Non-coding RNA species in heart failure

Chapter 7

Summary and general discussion

The aim of this thesis was to extend our knowledge on the role of different non-coding RNA species in the development of heart failure (HF). Over the last years it has been acknowledged that multiple cell types are critically involved in HF, including cardiomyocytes, cardiac fibroblasts, and resident or infiltrating immune cells. This thesis comprises research on the involvement of these three cell types in HF with a focus on microRNAs (miRNAs) and long non-coding RNAs (lncRNAs) as regulators of cell function. Only very recently, the cell type-specific effects of miRNAs in HF and their role in paracrine signaling have come to the center of attention[1-5]. Here we extend this knowledge by identifying miR-139 as regulator of cardiomyocyte function and the miR-221/222 family as repressor of fibroblast activation. In comparison, the role of lncRNAs in the different cardiac cell types is to date essentially unknown. We performed an in-depth analysis of the development of HF in mice lacking the lncRNA Malat-1 but surprisingly did not find a relevant function in any of the mentioned cell types. In contrast, we propose mascRNA, a processed product of Malat-1, to act as regulator of the immune system during VM. The overall conclusion is that ncRNAs play distinct roles in different cell types that could be exploited for targeted therapy strategies in cardiac disease.

MIRNAS AS MODULATORS OF CARDIOMYOCYTE FUNCTION AND MYOCARDIAL FIBROSIS

In chapter 2 of this thesis we have shown that miR-139 is differentially expressed in human aortic valve stenosis and affects calcium handling in cardiomyocytes, probably by interfering with cAMP/PDE signaling. Stimulation of β-adrenergic receptors (β-AR) is a central physiological response to increased cardiac demands that evokes positive inotropic and lusitropic effects in cardiomyocytes[6]. The intracellular effect of β-AR activation is initiated by production of cAMP by adenylate cyclase, which leads to activation of cAMP effector proteins such as PKA and EPAC1, whereas phosphodiesterases (PDEs) terminate β-AR signaling by degrading cAMP. In HF, the adrenergic system is activated but the downstream effects are altered due to reduced number and function of the β-AR[7] and deregulation of the intracellular signaling cascade, including PDEs[8-11]. We validated the phosphodiesterases 3a, 4a and 4d as targets of miR-139 and found indications for increased phosphorylation of the L-type calcium channel in rat cardiomyocytes overexpressing miR-139. At the same time, miR-139 reduced spontaneous calcium release from the SR. These findings indicate that miR-139 is involved in calcium entry and intracellular cycling, although more detailed studies are needed to unravel the precise mechanism involved. *In vivo* we found that overexpression of miR-139 aggravated LV dilation after pressure overload. Defining the link between PDE repression and long-term effects after pressure overload *in vivo* was beyond the scope of this project because of the complexity of cAMP regulation and the multitude of possible miR-139 targets involved. Of note, it is conceivable that apart from affecting PKA signaling as assessed *in vitro*, miR-139 also influences transcriptional programs involved in cardiac hypertrophy, such as cAMP/EPAC1 signaling[12]. Therefore, further studies are warranted to delineate the effect of miR-139 on cAMP/PDE signaling in the diseased heart.

Besides cardiomyocyte dysfunction, myocardial fibrosis is a hallmark of HF and negatively affects cardiac contraction, electrical conduction, and oxygen supply[13]. In chapter 3 we have reported that the miRNA-221/222 family correlates negatively with the level of cardiac fibrosis in patients with aortic valve stenosis. In a mouse model of cardiac pressure overload we showed that inhibition of miR-221/222 aggravates the development of cardiac dysfunction and fibrosis. These data indicate that high myocardial levels of miR-221/222 may protect against excessive fibrosis in cardiac disease. To unravel the mechanism behind this, we modulated expression of miR-221/222 in rat cardiac fibroblasts and found a repressive effect on TGFβ-induced activation of fibroblasts into collagen-producing myofibroblasts. This effect is possibly mediated by the miR-221/222 target Ets1, a pro-fibrotic transcription factor that synergizes with TGFβ[14,15]. While we show a detrimental role of miR-221/222 in HF by acting on fibroblasts, other groups have investigated cardiomyocyte-specific effects of miR-221/222: Liu et al. found that miR-222 is necessary for physiological cardiac hypertrophy and that its overexpression in cardiomyocytes attenuates ischemic injury and reduces fibrosis[16]. In contrast, Su et al. reported that cardiomyocyte-restricted overexpression of miR-221 leads to spontaneous development of heart failure due to cardiomyocyte dysfunction and death[17]. Taken together, the outcome of systemic miRNA inhibition as performed in our study differs from that of cell type-restricted intervention. The prevailing phenotype of systemic inhibition probably depends on the cell type with highest expression of the targeted miRNA, which calls for the development of more targeted delivery strategies as discussed further below.

