Minotti, G., Sarvazyan, N. and Sawyer, D.B. (2008) **Anthracycline Cardiotoxicity: From Bench to Bedside**. *Journal of Clinical Oncology* **26**, 3777-3784.

71.   Goodsaid, F. and Frueh, F. (2007) **Biomarker qualification pilot process at the US Food and Drug Administration**. *AAPS J* **9**, E105-E108.

72.   Goymer, P. (2007) **Introducing the mirtron**. *Nat Rev Mol Cell Biol* **8**, 597-597.

73.   Greco, S., De, S.M., Colussi, C., Zaccagnini, G., Fasanaro, P., Pescatori, M., Cardani, R., Perbellini, R., Isaia, E., Sale, P., Meola, G., Capogrossi, M.C., Gaetano, C. and Martelli, F. (2009) **Common micro-RNA signature in skeletal muscle damage and regeneration induced by Duchenne muscular dystrophy and acute ischemia**. *FASEB J* **23**, 3335-3346.

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

75.   Grimson, A., Farh, K.K.H., Johnston, W.K., Garrett-Engele, P., Lim, L.P. and Bartel, D.P. (2007) **MicroRNA targeting specificity in mammals: Determinants beyond seed pairing**. *Molecular Cell* **27**, 91-105.

76.   Guengerich, F.P. (2011) **Mechanisms of drug toxicity and relevance to pharmaceutical development**. *Drug Metab Pharmacokinet* **26**, 3-14.

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

78.   Hafner, M., Landthaler, M., Burger, L., Khorshid, M., Hausser, J., Berninger, P., Rothballer, A., Ascano, J., Jungkamp, A.C., Munschauer, M., Ulrich, A., Wardle, G.S., Dewell, S., Zavolan, M. and Tuschl, T. (2010) **Transcriptome-wide Identification of RNA-Binding Protein and MicroRNA Target Sites by PAR-CLIP**. *Cell* **141**, 129-141.

79.   Hamadeh, H.K., Bushel, P.R., Jayadev, S., Martin, K., DiSorbo, O., Sieber, S., Bennett, L., Tennant, R., Stoll, R., Barrett, J.C., Blanchard, K., Paules, R.S. and Afshari, C.A. (2002) **Gene Expression Analysis Reveals Chemical-Specific Profiles**. *Toxicological Sciences* **67**, 219-231.

80.   Han, M., Toli, J. and Abdellatif, M. (2011) **MicroRNAs in the cardiovascular system**. *Curr Opin Cardiol* **26**, 181-189.

81.   Hasinoff, B.B. and Herman, E.H. (2007) **Dexrazoxane: how it works in cardiac and tumor cells. Is it a prodrug or is it a drug?** *Cardiovasc Toxicol* **7**, 140-144.

82.   Hauck, L., Harms, C., An, J., Rohne, J., Gertz, K., Dietz, R., Endres, M. and von, H.R. (2008a) **Protein kinase CK2 links extracellular growth factor signaling with the control of p27(Kip1) stability in the heart**. *NatMed* **14**, 315-324.

83.   Hauck, L., Harms, C., An, J., Rohne, J., Gertz, K., Dietz, R., Endres, M. and von, H.R. (2008b) **Protein kinase CK2 links extracellular growth factor signaling with the control of p27(Kip1) stability in the heart**. *Nat Med* **14**, 315-324.

84.   He, L. and Hannon, G.J. (2004) **MicroRNAs: small RNAs with a big role in gene regulation**. *Nat Rev Genet* **5**, 522-531.

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

86.   Hendrickson, D.G., Hogan, D.J., Herschlag, D., Ferrell, J.E. and Brown, P.O. (2008) **Systematic Identification of mRNAs Recruited to Argonaute 2 by Specific microRNAs and Corresponding Changes in Transcript Abundance**. *PLoS ONE* **3**.

87.   Henke, J.I., Goergen, D., Zheng, J., Song, Y., Schuttler, C.G., Fehr, C., Junemann, C. and Niepmann, M. (2008) **microRNA-122 stimulates translation of hepatitis C virus RNA**. *EMBO J* **27**, 3300-3310.

88.   Hergenreider, E., Heydt, S., Tréguer, K., Boettger, T., Horrevoets, A.J.G., Zeiher, A.M., Scheffer, M.P., Frangakis, A.S., Yin, X., Mayr, M., Braun, T., Urbich, C., Boon, R.A. and Dimmeler, S. (2012) **Atheroprotective communication between endothelial cells and smooth muscle cells through miRNAs**. *Nature Cell Biology* **14**.

89.   Herman, E.H., el-Hage, A.N., Ferrans, V.J. and Ardalan, B. (1985) **Comparison of the severity of the chronic cardiotoxicity produced by doxorubicin in normotensive and hypertensive rats**. *ToxicolApplPharmacol* **78**, 202-214.

90.   Herman, E.H. and Ferrans, V.J. (1998) **Preclinical animal models of cardiac protection from anthracycline-induced cardiotoxicity**. *SeminOncol* **25**, 15-21.

91.   Hermeking, H. (2010) **The miR-34 family in cancer and apoptosis**. *Cell Death Differ* **17**, 193-199.

92.   Hill, J.A. and Olson, E.N. (2008) **Cardiac plasticity**. *NEnglJ Med* **358**, 1370-1380.

93.   Horie, T., Ono, K., Nishi, H., Nagao, K., Kinoshita, M., Watanabe, S., Kuwabara, Y., Nakashima, Y., Mori, R.T., Nishi, E., Hasegawa, K., Kita, T. and Kimura, T. (2010) **Acute Doxorubicin Cardiotoxicity Is Associated with miR-146a-induced Inhibition of the Neuregulin-ErbB Pathway**. *Cardiovascular Research*, -.

94.   Hoshijima, M. and Chien, K.R. (2002) **Mixed signals in heart failure: cancer rules**. *J Clin Invest* **109**, 849-855.

95.   Huang, Y., Zou, Q., Wang, S.P., Tang, S.M., Zhang, G.Z. and Shen, X.J. (2011) **The discovery approaches and detection methods of microRNAs**. *MolBiol Rep* **38**, 4125-4135.

96.   Hullinger, T.G., Montgomery, R.L., Seto, A.G., Dickinson, B.A., Semus, H.M., Lynch, J.M., Dalby, C.M., Robinson, K., Stack, C., Latimer, P.A., Hare, J.M., Olson, E.N. and van, R.E. (2012) **Inhibition of miR-15 Protects Against Cardiac Ischemic Injury**. *CircRes* **110**, 71-81.

97.   Ichimura, A., Ruike, Y., Terasawa, K., Shimizu, K. and Tsujimoto, G. (2010) **MicroRNA-34a Inhibits Cell Proliferation by Repressing Mitogen-Activated Protein Kinase Kinase 1 during Megakaryocytic Differentiation of K562 Cells**. *Molecular Pharmacology* **77**, 1016-1024.

98.   International Conference for Harmonization. (2011) ICH Guidelines. http://www.ich.org/products/guidelines.html 2012/03/12

99.   Ivey, K.N., Muth, A., Arnold, J., King, F.W., Yeh, R.F., Fish, J.E., Hsiao, E.C., Schwartz, R.J., Conklin, B.R., Bernstein, H.S. and Srivastava, D. (2008) **MicroRNA regulation of cell lineages in mouse and human embryonic stem cells**. *Cell Stem Cell* **2**, 219-229.

100.   Izzotti, A., Larghero, P., Cartiglia, C., Longobardi, M., Pfeffer, U., Steele, V.E. and De, F.S. (2010) **Modulation of microRNA expression by budesonide, phenethyl isothiocyanate and cigarette smoke in mouse liver and lung**. *Carcinogenesis* **31**, 894-901.

