may be another factor in suppressing the proliferative potential of cardiomyocytes (Porrello, Johnson, et al. 2011). Notably, members of this miRNA family have been shown to be upregulated in the injured heart (unpublished data chapter 3 and (van Rooij et al. 2006), suggesting active repression of regeneration. Indeed, in another study by Porrello et al., inhibition of the miR-15 family increased cardiomyocyte proliferation and improved cardiac function following MI in the adult heart (Porrello et al. 2013).

The above-mentioned observations and speculations lead to the question: Is the DLK1-DIO3 imprinted region active during the first week after birth? Although cardiac DIO3 levels have not been measured during the first week of life in rodents, we speculate that the observed reduction of DIO3 in the eye and ear (Ng et al. 2010; Ng et al. 2009) are indications that DIO3 expression in the majority of maturing tissues, including the heart (Pol et al. 2011), is lost at day 7. This implies that at postnatal day 7 (P7), cardiac T3 levels will be determined by the rising plasma T3 levels, which will restrict proliferation. However, no information has been published concerning the DLK1-DIO3-associated miRNAs. It would therefore be interesting to analyze the miRNA profiles during the first week after birth.

To complicate things further, Naqvi et al. recently described a burst of cardiomyocyte proliferation, as part of normal development, around postnatal day 15 (P15) (Naqvi et al. 2014). They suggest that this proliferation involves mitosis of adult cardiomyocytes, similar to the mechanism of regeneration proposed by Porello et al. In contrast, however, the brief period of activity in Naqvi’s study depended on the rise in plasma T3 levels that occurs around this time. It was speculated that this cardiomyocyte proliferative burst involves an orchestrated interplay between a permissive action of T3 and an increase in wall stress, which both are part of the fetal to adult shift. Expression levels of IGF-1 and the corresponding receptor IGFR-1 are locally induced by T3 (Xing et al. 2012; L. Wang, Shao, and Ballock 2010), and translocation of IGF-1-activated Akt to the nucleus is known to be involved in cell proliferation (Condorelli et al. 2002; Rota et al. 2005).

Combining the available data, we speculate that the initial proliferative capacity, which appears to require low T3 levels, is different from the proliferative burst that occurs around P15. It will be important to identify the full complement of factors responsible for both processes. This information will help to decide which approach might be taken to induce proliferation in the post-MI heart. For instance, keep T3 levels low and activate the missing factors, mimicking the first week of postnatal life, or restore T3 levels and possibly stimulate factors important for the proliferation observed at P15.

**A TH-dependent negative feedback mechanism aimed at regulating Dio3 expression after MI**

The re-expression of Dio3 in the heart following MI or pressure overload is now well accepted in the field (Pol et al. 2011; Olivares et al. 2007; Ueta et al. 2012; Trivieri et al. 2006). However, the mechanisms of regulation of Dio3 expression in the heart remain unclear. To this date, several signaling cascades, which include TGFβ, MAPK, SHH, and HIF-1α, have been implicated in cardiac remodeling, but none of these have yet definitely been shown to be involved in the re-expression of Dio3 in the heart following MI [see also chapter 1]. Given the strict spatiotemporal regulation of DIO3 activity during fetal development, such as in the retina and cochlea (Ng et al. 2010; Ng et al. 2009), Dio3 expression is considered a candidate for regulation by miRNAs. To gain insight in the role of miRNAs in regulating Dio3 expression, we performed an in silico analysis of miRNAs that were

