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MicroRNA 214 is a Potential Regulator of Thyroid Hormone Levels in the Mouse Heart Following Myocardial Infarction, by Targeting the Thyroid-hormone Inactivating Enzyme Deiodinase type III

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Cardiac thyroid-hormone signaling is a critical determinant of cellular metabolism and function in health and disease. A local hypothyroid condition within the failing heart in rodents has been associated with the re-expression of the fetally expressed thyroid-hormone inactivating enzyme deiodinase type III (DIO3). While this enzyme emerges as a common denominator in the development of heart failure, the mechanism underlying its regulation remains largely unclear. In the present study, we investigated the involvement of microRNAs (miRNAs) in the regulation of Dio3 mRNA expression in the remodeling left ventricle of the mouse heart following myocardial infarction (MI). In silico analysis indicated that of the miRNAs that are differentially expressed in the post-MI heart, miR-214 has the highest potential to target Dio3 mRNA. In accordance, a luciferase reporter assay including the full length 3’UTR of mouse Dio3 mRNA, showed a 30% suppression of luciferase activity by miR-214. In the post-MI mouse heart, miR-214 and Dio3 protein were shown to be co-expressed in cardiomyocytes, while time-course analysis revealed that Dio3 mRNA expression precedes miR-214 expression in the post-MI left ventricle. This suggests that a Dio3-induced decrease of T3 levels is involved in the induction of miR-214, which was supported by the finding that cardiac miR-214 expression is down regulated by T3 in mice. In vitro analysis of human DIO3 mRNA furthermore showed that miR-214 is able to suppress both mRNA and protein expression. Dio3 mRNA is a target of miR-214 and the Dio3-dependent stimulation of miR-214 expression in post-MI cardiomyocytes support the involvement of a negative feedback mechanism regulating Dio3 expression.
Myocardial infarction (MI) is one of the major causes of heart failure in Western society (Go et al. 2014). The sudden interruption of blood flow results in cardiomyocyte death by necrosis and subsequent fibrosis of the affected part of the left ventricle (LV). The associated increase in cardiac load of the non-infarcted region of the heart triggers functional and structural remodeling of the spared myocardium to reduce ventricular wall stress and to preserve cardiac output (Sutton and Sharpe 2000; Bernardo et al. 2010). The process of remodeling includes hypertrophy of cardiomyocytes and marked changes in gene expression, including re-expression of part of the fetal gene program. These changes are mediated by a multitude of signal transduction pathways involving transcriptional regulation, as well as non-coding transcripts such as microRNAs (miRNAs) that affect mRNA stability or translation efficiency (Bernardo et al. 2010; Thum et al. 2007; Thum, Catalucci, and Bauersachs 2008; Dirkx, da Costa Martins, and De Windt 2013).

One of the genes that is re-expressed as part of the fetal gene program in various models of cardiac hypertrophy and heart failure is the thyroid hormone inactivating enzyme, deiodinase type III (DIO3) (Trivieri et al. 2006; Pol et al. 2011; Simonides et al. 2008; Olivares et al. 2007; Wassen et al. 2002). Dio3 belongs to a family of 25 so called selenoproteins that require a mRNA stem loop structure located in the 3'UTR region, termed a SECIS-element, for incorporation of a selenocysteine critical for enzymatic activity (reviewed by Berry et al. (Berry 2005). Dio3 converts the active form of thyroid hormone, triiodothyronine (T3), to the inactive metabolite diiodothyronine (T2) (reviewed by Gereben et al. 2008). In both mouse and rat models of ventricular failure, the re-expression of Dio3 in the remodeling left ventricle is associated with a ~50% reduction of tissue T3 levels and a similarly reduced T3 transcriptional activity in cardiomyocytes in vivo (Simonides et al., 2008; Pol et al., 2011). This local impairment of T3 signaling is suggested to play a role in the development of cardiac dysfunction given the fact that T3 is an important regulator of cardiac contractility and metabolism (reviewed by Klein and Ojamaa 2001).

Expression of Dio3 has been shown to be regulated by transforming growth factor β (TGFβ), mitogen-activated protein kinases (MAPK), sonic hedgehog (SHH) and hypoxia-inducible factor-1α (HIF-1α) (Simonides et al. 2008; S. A. Huang et al. 2005; Dentice et al. 2007). Although the signaling cascades involving these factors are all implicated in cardiac remodeling (reviewed by Pol et al. Pol, Muller, and Simonides 2010), none of these have as yet been shown to be involved in the re-expression of Dio3 in the heart. Given the strict spatiotemporal regulation of Dio3 activity during fetal development, such as in the retina and cochlea (Ng et al. 2009; Ng et al. 2010), Dio3 expression is considered a likely candidate for regulation by miRNAs.

MiRNAs are evolutionary conserved small non-coding RNA molecules of approximately 22 nucleotides long that are encoded within the genomes of almost all eukaryotes. In general, miRNAs post-transcriptionally regulate protein synthesis through base pairing with sufficiently complementary sequences in the 3' untranslated region (3'UTR) of target mRNAs (Bartel 2009). Recent studies have shown 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). Although up or down regulation of miRNAs is generally considered to fine-tune gene expression, several studies have provided insight in the critical roles of individual miRNAs in the different processes associated with development and disease, and in particular the progression of heart failure (Aurora et al. 2012; Callis et al. 2009; van Rooij et al. 2006; da Costa Martins et al. 2010).