101.   Janero, D.R., Hreniuk, D. and Sharif, H.M. (1994) **Hydroperoxide-induced oxidative stress impairs heart muscle cell carbohydrate metabolism**. *AJP - Cell Physiology* **266**, C179-C188.

102.   Jentzsch, C., Leierseder, S., Loyer, X., Flohrschutz, I., Sassi, Y., Hartmann, D., Thum, T., Laggerbauer, B. and Engelhardt, S. (2011) **A phenotypic screen to identify hypertrophy-modulating microRNAs in primary cardiomyocytes**. *JMolCell Cardiol*.

103.   Jeyaraj, S.C., Unger, N.T. and Chotani, M.A. (2011) **Rap1 GTPases: an emerging role in the cardiovasculature**. *Life Sci* **88**, 645-652.

104.   Ji, J., Zhang, J., Huang, G., Qian, J., Wang, X. and Mei, S. (2009a) **Over-expressed microRNA-27a and 27b influence fat accumulation and cell proliferation during rat hepatic stellate cell activation**. *FEBS Lett* **583**, 759-766.

105.   Ji, X., Takahashi, R., Hiura, Y., Hirokawa, G., Fukushima, Y. and Iwai, N. (2009b) **Plasma miR-208 as a Biomarker of Myocardial Injury**. *Clinical Chemistry*, clinchem-.

106.   Jiang, Y., Wu, Y., Greenlee, A.R., Wu, J., Han, Z., Li, X. and Zhao, Y. (2011) **miR-106a-mediated malignant transformation of cells induced by anti-benzo[a]pyrene-trans-7,8-diol-9,10-epoxide**. *ToxicolSci* **119**, 50-60.

107.   Jin, D., Hidaka, K., Shirai, M. and Morisaki, T. (2010) **RNA-binding motif protein 24 regulates myogenin expression and promotes myogenic differentiation**. *Genes Cells* **15**, 1158-1167.

108.   Jones, R.L., Swanton, C. and Ewer, M.S. (2006) **Anthracycline cardiotoxicity**. *Expert Opinion on Drug Safety* **5**, 791-809.

109.   Kalscheuer, S., Zhang, X., Zeng, Y. and Upadhyaya, P. (2008) **Differential expression of microRNAs in early-stage neoplastic transformation in the lungs of F344 rats**

chronically treated with the tobacco carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone. *Carcinogenesis* **29**, 2394-2399.

110.   Kandalam, V., Basu, R., Abraham, T., Wang, X., Awad, A., Wang, W., Lopaschuk, G.D., Maeda, N., Oudit, G.Y. and Kassiri, Z. (2010) **Early activation of matrix metalloproteinases underlies the exacerbated systolic and diastolic dysfunction in mice lacking TIMP3 following myocardial infarction**. *AmJPhysiol Heart CircPhysiol* **299**, H1012-H1023.

111.   Karginov, F.V., Conaco, C., Xuan, Z., Schmidt, B.H., Parker, J.S., Mandel, G. and Hannon, G.J. (2007) **A biochemical approach to identifying microRNA targets**. *P Natl Acad Sci USA* **104**, 19291-19296.

112.   Kato, M., Putta, S., Wang, M., Yuan, H., Lanting, L., Nair, I., Gunn, A., Nakagawa, Y., Shimano, H., Todorov, I., Rossi, J.J. and Natarajan, R. (2009) **TGF-beta activates Akt kinase through a microRNA-dependent amplifying circuit targeting PTEN**. *NatCell Biol* **11**, 881-889.

113.   Kim, D.H., Saetrom, P., Snove, O. and Rossi, J.J. (2008) **MicroRNA-directed transcriptional gene silencing in mammalian cells**. *P Natl Acad Sci USA* **105**, 16230-16235.

114.   Kim, D.J., Linnstaedt, S., Palma, J., Park, J.C., Ntrivalas, E., Kwak-Kim, J.Y., Gilman-Sachs, A., Beaman, K., Hastings, M.L., Martin, J.N. and Duelli, D.M. (2012) **Plasma Components Affect Accuracy of Circulating Cancer-Related MicroRNA Quantitation**. *J Mol Diagn* **14**, 71-80.

115.   Kinet, V., Dirkx, E. and De Windt, L.J. (2012) **Quaero muneris: Exploring microRNA function in cardiovascular disease**. *J Mol Cell Cardiol* **52**, 1-2.

116.   Kobayashi, S., Volden, P., Timm, D., Mao, K., Xu, X.M. and Liang, Q.R. (2010) **Transcription Factor GATA4 Inhibits Doxorubicin-induced Autophagy and Cardiomyocyte Death**. *Journal of Biological Chemistry* **285**, 793-804.

117.   Kola, I. and Landis, J. (2004) **Can the pharmaceutical industry reduce attrition rates?** *NatRevDrug Discov* **3**, 711-715.

118.   Komagata, S., Nakajima, M., Takagi, S., Mohri, T., Taniya, T. and Yokoi, T. (2009) **Human CYP24 catalyzing the inactivation of calcitriol is post-transcriptionally regulated by miR-125b**. *Mol Pharmacol* **76**, 702-709.

119.   Kosaka, N., Iguchi, H., Yoshioka, Y., Takeshita, F., Matsuki, Y. and Ochiya, T. (2010) **Secretory Mechanisms and Intercellular Transfer of MicroRNAs in Living Cells**. *Journal of Biological Chemistry* **285**, 17442-17452.

120.   Krejsa, C.M., Horvath, D., Rogalski, S.L., Penzotti, J.E., Mao, B., Barbosa, F. and Migeon, J.C. (2003) **Predicting ADME properties and side effects: the BioPrint approach**. *Curr Opin Drug Discov Devel* **6**, 470-480.

121.   Krol, J., Loedige, I. and Filipowicz, W. (2010) **The widespread regulation of microRNA biogenesis, function and decay**. *Nat Rev Genet* **11**, 597-610.

122.   Kuwabara, Y., Ono, K., Horie, T., Nishi, H., Nagao, K., Kinoshita, M., Watanabe, S., Baba, O., Kojima, Y., Shizuta, S., Imai, M., Tamura, T., Kita, T. and Kimura, T. (2011) **Increased microRNA-1 and microRNA-133a levels in serum of patients with cardiovascular disease indicate myocardial damage**. *Circ Cardiovasc Genet* **4**, 446-454.

123.   Langmead, B., Trapnell, C., Pop, M. and Salzberg, S.L. (2009) **Ultrafast and memory-efficient alignment of short DNA sequences to the human genome**. *Genome Biol* **10**.

124.   Laterza, O.F., Lim, L., Garrett-Engele, P.W., Vlasakova, K., Muniappa, N., Tanaka, W.K., Johnson, J.M., Sina, J.F., Fare, T.L., Sistare, F.D. and Glaab, W.E. (2009) **Plasma MicroRNAs as Sensitive and Specific Biomarkers of Tissue Injury**. *Clinical Chemistry* **55**, 1977-1983.

125.   Latronico, M.V. and Condorelli, G. (2011) **microRNAs in hypertrophy and heart failure**. *Exp Biol Med* **236**, 125-131.

126.   Latronico, M.V.G. and Condorelli, G. (2009) **MicroRNAs and cardiac pathology**. *Nat Rev Cardiol* **6**, 418-429.