**Note** In contrast to the observation of Naqvi et al., a very recent paper suggested that cardiomyocyte number expansion was solely limited to the early neonatal period, and that TH-induced physiological hypertrophy via the activation of the IGF-1/IGF-1R/Akt pathway is most likely the cause of the increase in heart size observed by Naqvi et al. (Alkass et al. 2015). This issue is now a matter of intense debate (Alkass et al., 2015; Soonpaa et al., 2015), but whether T3 has a permissive effect on cardiomyocyte hyperplasia or induces physiological hypertrophy, its role is undisputable in both.
found to be differentially expressed following MI [chapter 3]. In the studies described in chapter 4 we identified only one miRNA, miR-214, as potentially targeting a highly conserved region within the SECIS-element of Dio3. Besides studies that have shown a role for miR-214 in developing cardiac hypertrophy, this miRNA has been shown to protect the heart from ischemic injury (Aurora et al. 2012). We confirmed the binding of miR-214 to the 3'UTR of Dio3, which resulted in decreased translation efficiency. This was in line with pilot data obtained using LV tissue from a study in which a miR-214 antagomiR was used in a model of pressure overload. Our analyses showed a border-line significant increase in Dio3 expression after blocking miR-214 and an associated increase of Myh7 expression, which suggests a further reduction of cardiac T3 levels (unpublished data). However, having confirmed the suppressive effect of miR-214 on Dio3 expression in vitro, it was unexpected that the expression of miR-214 was increased in the post-MI mouse heart, given the marked upregulation of cardiac DIO3 activity in this model (Pol et al. 2011; Olivares et al. 2007). Therefore, the expression patterns of miR-214 and its target were studied in individual cardiomyocytes in sections of LV tissue. We concluded that miR-214 and Dio3 were co-expressed within the same regions of the spared myocardium, indicating either that Dio3 and miR-214 are independently upregulated, or that the expressions are mechanistically linked. The latter was suggested after time-course analysis of up to post-MI day 56. This revealed that increased DIO3 expression and activity levels preceded the increase in miR-214. Furthermore, we showed that miR-214 expressions were reduced when mice received a short-term T3 treatment. Taken together, we hypothesized that Dio3 expression is limited by miR-214 via a negative feed-back loop involving T3.

Aurora et al., using a homozygous miR-214KO mouse model, showed that post-MI survival is in part dependent on miR-214 expression (Aurora et al. 2012). This result is in line with the effect of miR-214 on Dio3 expression and our hypothesis that
this limits the deleterious effects of cardiac hypothyroidism. To test the interaction between Dio3 and miR-214 in vivo, we analyzed the effect of treatment with antagomiR-214 on post-MI LV tissue, expecting an increase in Dio3 expression. Using the antagomiR approach we observed no changes in either Dio3, or expression of the already established miR-214 target Ncx1 (Aurora et al. 2012). In our experiments we started the antagomiR treatment at 7 days following MI, when miR-214 levels started to increase, and analyzed the effect at 14 days post-MI. The treatment schedule and antagomiR dosage used were apparently sufficient to suppress miR-214 levels at day 14. However, the time course of the effect of the antagomiR is not yet evident at this time point. Subsequent studies should therefore include additional time points.

Deiodinase type III expression in human ischemic heart disease

In chapter 5 we showed DIO3 activity in human fetal cardiac tissue, which decreased dramatically in the first weeks of postnatal life. DIO3 activity has been shown in various human fetal as well as some adult tissues (Gereben et al. 2008), but to the best of our knowledge this is the first time that activity of this deiodinase is reported for human cardiac tissue. The transient nature of this expression fits the general idea that DIO3 expression is part of the fetal gene program in most tissues, keeping T3 levels low during early development. Re-expression of DIO3 as part of pathological ventricular remodeling has been shown in various animal models of heart failure (Pol et al. 2011; Olivares et al. 2007; Simonides et al. 2008; Wassen et al. 2002) and the data on DIO3 expression in the LV of ISHD patients presented in chapter 5 suggest that this is also the case in human myocardium. Although the results of the quantitative immunohistochemistry of DIO3 as well as the mRNA analyses make a strong case for increased DIO3 activity, we were unable to detect enzyme activity. As pointed out earlier, the age of the samples in combination with the expected low levels of activity and the present detection limit of the assay, may provide an explanation. Obviously, this is a shortcoming of the study and additional analyses on freshly procured samples are required to substantiate our claim. Nevertheless, the analysis of LV and RV T3 content and the observed 25% reduction in LV ISHD samples is at least in line with an LV-specific increase in DIO3 activity. Furthermore, the observed lower T3 content may in part explain the hypothyroid-like profile of MHC mRNA expression that is also a hallmark of pathological remodeling, i.e., a decrease in MYH6 and an increase in MYH7 levels.

The marked increase in tissue T4 levels of LV and RV in ISHD patients is a puzzle. The fact that both ventricles show the same, highly significant, increase suggests that plasma T4 levels may be increased, but, if anything, a decrease in plasma T4 levels may be expected in these critically ill patients. Peri-operative treatment of transplant patients with T4 could explain these findings, but it is unlikely that all patients received this treatment. Plasma TH data need to be obtained in subsequent studies to elucidate this. In any case, the increased T4 levels do not seem to compensate for the reduced T3 levels in the LV, as indicated by the MYH6 and MYH7 mRNA expression levels.