LNCRNAS AS NOVEL PLAYERS IN HEART DISEASE

Several studies describe an important role for lncRNAs especially in cardiac development but also in heart disease as summarized in chapter 4. However, it remains challenging to identify lncRNAs with relevant functions in the heart and especially to define the precise pathway and binding partners mediating this function. Several lncRNAs have functions in the immune system[18] but their role in cardiac inflammation had not been investigated so far. In chapter 6 we have provided first evidence for a protective role of mascRNA, a lncRNA-derived small ncRNA, in the regulation of the immune response during VM. We found mascRNA to induce cellular defense mechanisms against viral entry and replication by upregulating IFITM and IFIT genes. Above that, systemic manipulation of mascRNA affected circulating immune cell populations. MascRNA is therefore a novel player in the pathophysiology of VM and an interesting study object in immunology and infection research in general.

Surprisingly, we found the host transcript of mascRNA, the lncRNA Malat-1, to be dispensable for the development of pressure overload-induced HF. Malat-1 is a highly abundant nuclear lncRNA with remarkable evolutionary conservation. It has been reported to affect vascularization[19], to regulate the abundance of the muscle-specific miR-133[20,21], and to activate the pro-hypertrophic ERK/MAPK pathway[22]. These reports notwithstanding, cardiac pressure overload in Malat-1 knockout mice had largely the same effect as in wild

type mice (chapter 5). We performed in-depth phenotyping including analysis of LV morphology, function, histology, and gene expression, all of which are known to be perturbed in HF. However, we only found an effect of Malat-1 ablation on splicing of Ndrg2, which was apparently irrelevant for the clinical outcome. These unexpected observations suggest that the reported functions of Malat-1 as regulator of vascularization, scavenger of miR-133, and activator of ERK/MAPK signaling may be context-dependent and do not sum up to an important role of Malat-1 in cardiac hypertrophy and failure. Our results therefore stress that individually reported lncRNA functions need to be validated in complex disease models and highlight that sequence conservation and high expression level of a lncRNA do not necessarily indicate important (patho-)physiological functions.

NOVEL TOOLS FOR NOVEL RNA

MiRNAs are known to function primarily via target gene repression (except for anecdotal reports about enhanced target gene translation[23-25]), and there are established tools to predict and validate miRNA targets and to modulate miRNA levels *in vitro* and *in vivo* by use of miRNA mimics, inhibitors, viral vectors or antagomirs[26-29]. LncRNAs, in strong contrast to miRNAs, are a very heterogeneous class of RNA molecules with variable length, post-transcriptional processing, three-dimensional structure, intracellular localization, and mode of action as outlined in chapter 4. A first bioinformatic method to predict the function of lncRNAs has very recently been developed but is limited to effects on gene regulation[30]. Therefore, research into lncRNA function so far relies on loss-of-function experiments by post-transcriptional knockdown or genomic deletion. Both approaches have their values but also some limitations, and novel approaches are needed to investigate this novel class of RNA.

LncRNAs may act in the cytoplasm or nucleus, and it is important to know the predominant location to design appropriate knockdown strategies. For example, siRNA-mediated knockdown is very efficient in the cytoplasm but its efficacy in the nucleus in controversial[31-33] and may relate to repression of transcription rather than post-transcriptional target degradation[34,35]. Therefore, we made use of gapmers to knock down nuclear Malat-1 (chapter 6). Gapmers are antisense oligonucleotides with an LNA-DNA-LNA backbone that potently induces cleavage of nuclear lncRNAs by RNAseH[36,37]. However, even with the correct approach, knockdown efficiency can be impaired by lncRNA secondary structure or binding partners that block the target site. For example, in chapter 6 we discussed difficulties to knock down the cytoplasmic mascRNA, probably due to its secondary structure. Nuclear lncRNAs can have functions in organizing nuclear structures (e.g. Neat1 in paraspeckles[38]), affect mRNA processing (e.g. Malat-1 in splicing[39,40]), or regulate gene expression in *cis* or in *trans* (e.g. XIST and HOTAIR[41]). Importantly, gene regulatory effects may even be independent of the lncRNA transcript and emerge from the mere transcriptional activity at the lncRNA gene locus[42-45]. In that case, post-transcriptional knockdown of the lncRNA will either way fail to identify the function of the gene locus.