127.   Laverty, H., Benson, C., Cartwright, E., Cross, M., Garland, C., Hammond, T., Holloway, C., McMahon, N., Milligan, J., Park, B., Pirmohamed, M., Pollard, C., Radford, J., Roome, N., Sager, P., Singh, S., Suter, T., Suter, W., Trafford, A., Volders, P., Wallis, R., Weaver, R., York, M. and Valentin, J. (2011) **How can we improve our understanding of cardiovascular safety liabilities to develop safer medicines?** *BrJPharmacol* **163**, 675-693.

128.   Le Brigand, K., Robbe-Sermesant, K., Mari, B. and Barbry, P. (2010) **MiRonTop: mining microRNAs targets across large scale gene expression studies**. *Bioinformatics* **26**, 3131-3132.

129.   Le, M.T., Shyh-Chang, N., Khaw, S.L., Chin, L., Teh, C., Tay, J., O'Day, E., Korzh, V., Yang, H., Lal, A., Lieberman, J., Lodish, H.F. and Lim, B. (2011) **Conserved Regulation of p53 Network Dosage by MicroRNA-125b Occurs through Evolving miRNA-Target Gene Pairs**. *PLoSGenet* **7**.

130.   Lee, J.W., Devanarayan, V., Barrett, Y.C., Weiner, R., Allinson, J., Fountain, S., Keller, S., Weinryb, I., Green, M., Duan, L., Rogers, J.A., Millham, R., O'Brien, P.J., Sailstad, J., Khan, M., Ray, C. and Wagner, J.A. (2006) **Fit-for-purpose method development and validation for successful biomarker measurement**. *Pharm Res* **23**, 312-328.

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

132.   Lee, Y.S., Kim, H.K., Chung, S., Kim, K.S. and Dutta, A. (2005) **Depletion of human micro-RNA miR-125b reveals that It Is critical for theproliferation of differentiated cells**

but not for the down-regulation of putative targets during differentiation. *J Biol Chem* **280**, 16635-16641.

133.   Lehnart, S.E., Maier, L.S. and Hasenfuss, G. (2009) **Abnormalities of calcium metabolism and myocardial contractility depression in the failing heart**. *Heart Failure Reviews* **14**, 213-224.

134.   Lema, C. and Cunningham, M.J. (2010) **MicroRNAs and their implications in toxicological research**. *Toxicology Letters* **198**, 100-105.

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

136.   Li, L.C., Okino, S.T., Zhao, H., Pookot, D., Place, R.F., Urakami, S., Enokida, H. and Dahiya, R. (2006) **Small dsRNAs induce transcriptional activation in human cells**. *ProcNatlAcadSciUSA* **103**, 17337-17342.

137.   Lian, J., Zhang, X., Tian, H., Liang, N., Wang, Y., Liang, C., Li, X. and Sun, F. (2009) **Altered microRNA expression in patients with non-obstructive azoospermia**. *Reproductive Biology and Endocrinology* **7**, 13-.

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

139.   Limana, F., Esposito, G., D'Arcangelo, D., Di, C.A., Romani, S., Melillo, G., Mangoni, A., Bertolami, C., Pompilio, G., Germani, A. and Capogrossi, M.C. (2011) **HMGB1 attenuates cardiac remodelling in the failing heart via enhanced cardiac regeneration and miR-206-mediated inhibition of TIMP-3**. *PLoS One* **6**.

140.   Lin, Z., Murtaza, I., Wang, K., Jiao, J., Gao, J. and Li, P.F. (2009) **miR-23a functions downstream of NFATc3 to regulate cardiac hypertrophy**. *Proceedings of the National Academy of Sciences* **106**, 12103-12108.

141.   Linsen, S.E.V., de Wit, E., Janssens, G., Heater, S., Chapman, L., Parkin, R.K., Fritz, B., Wyman, S.K., de Bruijn, E., Voest, E.E., Kuersten, S., Tewari, M. and Cuppen, E. (2009) **Limitations and possibilities of small RNA digital gene expression profiling**. *Nat Meth* **6**, 474-476.

142.   Litchfield, D.W. (2003) **Protein kinase CK2: structure, regulation and role in cellular decisions of life and death**. *Biochem J* **369**, 1-15.

143.   Liu, D.Z., Tian, Y., Ander, B.P., Xu, H., Stamova, B.S., Zhan, X., Turner, R.J., Jickling, G. and Sharp, F.R. (2009a) **Brain and blood microRNA expression profiling of ischemic stroke, intracerebral hemorrhage, and kainate seizures**. *J Cereb Blood Flow Metab* **30**, 92-101.

144.   Liu, H., Chen, S.E., Jin, B., Carson, J.A., Niu, A., Durham, W., Lai, J.Y. and Li, Y.P. (2010) **TIMP3: a physiological regulator of adult myogenesis**. *J Cell Sci* **123**, 2914-2921.

145.   Liu, N., Bezprozvannaya, S., Williams, A.H., Qi, X., Richardson, J.A., Bassel-Duby, R. and Olson, E.N. (2008) **microRNA-133a regulates cardiomyocyte proliferation and suppresses smooth muscle gene expression in the heart**. *Genes Dev* **22**, 3242-3254.

146.   Liu, N., Williams, A.H., Kim, Y., McAnally, J., Bezprozvannaya, S., Sutherland, L.B., Richardson, J.A., Bassel-Duby, R. and Olson, E.N. (2007) **An intragenic MEF2-dependent enhancer directs muscle-specific expression of microRNAs 1 and 133**. *ProcNatlAcadSci USA* **104**, 20844-20849.

147.   Liu, X., Cheng, Y., Zhang, S., Lin, Y., Yang, J. and Zhang, C. (2009b) **A necessary role of miR-221 and miR-222 in vascular smooth muscle cell proliferation and neointimal hyperplasia**. *CircRes* **104**, 476-487.

148.   Lu, M., Zhang, Q., Deng, M., Miao, J., Guo, Y., Gao, W. and Cui, Q. (2008) **An analysis of human microRNA and disease associations**. *PLoS ONE* **3**, e3420.

149.   Ma, F., Liu, X., Li, D., Wang, P., Li, N., Lu, L. and Cao, X. (2010) **MicroRNA-466l upregulates IL-10 expression in TLR-triggered macrophages by antagonizing RNA-binding protein tristetraprolin-mediated IL-10 mRNA degradation**. *JImmunol* **184**, 6053-6059.

150.   Maroney, P.A., Yu, Y., Fisher, J. and Nilsen, T.W. (2006) **Evidence that microRNAs are associated with translating messenger RNAs in human cells**. *NatStruct MolBiol* **13**, 1102-1107.

151.   Marrer, E. and Dieterle, F. (2010) **Impact of biomarker development on drug safety assessment**. *Toxicol Appl Pharmacol* **243**, 167-179.

152.   Marsit, C.J., Eddy, K. and Kelsey, K.T. (2006a) **MicroRNA responses to cellular stress**. *Cancer Research* **66**, 10843-10848.

153.   Marsit, C.J., Eddy, K. and Kelsey, K.T. (2006b) **MicroRNA responses to cellular stress**. *Cancer Res* **66**, 10843-10848.

154.   Marson, A., Levine, S.S., Cole, M.F., Frampton, G.M., Brambrink, T., Johnstone, S., Guenther, M.G., Johnston, W.K., Wernig, M., Newman, J., Calabrese, J.M., Dennis, L.M., Volkert, T.L., Gupta, S., Love, J., Hannett, N., Sharp, P.A., Bartel, D.P., Jaenisch, R. and Young, R.A. (2008) **Connecting microRNA genes to the core transcriptional regulatory circuitry of embryonic stem cells**. *Cell* **134**, 521-533.