These are the first studies in which TH levels human myocardium are determined and the data on T3 content in healthy donors show an almost 2 fold higher level in LV compared to the RV in the same heart. We do not know whether this reflects ventricle-specific differences in uptake and/or metabolism of T3 and T4, but this observation may provide an explanation for an earlier finding in human myocardium on MYH7 expression, the MHC isoform that is downregulated by T3. This study by Sharma et al. (Sharma et al. 2003) showed 60% lower mRNA expression levels of MYH7 in the LV compared to the RV. The authors suggest that the RV requires a different gene profile than the LV because of the differences in workload between both ventricles. Our present data suggest that differences in local metabolism of T3 may play a role in
determining this ventricle-specific gene profile in the healthy heart. This unexpected finding warrants further investigation.

**Novel therapeutic options for the treatment of heart failure**

There has been a long-standing interest in the use of TH in the treatment of heart failure because of the similarities in cardiac gene expression and function in this syndrome and hypothyroidism. However, early attempts to treat heart failure using TH preparations of unknown composition proved to be anything but therapeutic and in a few cases even fatal. These results still cast a shadow over this field despite an increasing number of studies showing the potential of TH treatment without adverse side effects. Gerdes and Iervasi recently summarized the background and current status of this area of research, where the focus now is on normalizing the reduced levels of plasma TH levels that is associated with the progression of heart failure (Gerdes and Iervasi 2010). This reduction is a general feature of critical illness and is referred to as the Non-Thyroidal Illness Syndrome (NTIS). This syndrome is characterized by normal or decreased plasma T4 levels, low T3 levels, increased rT3 levels, and usually unaltered TSH levels. The magnitude of these changes is correlated with the severity of the disease. It is generally accepted that changes that occur during the initial phase of illness are beneficial, as they reduce energy turnover, but in the long run become maladaptive. Indeed, the extent of NTIS is an independent predictor of mortality, which was already established three decades ago for chronic heart failure (Wiersinga, Lie, and Touber 1981). To date, several mechanisms have been proposed to contribute to the development of NTIS, including altered TH clearance and TH secretion by the thyroid gland. The latter reflects an apparent shift in the set point within the hypothalamus as a result of higher hypothalamic DIO2 activity and associated local T3 levels. This leads to reduced TRH release and subsequently reduced TSH production, which results in lower systemic T3 levels. Increased DIO3 expression in the liver causes an additional decrease in plasma T3 levels (reviewed by (Boelen, Kwakkel, and Fliers 2011; de Vries, Fliers, and Boelen 2015). Recently, evidence was found that changes in local TH metabolism also occur as part of NTIS during prolonged critical illness. Analyzing tissues obtained from critically ill patients at the time of death revealed a significant induction of DIO3 activity in various tissues. This suggests that DIO3-mediated TH inactivation contributes to NTIS (Peeters et al. 2005; Peeters et al. 2003; Rodriguez-Perez et al. 2008). Although it cannot be excluded that cardiac DIO3 activity also contributes to NTIS, as was suggested by Olivares et al. (Olivares et al. 2007), the early onset of Dio3 expression following MI in rat and mouse models, and the fact that absolute activity levels are low, makes such a contribution unlikely. The potential relevance of cardiac Dio3 activity lies in the fact that normalizing plasma T3 may not be sufficient to affect cardiac gene expression, which is the ultimate therapeutic target. Nevertheless, recent studies using a rat model of diabetes mellitus, in which cardiac Dio3 activity is also increased, have shown that physiological replacement dosages of T3 normalized cardiac T3 levels (Weltman et al. 2014). An unexplained aspect of this study was the fact that plasma T3 levels were not significantly elevated. An earlier study from Mourouzis et al. also reported beneficial effects on cardiac function of low-dose TH treatment in post-MI mice (Mourouzis et al. 2012). However, cardiac T3 levels were not analyzed in this study and improvement was suggested to be mediated in part by TH-induced physiological hypertrophy following activation of the Akt pathway. Therefore, it remains unclear whether increasing systemic TH levels can overcome the local cardiac hypothyroid condition, which is assumed to be due to the induction of DIO3 activity (Pol et al. 2011). It should be noted that this causal relationship between DIO3 activity and impaired TH signaling has not yet been established. To address this issue we recently developed in collaboration with the group of Prof. Salvatore (Naples, Italy) a cardiomyocyte-specific, conditional DIO3 knockout model. Experiments are currently underway to definitively determine the role of
DIO3 activity in the impairment of TH signaling in post-MI remodeling, at least in mice. The results of these studies will also shed light on the possible relevance of the expression of DIO3 in ischemic heart disease in patients and the therapeutic use of TH [chapter 5]. The outcome of an ongoing phase II trial entitled Thyroid Hormone Replacement Therapy in ST Elevation MI (THiRST) is therefore particularly interesting (Mourouzis et al. 2011) (http://www.ponte-project.eu). Preliminary data of this randomized, double-blind, placebo-controlled trial, which was started by Dr. Iervasi in 2009, suggest that TH treatment after MI is safe and the benefits may be substantial.