Next to antisense-mediated knockdown strategies, genomic deletion of lncRNAs is widely employed. However, interfering with genomic integrity at a lncRNA locus may remove or reorganize binding sites for regulatory factors, leading to off-target effects[46]. For example, it has been shown that blocking transcription of the lncRNA *Fendrr* has different consequences than replacing the gene while keeping the locus transcriptionally active[47,48]. Therefore, a careful study design including appropriate controls is mandatory in lncRNA research and elaborate considerations about the interpretation of *in vivo* studies have been summarized recently[46]. Of note, positional off-target effects may also occur after genomic manipulation of coding genes, which has been carried out for several years. The lessons learned from lncRNA research may thus also have important implications for established methods in research on coding RNA.

Another layer of complexity is added by lncRNA processing and Malat-1 is a good example for this: The primary Malat-1 transcript is processed into a long nuclear-retained fragment with reported functions in gene transcription and mRNA splicing[39,49-51], but its 3' terminus also gives rise to mascRNA, a small cytoplasmic ncRNA for which no function has been published previosuly[52]. In chapter 6 we have described a role for mascRNA in VM, but disentangling the functions of Malat-1 and mascRNA remains challenging. Complete removal of the Malat-1 locus abolishes expression of mascRNA, whereas transcriptional blockade of Malat-1 may still allow for residual expression of downstream regions from an unknown promoter. Also post-transcriptional knockdown of a lncRNA may or may not lead to simultaneous depletion of its processed products, depending on the time frame between transcription and processing. Novel methods of genome engineering, such as the CRISPR/Cas-system, may be suitable for targeted removal of a lncRNA and/or its processed product from the genome.

In contrast to loss-of-function studies, overexpression of lncRNAs is rarely employed to date and is much more challenging than overexpression or mimicking miRNAs. The use of adeno-associated virus (AAV) vectors *in vivo* is well-established and different serotypes allow for some organ specificity in transgene delivery. In chapter 2 and 6 we employed AAV9 vectors for cardiomyocyte-specific overexpression, but these vectors are limited to transgenes smaller than 4.5 kb[29] and some lncRNAs including Malat-1 are simply too large to fit into viral vectors. Above that, transgene delivery is futile for lncRNAs with a *cis*-regulatory function that depends on correct chromosomal location. Interestingly, methods to enhance expression of endogenous genes by modified zinc finger proteins[53,54] and TALEs[55,56] have been developed in the last years. Especially the recently described CRIPR/Cas-mediated gene activation system may provide an easily applicable tool to overexpress lncRNAs from the endogenous locus[57].

THE BROAD FIELD OF NCRNA RESEARCH: WHICH WAY TO GO?

Our growing knowledge about miRNA function and the impressive potency of miRNA modulation in animal models has quickly elicited interest of pharmaceutical companies, and first miRNA-based therapeutics are already being developed and tested[58-60]. The transition from discovery to clinical testing has happened with remarkable speed: The first miRNA, lin-4, was discovered in 1993[61] and 5 years later the concept or RNAi was introduced[62], although the term "microRNA" was only coined in 2001[63-65]. Already in 2008, the applicability of miRNA inhibitors in primates was tested[66] and soon followed by a clinical phase 2a trial in humans, which showed promising results for the treatment of hepatitis[67]. To date, we are clearly able to effectively inhibit miRNAs *in vivo*, and by now also the first miRNA mimic has entered a phase 1 clinical trial[68]. However, this haste in exploiting the therapeutic potential of miRNAs also calls for a sober view on the potential limitations and dangers. Possible adverse effects include activation of the immune system by exogenous RNA and off-target effects by unintentional modulation of unrelated miRNAs[60]. Above that, our results and the growing knowledge about cell type- and context-specific miRNA functions highlight the possibility of side effects by manipulating the intended miRNA in unintended cell types or organs. In chapter 3 we described anti-fibrotic effects of the miR-221/222 family in cardiac pressure overload, putting miRNA mimics forward as therapeutic option to dampen fibroblast activation. However, another group has shown that overexpression of miR-221 in cardiomyocytes causes cell death and leads to HF[17]. Above that, the miR-221/222 family can suppress or promote malignant diseases, depending on the type of tumor[69]. In view of the Janus-faced function of miRNAs, possible off-target effects need to be carefully assessed and advances in targeted delivery methods are much-needed before the full potential of miRNA-based medicine can safely be harnessed[60,70].