155.   Martinez, N.J., Ow, M.C., Reece-Hoyes, J.S., Barrasa, M.I., Ambros, V.R. and Walhout, A.J. (2008) **Genome-scale spatiotemporal analysis of Caenorhabditis elegans microRNA promoter activity**. *Genome Res* **18**, 2005-2015.

156. Matkovich, S.J., Van Booven, D.J., Eschenbacher, W.H. and Dorn, G.W., II. (2011) **RISC RNA Sequencing for Context-Specific Identification of In Vivo MicroRNA Targets**. *Circulation Research* **108**, 18-26.

157. McManus, D.D. and Ambros, V. (2011) **Circulating MicroRNAs in cardiovascular disease**. *Circulation* **124**, 1908-1910.

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

159. Migrino, R.Q., Aggarwal, D., Konorev, E., Brahmbhatt, T., Bright, M. and Kalyanaraman, B. (2008) **Early detection of doxorubicin cardiomyopathy using two-dimensional strain echocardiography**. *UltrasoundMedBiol* **34**, 208-214.

160. Mikhailov, A.T. and Torrado, M. (2008) **The enigmatic role of the ankyrin repeat domain 1 gene in heart development and disease**. *International Journal of Developmental Biology* **52**, 811-821.

161. Milewicz, D.M. (2012) **MicroRNAs, fibrotic remodeling, and aortic aneurysms**. *JClinInvest* **122**, 490-493.

162. Minato, N. and Hattori, M. (2009) **Spa-1 (Sipa1) and Rap signaling in leukemia and cancer metastasis**. *Cancer Science* **100**, 17-23.

163. Miyamoto, S., Hidaka, K., Jin, D. and Morisaki, T. (2009) **RNA-binding proteins Rbm38 and Rbm24 regulate myogenic differentiation via p21-dependent and -independent regulatory pathways**. *Genes Cells* **14**, 1241-1252.

164. Mohri, T., Nakajima, M., Fukami, T., Takamiya, M., Aoki, Y. and Yokoi, T. (2010) **Human CYP2E1 is regulated by miR-378**. *BiochemPharmacol* **79**, 1045-1052.

165. Mohri, T., Nakajima, M., Takagi, S., Komagata, S. and Yokoi, T. (2009) **MicroRNA regulates human vitamin D receptor**. *IntJCancer* **125**, 1328-1333.

166. Momparler, R.L., Karon, M., Siegel, S.E. and Avila, F. (1976) **Effect of Adriamycin on DNA, RNA, and Protein Synthesis in Cell-free Systems and Intact Cells**. *Cancer Research* **36**, 2891-2895.

167. Montgomery, R.L., Hullinger, T.G., Semus, H.M., Dickinson, B.A., Seto, A.G., Lynch, J.M., Stack, C., Latimer, P.A., Olson, E.N. and van, R.E. (2011) **Therapeutic Inhibition of miR-208a Improves Cardiac Function and Survival During Heart Failure**. *Circulation* **124**, 1537-1547.

168. Mori, Y., Kondo, C., Tonomura, Y., Torii, M. and Uehara, T. (2010) **Identification of potential genomic biomarkers for early detection of chemically induced cardiotoxicity in rats**. *Toxicology* **271**, 36-44.

169.   Muller, P.Y. and Dieterle, F. (2009) **Tissue-specific, non-invasive toxicity biomarkers: translation from preclinical safety assessment to clinical safety monitoring**. *Expert Opinion on Drug Metabolism & Toxicology* **5**, 1023-1038.

170.   Murphy, A.J., Guyre, P.M. and Pioli, P.A. (2010) **Estradiol suppresses NF-kappa B activation through coordinated regulation of let-7a and miR-125b in primary human macrophages**. *JImmunol* **184**, 5029-5037.

171.   Nakanishi, N., Nakagawa, Y., Tokushige, N., Aoki, N., Matsuzaka, T., Ishii, K., Yahagi, N., Kobayashi, K., Yatoh, S., Takahashi, A., Suzuki, H., Urayama, O., Yamada, N. and Shimano, H. (2009) **The up-regulation of microRNA-335 is associated with lipid metabolism in liver and white adipose tissue of genetically obese mice**. *Biochemical and Biophysical Research Communications* **385**, 492-496.

172.   Natsoulis, G., El, G.L., Lanckriet, G.R., Tolley, A.M., Leroy, F., Dunlea, S., Eynon, B.P., Pearson, C.I., Tugendreich, S. and Jarnagin, K. (2005) **Classification of a large microarray data set: algorithm comparison and analysis of drug signatures**. *Genome Res* **15**, 724-736.

173.   Nuovo, G.J. (2008) **In situ detection of precursor and mature microRNAs in paraffin embedded, formalin fixed tissues and cell preparations**. *Methods* **44**, 39-46.

174.   Nuovo, G.J. (2010) **In situ detection of microRNAs in paraffin embedded, formalin fixed tissues and the co-localization of their putative targets**. *Methods* **52**, 307-315.

175.   Nuovo, G.J., Lee, E.J., Lawler, S., Godlewski, J. and Schmittgen, T.D. (2009) **In situ detection of mature microRNAs by labeled extension on ultramer templates**. *Biotechniques* **46**, 115.

176.   Obernosterer, G., Martinez, J. and Alenius, M. (2007) **Locked nucleic acid-based in situ detection of microRNAs in mouse tissue sections**. *NatProtocols* **2**, 1508-1514.

177.   Ohshima, K., Inoue, K., Fujiwara, A., Hatakeyama, K., Kanto, K., Watanabe, Y., Muramatsu, K., Fukuda, Y., Ogura, S., Yamaguchi, K. and Mochizuki, T. (2010) **Let-7 microRNA family is selectively secreted into the extracellular environment via exosomes in a metastatic gastric cancer cell line**. *PLoSOne* **5**.

178.   Olson, H., Betton, G., Robinson, D., Thomas, K., Monro, A., Kolaja, G., Lilly, P., Sanders, J., Sipes, G., Bracken, W., Dorato, M., Van, D.K., Smith, P., Berger, B. and Heller, A. (2000) **Concordance of the toxicity of pharmaceuticals in humans and in animals**. *RegulToxicolPharmacol* **32**, 56-67.

179.   Orom, U.A. and Lund, A.H. (2007) **Isolation of microRNA targets using biotinylated synthetic microRNAs**. *Methods* **43**, 162-165.

180.   Orom, U.A., Nielsen, F.C. and Lund, A.H. (2008) **MicroRNA-10a binds the 5'UTR of ribosomal protein mRNAs and enhances their translation**. *MolCell* **30**, 460-471.

181.   Osada, H. and Takahashi, T. (2011) **let-7 and miR-17-92: small-sized major players in lung cancer development**. *Cancer Sci* **102**, 9-17.

182.   Pan, Y.Z., Gao, W. and Yu, A.M. (2009) **MicroRNAs regulate CYP3A4 expression via direct and indirect targeting**. *Drug Metab Dispos* **37**, 2112-2117.

183.   Park, Y.G., Zhao, X.H., Lesueur, F., Lowy, D.R., Lancaster, M., Pharoah, P., Qian, X.L. and Hunter, K.W. (2005) **Sipa1 is a candidate for underlying the metastasis efficiency modifier locus Mtes1**. *Nat Genet* **37**, 1055-1062.

184.   Pigati, L., Yaddanapudi, S.C., Iyengar, R., Kim, D.J., Hearn, S.A., Danforth, D., Hastings, M.L. and Duelli, D.M. (2010) **Selective release of microRNA species from normal and malignant mammary epithelial cells**. *PLoS One* **20;5**, e13515-.