**miRNA 208** Therapies using miRNA mimics or antagomiRs are currently under investigation, which includes the TH-regulated miR-208a (van Rooij and Kauppinen 2014). This miRNA, and its closely related homolog miR-208b, are so far the most intriguing TH-regulated miRNAs. These miRNAs are involved in a complex regulatory signaling pathway, which to this date has not been fully resolved. In chapter 1 we discussed that these miRNAs are located within the TH-regulated genes *Myh6* (208a) and *Myh7* (208b). Like their host genes, these miRNAs display a distinct regulation in the mouse heart with miR-208b being the predominantly expressed miRNA during early mouse cardiac development. After birth, following the fetal to adult switch, miR-208a expression rises, while miR-208b levels decrease (Callis et al. 2009). Inhibition of T3 synthesis by exposing mice to PTU resulted in increased *Myh7* expression [see also chapter 2], which could be reversed by T3 supplementation (Callis et al. 2009; van Rooij et al. 2007; van Rooij et al. 2009). MiR-208a is required for the increase in *Myh7* expression during the development of pathological cardiac hypertrophy (Callis et al. 2009; van Rooij et al. 2007). Evidence for this was found in a miR-208a KO mouse model showing reduced fibrosis and cardiac hypertrophy in response to stress (van Rooij et al. 2007). These results were confirmed in a study that applied prolonged therapeutic inhibition of miR-208a, which resulted in reduced *Myh7* expression and improved cardiac function in a rat model of congestive heart failure (Montgomery et al. 2011). The latter observation is consistent with an earlier study in which it was shown that overexpression of miR-208a in a transgenic mouse model resulted in an increase of *Myh7* expression regions within the heart that also developed fibrosis (Callis et al. 2009). This observation is interesting, because cardiac fibrosis occurs in regions containing low levels of T3, and can be reversed by treating animals with TH (Yao and Eghbali 1992; Ghose Roy et al. 2007; H. W. Lee et al. 1998). In other words, overexpression of miR-208a induced a condition equivalent to low T3 signaling. This can be understood by looking at the target of miR-208a, i.e., the mRNA encoding the thyroid hormone receptor associated protein 1 (THRAP1/MED13), THRAP1 is a component of the mediator complex that connects RNA polymerase II with accessory factors at gene promoters with distal enhancers (Grueter et al. 2012; Callis et al. 2009; van Rooij et al. 2007), and mediates TH-dependent transcription (Ito and Roeder 2001). MiR-208a suppresses *Thrap1* expression and consequently suppresses the stimulating effect of T3 on positively regulated genes (e.g. *Myh6*) and suppresses the inhibitory effect of T3 on negatively regulated genes (e.g. *Myh7*). This explains the upregulation of *Myh7* in the miR-208a over-expression studies mentioned above.