While the first miRNAs enter the clinic, the involvement of lncRNAs in (patho-)physiological processes in the heart has only been investigated in the last few years. In chapter 4 we have summarized the progress that has been made in assigning functions to individual lncRNAs and their involvement in cardiac development, function, and disease. We showed that research on lncRNAs in cardiac disease is still in its infancy but offers a new opportunity to develop biomarkers and treatment options in heart failure. In the years while I was preparing this thesis, the number of publications on lncRNAs has steeply increased from about 100 publications indexed in PubMed in 2011 to more than 700 in 2014. LncRNAs are clearly a "hot topic", and interest in this area was boosted in 2012 by multiple publications from the ENCODE consortium (Encyclopedia of DNA Elements), stating that the vast majority of the genome is not silent "junk" as previously considered[71] but transcribed and functional[72,73]. However, it did not take long until a debate about the interpretation of ENCODE arose[74,75], although part of the controversy may relate to definitions and semantics. The current interest in lncRNAs exemplifies the problem of "hypes" in scientific research, leading to high expectations and possibly a one-sided and misleading interpretation of novel observations. The lncRNA Malat-1 is remarkably well conserved, highly expressed in most

tissues, and has a function in synaptogenesis[40]. Therefore, expectations were high that genomic deletion of Malat-1 would have severe consequences. Three independent groups made an effort to generate Malat-1 knockout mice, but surprisingly all three strains develop and breed normally[51,76,77]. Somewhat later, several reports indicated a role for Malat-1 in cardiac hypertrophy but we found Malat-1 to be dispensable for the development of pressure overload-induced cardiac hypertrophy and failure (chapter 5).

Obviously, several highly important lncRNAs have been identified to date, but it remains questionable if they constitute the "tip of the iceberg" of functional lncRNAs, or if most lncRNAs are in fact redundant or even non-functional. A publication from Sauvageau et al. exemplifies this conundrum and illustrates the problem of "misleading interpretation" as mentioned above. In their methodologically remarkable and insightful study "Multiple knockout mouse models reveal lincRNAs are required for life and brain development", the authors generated 18 lncRNA knockout mice and found clear abnormalities in 5 of them, indeed suggesting that some lncRNAs are crucial for normal development[48]. However, in a detailed comment, Claudiu Bandea criticizes the misleading title and emphasizes that actually "most lncRNAs (i.e. 13 out of 18) do not appear to play critical roles *in vivo*"[78]. In conclusion, research of the last years has identified several highly interesting lncRNAs but the true potential of lncRNAs to revolutionize biomedical research is still difficult to assess.

CONCLUDING REMARKS

This thesis provides a comprehensive picture of the cell types involved in the development of HF and highlights how ncRNAs can affect cell type-specific pathological mechanisms. It is evident from our data and from the large amount of work done by other groups that ncRNAs are crucial regulators of cell function and consequently important players in heart failure. The levels of knowledge, however, differ greatly when it comes to compare miRNAs and lncRNAs. Several miRNAs are known to play important roles in the development of HF, and we have contributed some more puzzle pieces to this knowledge. The utilization of miRNA-based therapeutics for HF appears to be a matter of time despite some hurdles that still need to be taken. In contrast, research on lncRNAs in heart failure is still in its infancy and is hampered by methodological challenges and the heterogeneity of lncRNA function. However, progress in science depends on sailing unknown seas and with the discovery of lncRNAs there is now a New World to explore.

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