185.   Pineau, P., Volinia, S., McJunkin, K., Marchio, A.s., Battiston, C., Terris, B.t., Mazzaferro, V., Lowe, S.W., Croce, C.M. and Dejean, A. (2010) **miR-221 overexpression contributes to liver tumorigenesis**. *Proceedings of the National Academy of Sciences* **107**, 264-269.

186.   Place, R.F., Li, L.C., Pookot, D., Noonan, E.J. and Dahiya, R. (2008) **MicroRNA-373 induces expression of genes with complementary promoter sequences**. *P Natl Acad Sci USA* **105**, 1608-1613.

187.   Pogribny, I.P., Tryndyak, V.P., Boyko, A., Rodriguez-Juarez, R., Beland, F.A. and Kovalchuk, O. (2007) **Induction of microRNAome deregulation in rat liver by long-term tamoxifen exposure**. *MutatRes* **619**, 30-37.

188.   Poliseno, L., Salmena, L., Zhang, J., Carver, B., Haveman, W.J. and Pandolfi, P.P. (2010) **A coding-independent function of gene and pseudogene mRNAs regulates tumour biology**. *Nature* **465**, 1033-1038.

189.   Pradervand, S., Weber, J., Lemoine, F., Consales, F., Paillusson, A., Dupasquier, M., Thomas, J., Richter, H., Kaessmann, H., Beaudoing, E., Hagenbuchle, O. and Harshman, K. (2010) **Concordance among digital gene expression, microarrays, and qPCR when measuring differential expression of microRNAs**. *Biotechniques* **48**, 219-222.

190.   Rangrez, A.Y., Massy, Z.A., Metzinger-Le, M., V and Metzinger, L. (2011) **miR-143 and miR-145: molecular keys to switch the phenotype of vascular smooth muscle cells**. *Circ Cardiovasc Genet* **4**, 197-205.

191.   Rao, P.K., Toyama, Y., Chiang, H.R., Gupta, S., Bauer, M., Medvid, R., Reinhardt, F., Liao, R., Krieger, M., Jaenisch, R., Lodish, H.F. and Blelloch, R. (2009) **Loss of cardiac microRNA-mediated regulation leads to dilated cardiomyopathy and heart failure**. *Circ Res* **105**, 585-594.

192.   Ratajczak, J., Wysoczynski, M., Hayek, F., Janowska-Wieczorek, A. and Ratajczak, M.Z. (2006) **Membrane-derived microvesicles: important and underappreciated mediators of cell-to-cell communication**. *Leukemia* **20**, 1487-1495.

193.   Rau, F., Freyermuth, F., Fugier, C., Villemin, J.P., Fischer, M.C., Jost, B., Dembele, D., Gourdon, G., Nicole, A., Duboc, D., Wahbi, K., Day, J.W., Fujimura, H., Takahashi, M.P., Auboeuf, D., Dreumont, N., Furling, D. and Charlet-Berguerand, N. (2011) **Misregulation of miR-1 processing is associated with heart defects in myotonic dystrophy**. *Nature Structural & Molecular Biology* **18**, 840-U120.

194.   Redfern, W., Ewart, L., Hammond, T., Bialecki, R., Kinter, L., Lindgren, S., Pollard, C., Roberts, R., Rolf, M. and Valentin, Y. (2011) **Impact and frequency of different toxicities throughout the pharmaceutical life cycle**. *The Toxicologist* **114**, 1081-.

195.   Redfern, W.S., Carlsson, L., Davis, A.S., Lynch, W.G., MacKenzie, I., Palethorpe, S., Siegl, P.K., Strang, I., Sullivan, A.T., Wallis, R., Camm, A.J. and Hammond, T.G. (2003) **Relationships between preclinical cardiac electrophysiology, clinical QT interval prolongation and torsade de pointes for a broad range of drugs: evidence for a provisional safety margin in drug development**. *CardiovascRes* **58**, 32-45.

196.   Rodriguez, A., Vigorito, E., Clare, S., Warren, M.V., Couttet, P., Soond, D.R., van Dongen, S., Grocock, R.J., Das, P.P., Miska, E.A., Vetrie, D., Okkenhaug, K., Enright, A.J., Dougan, G., Turner, M. and Bradley, A. (2007) **Requirement of bic/microRNA-155 for Normal Immune Function**. *Science* **316**, 608-611.

197.   Ronchetti, D., Lionetti, M., Mosca, L., Agnelli, L., Andronache, A., Fabris, S., Deliliers, G. and Neri, A. (2008) **An integrative genomic approach reveals coordinated expression of intronic miR-335, miR-342, and miR-561 with deregulated host genes in multiple myeloma**. *BMC Medical Genomics* **1**, 37-.

198.   Rossi, R.L., Rossetti, G., Wenandy, L., Curti, S., Ripamonti, A., Bonnal, R.J., Birolo, R.S., Moro, M., Crosti, M.C., Gruarin, P., Maglie, S., Marabita, F., Mascheroni, D., Parente, V., Comelli, M., Trabucchi, E., De, F.R., Geginat, J., Abrignani, S. and Pagani, M. (2011) **Distinct microRNA signatures in human lymphocyte subsets and enforcement of the naive state in CD4+ T cells by the microRNA miR-125b**. *Nat Immunol* **12**, 796-803.

199.   Roy, S., Khanna, S., Hussain, S.R., Biswas, S., Azad, A., Rink, C., Gnyawali, S., Shilo, S., Nuovo, G.J. and Sen, C.K. (2009) **MicroRNA expression in response to murine myocardial infarction: miR-21 regulates fibroblast metalloprotease-2 via phosphatase and tensin homologue**. *Cardiovascular Research* **82**, 21-29.

200.   Ruby, J.G., Jan, C.H. and Bartel, D.P. (2007) **Intronic microRNA precursors that bypass Drosha processing**. *Nature* **448**, 83-86.

201.   Ruepp, S., Boess, F., Suter, L., de Vera, M.C., Steiner, G., Steele, T., Weiser, T. and Albertini, S. (2005) **Assessment of hepatotoxic liabilities by transcript profiling**. *ToxicolApplPharmacol* **207**, 161-170.

202.   Saeed, A.I., Bhagabati, N.K., Braisted, J.C., Liang, W., Sharov, V., Howe, E.A., Li, J., Thiagarajan, M., White, J.A. and Quackenbush, J. (2006) **TM4 microarray software suite**. *Methods Enzymol* **411:134-93.**, 134-193.

203.   Saini, S., Yamamura, S., Majid, S., Shahryari, V., Hirata, H., Tanaka, Y. and Dahiya, R. (2011) **MicroRNA-708 induces apoptosis and suppresses tumorigenicity in renal cancer cells**. *Cancer Res* **71**, 6208-6219.

204.   Sarkar, D., Parkin, R., Wyman, S., Bendoraite, A., Sather, C., Delrow, J., Godwin, A.K., Drescher, C., Huber, W., Gentleman, R. and Tewari, M. (2009) **Quality Assessment and Data Analysis for microRNA Expression Arrays**. *Nucl Acids Res* **37**.

205.   Sathyan, P., Golden, H.B. and Miranda, R.C. (2007) **Competing interactions between micro-RNAs determine neural progenitor survival and proliferation after ethanol exposure: evidence from an ex vivo model of the fetal cerebral cortical neuroepithelium**. *JNeurosci* **27**, 8546-8557.

206.   Sato, F., Tsuchiya, S., Terasawa, K. and Tsujimoto, G. (2009) **Intra-platform repeatability and inter-platform comparability of microRNA microarray technology**. *PLoS ONE* **4**, e5540-.