The positive effect on cardiac function of blocking miR-208a, as shown by Montgomery et al. (Montgomery et al. 2011), underscores a role of impaired TH signaling in pathological remodeling, be it at the level of a transcription factor or T3 itself. However, many questions remain concerning the interactions of miR-208a and miR-208b, since both are equally capable of suppressing THRAP1 expression (Callis et al. 2009), but one is stimulated by T3 (miR-208a) and one is suppressed (miR-208b). For instance, how is it possible that cardiac stress decreases *Myh6*, while its corresponding miRNA, miR-208a, is required for the increase in *Myh7* expression during remodeling induced by this stress? The answer may lie in the cellular levels of T3.
Together with the availability of TRs, THRAP1 and other co-factors, T3 will to a large extent determine the degree of transcription stimulation or inhibition. For example, like its host gene Myh6, miR-208a is expressed in the healthy rodent heart, but Myh7 expression is suppressed by the prevailing T3 levels. Also in chapter 2 we showed that although miR-208a levels were significantly increased by T3 treatment, this did not increase either Myh7 or miR-208b expression levels. These results seem to be consistent with those of Callis et al. that showed uniform overexpression of miR-208a in a transgenic mouse model, but Myh7 expression only in a subset of stressed cardiomyocytes (Callis et al. 2009). It may be speculated that other factors affecting TH signaling, e.g., DIO3 activity, are involved in this subset of cardiomyocytes, tipping the balance towards impaired TH action.

miRNA 25 A potential candidate for miRNA-based therapy has recently been identified in a study that showed that the upregulated miR-25 in myocardial samples of heart failure patients and animal models of pressure overload (TAC) is a critical repressor of Serca2a (Wahlquist et al. 2014). Reduced expression of SERCA2a protein is generally considered to be a critical aspect of pathological cardiac hypertrophy and heart failure induced by either pressure overload, volume overload or MI. Apart from contributing to the slowing of calcium transients and affecting cardiac contraction, crucial calcium-dependent hypertrophic signal-transduction pathways are also affected by reduced SERCA2a activity and normalizing SERCA2a expression is a prime therapeutic target (Muller and Simonides 2005; Sikkeli et al. 2014; Periasamy and Kalyanasundaram 2008). Indeed, treating TAC mice with anti-miR-25 resulted in increased SERCA2a levels and improved cardiac function (Wahlquist et al. 2014). Expression of SERCA2a is transcriptionally regulated by T3 and it has been suggested that impaired TH-signaling alone could account for the observed SERCA2a reduction seen in heart failure (reviewed by Muller and Simonides 2005). Yet, the recent data would suggest that miR-25 is perhaps a more important independent factor in the suppression of SERCA2a expression. However, re-examination of the miRNA expression analysis presented in chapter 2 showed that T3 significantly reduced miR-25 expression. This implies that T3 may stimulate expression of SERCA2a at both the transcriptional and translational level. The T3-responsiveness of miR-25 would also provide a link between the upregulation of miR-25 and impaired TH signaling in pathological remodeling, underscoring the therapeutic potential of restoring cardiac TH action. Clearly, additional research is needed to substantiate the suggested interaction of this important miRNA and T3 in pathological remodeling. In terms of therapeutic applicability, cardiospecific modulation of a miRNA is perhaps more challenging than increasing TH signaling. MiRNA-therapeutics are potentially taken up by multiple organs. However, catheter-based delivery may be an option to enhance the specificity of the treatment. In a porcine model of ischemia and reperfusion this technique was reported to give a reduction in infarct size and improved cardiac function when compared to systemic delivery (Hinkel et al. 2013).

For both TH and miRNA-based therapies, timing is probably of great importance, and this will also depend on the specific form of cardiac remodeling. For example, it is speculated that the initial reduction of cardiac TH action seen following MI is an adaptive, energy-saving response and that treatment will only have a positive effect during later stages of pathological remodeling. On the other hand, the data presented in chapter 2 suggest that any reduction of cardiac T3 levels will increase pathological signaling by releasing inhibition by specific miRNAs, suggesting that early treatment may be beneficial. In miRNA-based interventions the spatiotemporal differences in miRNA expression need to be taken into account. For example, besides the reported upregulation of miR-25 in human and a mouse model of pressure overload, this miRNA has also been reported to be decreased after aortic constriction in mice (da Costa
Martins et al. 2010; Dirkx et al. 2013). It was suggested that these differences reflect different stages during heart failure development (Wahlquist et al. 2014). The same holds for the different cell types within the failing heart. Where miR-214 expression in cardiomyocytes is essential for post-MI-survival (Aurora et al. 2012), it inhibits angiogenesis in ischemic tissue (van Mil et al. 2012).