207.   Schaap-Oziemlak, A.M., Raymakers, R.A., Bergevoet, S.M., Gilissen, C., Jansen, B.J., Adema, G.J., Kogler, G., le, S.C., Agami, R., van der Reijden, B.A. and Jansen, J.H. (2010) **MicroRNA hsa-miR-135b regulates mineralization in osteogenic differentiation of human unrestricted somatic stem cells**. *Stem Cells Dev* **19**, 877-885.

208.   Schembri, F., Sridhar, S., Perdomo, C., Gustafson, A.M., Zhang, X., Ergun, A., Lu, J., Liu, G., Zhang, X., Bowers, J., Vaziri, C., Ott, K., Sensinger, K., Collins, J.J., Brody, J.S., Getts, R., Lenburg, M.E. and Spira, A. (2009) **MicroRNAs as modulators of smoking-induced gene expression changes in human airway epithelium**. *ProcNatlAcadSciUSA* **106**, 2319-2324.

209.   Schlesinger, J., Schueler, M., Grunert, M., Fischer, J.J., Zhang, Q., Krueger, T., Lange, M., Tonjes, M., Dunkel, I. and Sperling, S.R. (2011) **The Cardiac Transcription Network Modulated by Gata4, Mef2a, Nkx2.5, Srf, Histone Modifications, and MicroRNAs**. *PLoS Genet* **7**.

210.   Shah, Y.M., Morimura, K., Yang, Q., Tanabe, T., Takagi, M. and Gonzalez, F.J. (2007) **Peroxisome proliferator-activated receptor alpha regulates a microRNA-mediated signaling cascade responsible for hepatocellular proliferation**. *Mol Cell Biol* **27**, 4238-4247.

211.   Shimizu, Y., Hamazaki, Y., Hattori, M., Doi, K., Terada, N., Kobayashi, T., Toda, Y., Yamasaki, T., Inoue, T., Kajita, Y., Maeno, A., Kamba, T., Mikami, Y., Kamoto, T., Yamada, T., Kanno, T., Yoshikawa, K., Ogawa, O., Minato, N. and Nakamura, E. (2011) **SPA-1 controls the invasion and metastasis of human prostate cancer**. *Cancer Sci* **102**, 828-836.

212.   Shkumatava, A., Stark, A., Sive, H. and Bartel, D.P. (2009) **Coherent but overlapping expression of microRNAs and their targets during vertebrate development**. *Genes & Development* **23**, 466-481.

213. Si, M.L., Zhu, S., Wu, H., Lu, Z., Wu, F. and Mo, Y.Y. (2007) **miR-21-mediated tumor growth**. *Oncogene* **26**, 2799-2803.

214. Sibille, M., Deigat, N., Janin, A., Kirkesseli, S. and Durand, D.V. (1998) **Adverse events in phase-I studies: a report in 1015 healthy volunteers**. *EurJClinPharmacol* **54**, 13-20.

215. Sluijter, J.P.G., van Mil, A., van Vliet, P., Metz, C.H.G., Liu, J., Doevendans, P.A. and Goumans, M.J. (2010) **MicroRNA-1 and -499 Regulate Differentiation and Proliferation in Human-Derived Cardiomyocyte Progenitor Cells**. *Arteriosclerosis, Thrombosis, and Vascular Biology*, ATVBAHA-.

216. Small, E.M. and Olson, E.N. (2011) **Pervasive roles of microRNAs in cardiovascular biology**. *Nature* **469**, 336-342.

217. Stark, A., Brennecke, J., Bushati, N., Russell, R.B. and Cohen, S.M. (2005) **Animal MicroRNAs confer robustness to gene expression and have a significant impact on 3'UTR evolution**. *Cell* **123**, 1133-1146.

218. Starkey Lewis, P.J., Dear, J., Platt, V., Simpson, K.J., Craig, D.G., Antoine, D.J., French, N.S., Dhaun, N., Webb, D.J., Costello, E.M., Neoptolemos, J.P., Moggs, J., Goldring, C.E. and Park, B.K. (2011) **Circulating microRNAs as potential markers of human drug-induced liver injury**. *Hepatology* **54**, 1767-1776.

219. Steiner, G., Suter, L., Boess, F., Gasser, R., de Vera, M.C., Albertini, S. and Ruepp, S. (2004) **Discriminating different classes of toxicants by transcript profiling**. *EnvironHealth Perspect* **112**, 1236-1248.

220. Stevens, J.L. and Baker, T.K. (2009) **The future of drug safety testing: expanding the view and narrowing the focus**. *Drug Discov Today* **14**, 162-167.

221. Storm, G., van Hoesel, Q.G., de, G.G., Kop, W., Steerenberg, P.A. and Hillen, F.C. (1989) **A comparative study on the antitumor effect, cardiotoxicity and nephrotoxicity of doxorubicin given as a bolus, continuous infusion or entrapped in liposomes in the Lou/M Wsl rat**. *Cancer ChemotherPharmacol* **24**, 341-348.

222. Stummann, T.C., Beilmann, M., Duker, G., Dumotier, B., Fredriksson, J.M., Jones, R.L., Hasiwa, M., Kang, Y.J., Mandenius, C.F., Meyer, T., Minotti, G., Valentin, Y.J.P., Zunkler, B.J. and Bremer, S. (2009) **Report and Recommendations of the Workshop of the European Centre for the Validation of Alternative Methods for Drug-Induced Cardiotoxicity**. *Cardiovascular Toxicology* **9**, 107-125.

223. Suh, M.R., Lee, Y., Kim, J.Y., Kim, S.K., Moon, S.H., Lee, J.Y., Cha, K.Y., Chung, H.M., Yoon, H.S., Moon, S.Y., Kim, V.N. and Kim, K.S. (2004) **Human embryonic stem cells express a unique set of microRNAs**. *Developmental Biology* **270**, 488-498.

224. Sumazin, P., Yang, X., Chiu, H.S., Chung, W.J., Iyer, A., Llobet-Navas, D., Rajbhandari, P., Bansal, M., Guarnieri, P., Silva, J. and Califano, A. (2011) **An Extensive**

MicroRNA-Mediated Network of RNA-RNA Interactions Regulates Established Oncogenic Pathways in Glioblastoma. *Cell* **147**, 370-381.

225.   Suzuki, T., Hayashi, D., Yamazaki, T., Mizuno, T., Kanda, Y., Komuro, I., Kurabayashi, M., Yamaoki, K., Mitani, K., Hirai, H., Nagai, R. and Yazaki, Y. (1998) **Elevated B-type natriuretic peptide levels after anthracycline administration**. *Am Heart J* **136**, 362-363.

226.   Takagi, S., Nakajima, M., Kida, K., Yamaura, Y., Fukami, T. and Yokoi, T. (2010) **MicroRNAs regulate human hepatocyte nuclear factor 4alpha, modulating the expression of metabolic enzymes and cell cycle**. *JBiol Chem* **285**, 4415-4422.

227.   Takagi, S., Nakajima, M., Mohri, T. and Yokoi, T. (2008) **Post-transcriptional regulation of human pregnane X receptor by micro-RNA affects the expression of cytochrome P450 3A4**. *JBiol Chem* **283**, 9674-9680.

228.   Tan, L.P., Seinen, E., Duns, G., de Jong, D., Sibon, O.C.M., Poppema, S., Kroesen, B.J., Kok, K. and van den Berg, A. (2009) **A high throughput experimental approach to identify miRNA targets in human cells**. *Nucleic Acids Research* **37**, e137-.