The principal aim of this thesis was to gain insight in the relation between miRNAs and TH signaling in the heart. Based on the obtained results and the available literature we conclude that TH-signaling and TH-responsive miRNAs in the healthy and diseased heart may play an important, and previously unknown, role. Although knowledge in the field of heart failure is increasing continuously, translating this knowledge to effective therapies remains an enormous challenge. Based on the data described in this thesis, we advocate more research to elucidate the role of TH in pathological cardiac remodeling and heart failure and to explore the possibilities of increasing cardiac TH signaling as a therapeutic target to suppress disease progression and possibly even restore the failing heart.
Chapter 6

Summary and general discussion
Cardiac remodeling is the heart’s attempt to adapt to the changes in hemodynamic load, whether caused by for example, aortic stenosis, valvular dysfunction, or MI, the subject of this thesis. Several groups have focused attention on the role of TH in the heart, since it became evident that with respect to cardiac function and various aspects of gene expression, the failing and hypothyroid hearts are alike. As part of the re-expressed fetal gene program in response to MI, cardiac-specific induction of DIO3 is thought to account for the local hypothyroid state (Pol et al. 2011; Olivares et al. 2007) [see also chapter 3, and 4]. TH, or more specifically T3, transcriptionally regulates many cardiac genes. Thus, besides the reduction in energy turnover, impairment of T3 signaling leads to changes in gene expression that contribute to contractile dysfunction in pathologic remodeling (Pol, Muller, and Simonides 2010). In addition, recent studies showed an important role for miRNAs in the regulation of cardiac gene expression by virtue of their ability to induce degradation of specific target mRNAs or reduce the efficiency of translation (Thum, Catalucci, and Bauersachs 2008; van Rooij et al. 2006) [chapter 1]. The work described in this thesis was performed to gain more insight in T3 action and the possible involvement of miRNA regulation in the remodeling heart.

**TH-responsive miRNAs suppress pathways involved in pathological cardiac remodeling**

Analysis of miRNA expression levels in hearts of hypothyroid mice that were treated for three days with T3 revealed a miRNA signature of 52 regulated miRNAs, including three known T3-responsive miRNAs [chapter 2]. Pathway analyses predicted that most of the newly identified miRNAs suppress pathways involved in pathological cardiac hypertrophy, with some others enhancing pathways involved in physiological cardiac hypertrophy. The latter observation may be expected in view of the significant hypertrophy induced by the T3 treatment, but the suggested suppression of pathological signaling is remarkable. However, this study has certain limitations that need to be taken into account when considering the results. Firstly, it should be noted that the observed miRNA signature is the result of a relatively rapid shift from low to high T3 levels by three daily injections with supra-physiological doses of T3. We chose for this study design to find those miRNAs that most likely are directly regulated by T3. However, one may question whether these miRNAs will show the reverse response when cardiac T3 levels are gradually decreased, as would be the case during pathological remodeling. Secondly, because our conclusions in this chapter are solely based on *in silico* analyses, it needs to be investigated if modulating the expression of T3-regulated miRNAs that suppress the pathological pathways, like GPCR-signaling and NFAT-signaling, indeed dampen the pathological response in a model of pathological hypertrophy, e.g., TAC and MI.

**Activation of a miRNA cluster located within the DLK1-DIO3 imprinted region**

To study the possible involvement of TH-regulated miRNAs in remodeling following MI, we analyzed the miRNA signature of the post-MI LV one week after surgery [chapter 3]. The post-MI heart has been shown to be hypothyroid (Pol et al. 2011; Olivares et al. 2007) and our analyses indeed showed a differential expression of the known TH-regulated miRNAs miR-208a, 208b and 206, as well as a number of the TH-responsive miRNAs identified in chapter 2. Of the total of 641 known mouse miRNAs analyzed, 4% showed an expression profile of significantly regulated miRNAs similar in both in both the hypothyroid and post-MI heart. However, the effect of MI on most miRNAs showed only a trend. The heterogeneous re-expression of Dio3 following MI in the spared myocardium may provide a possible explanation for this (Pol et al. 2011). Homogenates used in the analyses consequently comprise a mixed population of hypothyroid cardiomyocytes expressing Dio3, and euthyroid cardiomyocytes. Any effect of cellular hypothyroidism on miRNA expression will therefore be ‘diluted’.