229.   Tang, R., Li, L., Zhu, D., Hou, D., Cao, T., Gu, H., Zhang, J., Chen, J., Zhang, C.Y. and Zen, K. (2011) **Mouse miRNA-709 directly regulates miRNA-15a/16-1 biogenesis at the posttranscriptional level in the nucleus: evidence for a microRNA hierarchy system**. *Cell Res*, 10-.

230.   Tang, Y.H., Zheng, J.Y., Sun, Y., Wu, Z.G., Liu, Z.M. and Huang, G.Z. (2009) **MicroRNA-1 Regulates Cardiomyocyte Apoptosis by Targeting Bd-2**. *International Heart Journal* **50**, 377-387.

231.   Taylor, E.L. and Gant, T.W. (2008) **Emerging fundamental roles for non-coding RNA species in toxicology**. *Toxicology* **246**, 34-39.

232.   Thomas, M., Lieberman, J. and Lal, A. (2010) **Desperately seeking microRNA targets**. *Nat Struct Mol Biol* **17**, 1169-1174.

233.   Thompson, K., Rosenzweig, B., Zhang, J., Knapton, A., Honchel, R., Lipshultz, S., Retief, J., Sistare, F. and Herman, E. (2009) **Early alterations in heart gene expression profiles associated with doxorubicin cardiotoxicity in rats**. *Cancer Chemotherapy and Pharmacology*.

234.   Thomson, D.W., Bracken, C.P. and Goodall, G.J. (2011) **Experimental strategies for microRNA target identification**. *Nucleic Acids Res* **39**, 6845-6853.

235.   Thum, T., Galuppo, P., Wolf, C., Fiedler, J., Kneitz, S., van Laake, L.W., Doevendans, P.A., Mummery, C.L., Borlak, J., Haverich, A., Gross, C., Engelhardt, S., Ertl, G. and Bauersachs, J. (2007) **MicroRNAs in the Human Heart: A Clue to Fetal Gene Reprogramming in Heart Failure**. *Circulation* **116**, 258-267.

236.   Thum, T., Gross, C., Fiedler, J., Fischer, T., Kissler, S., Bussen, M., Galuppo, P., Just, S., Rottbauer, W., Frantz, S., Castoldi, M., Soutschek, J., Koteliansky, V., Rosenwald, A., Basson, M.A., Licht, J.D., Pena, J.T., Rouhanifard, S.H., Muckenthaler, M.U., Tuschl, T., Martin, G.R., Bauersachs, J. and Engelhardt, S. (2008) **MicroRNA-21 contributes to myocardial disease by stimulating MAP kinase signalling in fibroblasts**. *Nature* **456**, 980-984.

237.   Tijsen, A.J., Creemers, E.E., Moerland, P.D., de Windt, L.J., van der Wal, A.C., Kok, W.E. and Pinto, Y.M. (2010) **MiR423-5p As a Circulating Biomarker for Heart Failure**. *Circulation Research*.

238.   Tsuchiya, Y., Nakajima, M., Takagi, S., Taniya, T. and Yokoi, T. (2006) **MicroRNA regulates the expression of human cytochrome P450 1B1**. *Cancer Res* **66**, 9090-9098.

239.   Turchinovich, A., Weiz, L., Langheinz, A. and Burwinkel, B. (2011) **Characterization of extracellular circulating microRNA**. *Nucleic Acids Res* **39**, 7223-7233.

240.   Uesugi, A., Kozaki, K., Tsuruta, T., Furuta, M., Morita, K., Imoto, I., Omura, K. and Inazawa, J. (2011) **The tumor suppressive microRNA miR-218 targets the mTOR component Rictor and inhibits AKT phosphorylation in oral cancer**. *Cancer Res* **71**, 5765-5778.

241.   Umbach, J.L. and Cullen, B.R. (2009) **The role of RNAi and microRNAs in animal virus replication and antiviral immunity**. *Genes & Development* **23**, 1151-1164.

242.   Valadi, H., Ekstrom, K., Bossios, A., Sjostrand, M., Lee, J.J. and Lotvall, J.O. (2007) **Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells**. *Nat Cell Biol* **9**, 654-659.

243.   Valastyan, S., Reinhardt, F., Benaich, N., Calogrias, D., Szβsz, A.M., Wang, Z.C., Brock, J.E., Richardson, A.L. and Weinberg, R.A. (2009) **A Pleiotropically Acting MicroRNA, miR-31, Inhibits Breast Cancer Metastasis**. *Cell* **137**, 1032-1046.

244.   van Dongen, S., Abreu-Goodger, C. and Enright, A.J. (2008) **Detecting microRNA binding and siRNA off-target effects from expression data**. *Nat Methods* **5**, 1023-1025.

245.   Van, H.P. and Sasaki, J. (2010) **State-of-the-art genomics approaches in toxicology**. *MutatRes* **705**, 165-171.

246.   van Rooij, E. and Olson, E.N. (2007) **MicroRNAs: powerful new regulators of heart disease and provocative therapeutic targets**. *J Clin Invest* **117**, 2369-2376.

247.   van Rooij, E., Quiat, D., Johnson, B.A., Sutherland, L.B., Qi, X., Richardson, J.A., Kelm, J. and Olson, E.N. (2009) **A Family of microRNAs Encoded by Myosin Genes Governs Myosin Expression and Muscle Performance**. *Developmental Cell* **17**, 662-673.

248.   van Rooij, E., Sutherland, L.B., Liu, N., Williams, A.H., McAnally, J., Gerard, R.D., Richardson, J.A. and Olson, E.N. (2006) **A signature pattern of stress-responsive**

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microRNAs that can evoke cardiac hypertrophy and heart failure. *Proc Natl Acad Sci U S A* **103**, 18255-18260.

249.   van Rooij, E., Sutherland, L.B., Qi, X., Richardson, J.A., Hill, J. and Olson, E.N. (2007) **Control of stress-dependent cardiac growth and gene expression by a microRNA**. *Science* **316**, 575-579.

250.   van Rooij, E., Sutherland, L.B., Thatcher, J.E., DiMaio, J.M., Naseem, R.H., Marshall, W.S., Hill, J.A. and Olson, E.N. (2008) **Dysregulation of microRNAs after myocardial infarction reveals a role of miR-29 in cardiac fibrosis**. *Proc Natl Acad Sci USA* **105**, 13027-13032.

251.   Vasudevan, S. (2011) **Posttranscriptional Upregulation by MicroRNAs**. *Wiley Interdiscip Rev RNA*.

252.   Vickers, K.C., Palmisano, B.T., Shoucri, B.M., Shamburek, R.D. and Remaley, A.T. (2011) **MicroRNAs are transported in plasma and delivered to recipient cells by high-density lipoproteins**. *Nat Cell Biol* **13**, 423-433.

253.   Wallace, K.B., Hausner, E., Herman, E., Holt, G.D., MacGregor, J.T., Metz, A.L., Murphy, E., Rosenblum, I.Y., Sistare, F.D. and York, M.J. (2004) **Serum troponins as biomarkers of drug-induced cardiac toxicity**. *ToxicolPathol* **32**, 106-121.

254.   Wang, D., Garcia-Bassets, I., Benner, C., Li, W., Su, X., Zhou, Y., Qiu, J., Liu, W., Kaikkonen, M.U., Ohgi, K.A., Glass, C.K., Rosenfeld, M.G. and Fu, X.D. (2011a) **Reprogramming transcription by distinct classes of enhancers functionally defined by eRNA**. *Nature* **474**, 390-394.