In chapter 3 we reported the unexpected upregulation of a miRNA cluster located in the DLK1-DIO3 genomic imprinted region, which coincides with the reported increased
expression of Dio3 (Pol et al. 2011; Olivares et al. 2007). Activation of the DLK1-DIO3 region has been reported for various types of tumors and has been associated with increased proliferative activity and disease progression (Hernández, García, and Obregón 2007; Liu et al. 2010). The concomitant increase in Dio3 activity appears to play a functional role, since low T3 favors proliferation. The miRNA-cluster located within the DLK1-DIO3 genomic imprinted region is the largest currently known miRNA-cluster (Seitz et al. 2004). Pathway analysis predicted a role for many of these miRNAs in signaling pathways like mTOR-, MAPK-, Wnt-, JAK-STAT- and p53-signaling (Benetatos et al. 2013). In recent years, numerous studies have shown the involvement of the DLK1-DIO3 miRNAs in regulating pluripotency. Studies have indicated that the level of stem cell pluripotency was positively correlated with the degree of DLK1-DIO3 activation (Liu et al. 2010; Stadtfeld et al. 2010). It has been suggested that miRNAs from this region target components of the polycomb repressive complex 2 (PRC2), namely HDAC2, RBAP48, and EED. Indeed, expression of the DLK1-DIO3 miRNAs was correlated with reduced expression levels of these three components. Targeting these components might release the methylation at the DLK1-DIO3 region, resulting in a feedback loop to further increase miRNA and mRNA expression from this region. These suggestions were supported by treatment of induced pluripotent stem cells that had silenced DLK1-DIO3. A histone deacetylase inhibitor reactivated the locus and restored full pluripotency.

The post-MI remodeling myocardium is hypothyroid, most likely as a consequence of the re-expression of Dio3, which favors proliferation in regenerative tissues like skeletal muscle (Dentice et al. 2010). However, in spite of the increased expression of pluripotency-related miRNAs, a regenerative process is perhaps initiated in the stressed heart, but obviously not completed. This suggests that in the post-MI myocardium, crucial factors must be missing. These may be factors located in the DLK1-DIO3 region itself. Where others reported upregulation of all aspects of the DLK1-DIO3 genomic region in highly proliferative tumors, including the three coding genes Dlk1, Rtl1 and Dio3 (Benetatos, Voulgaris, and Vartholomatos 2012), we only found Dio3 and the majority of miRNAs to be upregulated in the spared myocardium. Up till now only Dio3 expression has been shown to be crucial for proliferation and regeneration in skeletal muscle (Dentice et al. 2010), whereas Dlk1 expression does not appear to be essential in proliferation (Andersen et al. 2009). However, a role in regeneration of Rtl1, or some of the miRNAs that were not able to reach a threshold required for proliferation, in the post-MI myocardium cannot be ruled out. For example, it has been shown that the transcription factor myocyte enhancer factor 2A (MEF2A) regulates the imprinted noncoding RNA locus within the DLK1-DIO3 region during regeneration in skeletal muscle (Snyder et al. 2013). This factor is also expressed at higher levels during cardiac remodeling and heart failure development. Additional research showed that miR-410 and miR-495, both located within this noncoding region and upregulated after MI [chapter 3], are important factors initiating proliferation of neonatal cardiomyocytes (Clark and Naya 2015). It may be speculated that these miRNAs are insufficiently upregulated in our model and it would be interesting to examine the regenerative activity in myocardium after MI in mice that overexpress these specific miRNAs.

Porrello et al. showed in neonatal mice that a regenerative capacity of differentiated cardiomyocytes does exists in mammals after resection of part of the apex, but that this capacity is lost one week after birth (Porrello, Mahmoud, et al. 2011), coinciding with the postnatal rise of plasma TH levels. Whether this form of neonatal cardiomyocyte proliferation is part of late cardiac development or whether injury is required to initiate the process of myocardiogenesis remains unclear. In any case, evaluation of normal post-natal cardiomyocyte mitosis supports the conclusions drawn by Porello et al. that replication ceases by postnatal day seven (Walsh et al. 2010). In addition, it was shown that upregulation of the miR-15 family within the first week after birth