255.   Wang, F.E., Zhang, C., Maminishkis, A., Dong, L., Zhi, C., Li, R., Zhao, J., Majerciak, V., Gaur, A.B., Chen, S. and Miller, S.S. (2010a) **MicroRNA-204/211 alters epithelial physiology**. *FASEB J* **24**, 1552-1571.

256.   Wang, G., Wang, Y., Feng, W., Wang, X., Yang, J., Zhao, Y., Wang, Y. and Liu, Y. (2008) **Transcription factor and microRNA regulation in androgen-dependent and -independent prostate cancer cells**. *BMC Genomics* **9**, S22-.

257.   Wang, G.K., Zhu, J.Q., Zhang, J.T., Li, Q., Li, Y., He, J., Qin, Y.W. and Jing, Q. (2010b) **Circulating microRNA: a novel potential biomarker for early diagnosis of acute myocardial infarction in humans**. *European Heart Journal* **31**, 659-666.

258.   Wang, K. and Li, P.F. (2010a) **Foxo3a regulates apoptosis by negatively targeting miR-21**. *Journal of Biological Chemistry*.

259.   Wang, K. and Li, P.F. (2010b) **Foxo3a regulates apoptosis by negatively targeting miR-21**. *Journal of Biological Chemistry*, -.

260.	Wang, K., Zhang, S., Marzolf, B., Troisch, P., Brightman, A., Hu, Z., Hood, L.E. and Galas, D.J. (2009) **Circulating microRNAs, potential biomarkers for drug-induced liver injury**. *Proceedings of the National Academy of Sciences* **106**, 4402-4407.

261.	Wang, K., Zhang, S., Weber, J., Baxter, D. and Galas, D.J. (2010c) **Export of microRNAs and microRNA-protective protein by mammalian cells**. *Nucleic Acids Res* **38**, 7248-7259.

262.	Wang, R., Li, N., Zhang, Y., Ran, Y. and Pu, J. (2011b) **Circulating MicroRNAs are promising novel biomarkers of acute myocardial infarction**. *Intern Med* **50**, 1789-1795.

263.	Wang, Z., Zhao, Y., Smith, E., Goodall, G.J., Drew, P.A., Brabletz, T. and Yang, C. (2011c) **Reversal and prevention of arsenic-induced human bronchial epithelial cell malignant transformation by microRNA-200b**. *ToxicolSci* **121**, 110-122.

264.	Widera, C., Gupta, S.K., Lorenzen, J.M., Bang, C., Bauersachs, J., Bethmann, K., Kempf, T., Wollert, K.C. and Thum, T. (2011) **Diagnostic and prognostic impact of six circulating microRNAs in acute coronary syndrome**. *J Mol Cell Cardiol* **51**, 872-875.

265.	Woolcock, K.J., Gaidatzis, D., Punga, T. and Bühler, M. (2011) **Dicer associates with chromatin to repress genome activity in Schizosaccharomyces pombe**. *Nat Struct Mol Biol* **18**, 94-99.

266.	Wu, L. and Belasco, J.G. (2008) **Let Me Count the Ways: Mechanisms of Gene Regulation by miRNAs and siRNAs**. *Molecular Cell* **29**, 1-7.

267.	Xi, Y., Wu, G., Yang, L., Han, K., Du, Y., Wang, T., Lei, X., Bai, X. and Ma, A. (2009) **Increased late sodium currents are related to transcription of neuronal isoforms in a pressure-overload model**. *European Journal of Heart Failure* **11**, 749-757.

268.	Xiao, C., Calado, D.P., Galler, G., Thai, T.H., Patterson, H.C., Wang, J., Rajewsky, N., Bender, T.P. and Rajewsky, K. (2007) **MiR-150 controls B cell differentiation by targeting the transcription factor c-Myb**. *Cell* **131**, 146-159.

269.	Xu, C., Lu, Y., Pan, Z., Chu, W., Luo, X., Lin, H., Xiao, J., Shan, H., Wang, Z. and Yang, B. (2007) **The muscle-specific microRNAs miR-1 and miR-133 produce opposing effects on apoptosis by targeting HSP60, HSP70 and caspase-9 in cardiomyocytes**. *J Cell Sci* **120**, 3045-3052.

270.	Yang, B., Lin, H., Xiao, J., Lu, Y., Luo, X., Li, B., Zhang, Y., Xu, C., Bai, Y., Wang, H., Chen, G. and Wang, Z. (2007) **The muscle-specific microRNA miR-1 regulates cardiac arrhythmogenic potential by targeting GJA1 and KCNJ2**. *Nat Med* **13**, 486-491.

271.	Yano, M., Ikeda, Y. and Matsuzaki, M.U. (2005a) **Altered intracellular Ca2+ handling in heart failure**. *Journal of Clinical Investigation* **115**, 556-564.

272.	Yano, M., Yamamoto, T., Ikemoto, N. and Matsuzaki, M. (2005b) **Abnormal ryanodine receptor function in heart failure**. *Pharmacology & Therapeutics* **107**, 377-391.

273.   Yokoi, T. and Nakajima, M. (2011) **Toxicological implications of modulation of gene expression by microRNAs**. *Toxicological Sciences* **123**, 1-14.

274.   Yoshioka, W., Higashiyama, W. and Tohyama, C. (2011) **Involvement of microRNAs in dioxin-induced liver damage in the mouse**. *ToxicolSci* **122**, 457-465.

275.   Younger, S.T. and Corey, D.R. (2011) **Transcriptional gene silencing in mammalian cells by miRNA mimics that target gene promoters**. *Nucleic Acids Res* **39**, 5682-5691.

276.   Zernecke, A., Bidzhekov, K., Noels, H., Shagdarsuren, E., Gan, L., Denecke, B., Hristov, M., Koppel, T., Jahantigh, M.N., Lutgens, E., Wang, S., Olson, E.N., Schober, A. and Weber, C. (2009) **Delivery of microRNA-126 by apoptotic bodies induces CXCL12-dependent vascular protection**. *Sci Signal* **2**.

277.   Zhang, B. and Pan, X. (2009) **RDX induces aberrant expression of microRNAs in mouse brain and liver**. *EnvironHealth Perspect* **117**, 231-240.

278.   Zhang, L., Liu, Y., Song, F., Zheng, H., Hu, L., Lu, H., Liu, P., Hao, X., Zhang, W. and Chen, K. (2011) **Functional SNP in the microRNA-367 binding site in the 3'UTR of the calcium channel ryanodine receptor gene 3 (RYR3) affects breast cancer risk and calcification**. *Proc Natl Acad Sci USA* **108**, 13653-13658.

279.   Zhang, Y., Liu, D., Chen, X., Li, J., Li, L., Bian, Z., Sun, F., Lu, J., Yin, Y., Cai, X., Sun, Q., Wang, K., Ba, Y., Wang, Q., Wang, D., Yang, J., Liu, P., Xu, T., Yan, Q., Zhang, J., Zen, K. and Zhang, C.Y. (2010) **Secreted monocytic miR-150 enhances targeted endothelial cell migration**. *Mol Cell* **39**, 133-144.

280.   Zhao, Y., Ransom, J.F., Li, A., Vedantham, V., von Drehle, M., Muth, A.N., Tsuchihashi, T., McManus, M.T., Schwartz, R.J. and Srivastava, D. (2007) **Dysregulation of cardiogenesis, cardiac conduction, and cell cycle in mice lacking miRNA-1-2**. *Cell* **129**, 303-317.

281.   Zhao, Y., Samal, E. and Srivastava, D. (2005) **Serum response factor regulates a muscle-specific microRNA that targets Hand2 during cardiogenesis**. *Nature* **436**, 214-220.