In this study we therefore investigated whether miRNAs play a role in the regulation of Dio3 expression in the remodeling heart, using the post-MI model in the mouse. In a
previous study using this model, we identified miRNAs that were differentially modulated (Janssen et al. 2013). In silico analysis performed in the present study identified only one of these miRNAs as potentially targeting Dio3. However, this miR-214-3p (hereafter referred to as miR-214) was upregulated in MI, implying suppression of Dio3 expression. Upregulated expression of the highly conserved miR-214 in cardiac hypertrophy and heart failure has been demonstrated in several cardiac miRNA profiling studies in both human and experimental animal models (Janssen et al. 2013; da Costa Martins et al. 2010; van Rooij et al. 2006; van Rooij et al. 2008). It has been shown that elevated expression levels of miR-214 induces hypertrophy (el Azzouzi et al. 2013; van Rooij et al. 2006) by suppressing peroxisome proliferator-activated receptor δ (Pparδ) (el Azzouzi et al. 2013). In contrast to the apparently unfavorable effects of miR-214 in cardiac hypertrophy and heart failure, miR-214 has been shown to protect the heart from ischemic injury in a knock-out model, by suppressing the expression of the sodium calcium exchanger Ncx1 and consequently preventing Ca$^{2+}$ overload of cardiomyocytes and subsequent cell death (Aurora et al. 2012).

Given the observed role of miR-214 in cardiac remodeling we tested its possible relevance for the regulation of Dio3 expression. Our results show that Dio3 is a target of miR-214 and suggest that a negative feedback mechanism exists, in which the upregulation of miR-214 dampens the MI-induced upregulation of Dio3, limiting the reduction of cardiac T3 signaling.
**Materials and methods**

**Prediction of miRNAs targeting Dio3** Putative miRNAs targeting Dio3 were identified by combining the results of different prediction databases: Targetscan v5.2, PicTar v03-2007, mirDB, and MicroCosm v5. Only miRNAs that were called by all four databases were considered for further analysis.

**Luciferase reporter experiments** Approximately \(7.5 \times 10^4\) HEK293 cells were seeded per well in 48-well plates in growth medium (DMEM containing 10% FBS, 1% NEAA, 1% Sodium Pyruvate and 1% P/S). The next day, at 50% confluence, medium was changed to 200 µl DMEM per well. To increase the accuracy of the determination of miR-214 dependent luciferase activity, cells were transfected with 50 ng of a single dual luciferase reporter plasmid containing the Dio3-3′UTR downstream of the Renilla luciferase gene (RLuc). Firefly luciferase activity was used to correct for differences in transfection efficiency. To mimic or inhibit miRNA action, chemically modified double stranded RNA molecules were added. These included Pre-miR-214 (100 nM); a combination of Pre-miR-214 (50 nM) and Anti-miR-214 (50 nM); or Negative control miR#1 (100 nM) (Ambion, Foster City, CA, USA). Both plasmids and miRNAs were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After 6 hours, the transfection medium was replaced by growth medium. Forty-eight hours after the start of transfection, medium was removed and cells were lysed for 15 min in 50 µl lysis buffer (Promega, Madison, WI, USA) and the activity of RLuc and Firefly (FLuc) luciferase activity was assessed using the Dual-Luciferase® Reporter Assay System (Promega, Madison, WI, USA), according to manufacturer’s instructions.

**Mouse model of myocardial infarction** A total of 12 C57Bl/6J mice (Harlan, 10-12 weeks old) of either sex were randomly assigned to the sham-operated group or MI group, weighed and anesthetized. MI was induced by ligation of the left anterior descending (LAD) coronary artery and echocardiography measurements were performed as previously described (de Waard et al. 2007; van den Bos et al. 2005). Briefly, under anesthesia (2.5% isoflurane in a mixture of air and O2), a thoracotomy was performed at the fourth left intercostal space and the LAD was permanently ligated. The occlusion was confirmed by the slight change in color of the anterior wall of the LV downstream of the ligature. Sham-operated mice underwent the same procedure except for the occlusion of the LAD. After 1 week, echocardiography was used to establish the reduced contraction of the LV free wall. LV tissue from sham animals and non-infarcted, i.e., spared LV tissue from MI animals was collected, frozen in liquid nitrogen and stored at \(-80^\circ C\). In addition we used LV tissue (post-MI day 3, 5, 7, 14, 35, or 56) from previous experiments (Pol et al. 2011), which were performed according to above described protocol.

**Mouse model of hyperthyroidism** Left ventricular samples from a previous study were used to assess miR-214 expression (Janssen et al. 2014). Briefly, in this study two groups of six male C57BL/6 mice (age 10-12 weeks, Charles River) were used. Both groups were allowed ad libitum access to food containing propylthiouracil (PTU) (Teklad+0.15% PTU) for 41 days to induce hypothyroidism. Animals were maintained on this diet and six mice were injected intraperitoneally with a supra-physiological dose of 5 µg T3 in 20 µl saline (corresponding to 0.21-0.24 µg T3/g BW) at day 42, 43, and 44, while the remaining six mice were injected with 20 µl saline. Animals were sacrificed at day 45. Left ventricular tissue from hypothyroid and hyperthyroid mice was collected, frozen in liquid nitrogen and stored at \(-80^\circ C\) until analysis.
AntagomiR administration  AntagomiR against miR-214, conjugated to cholesterol to facilitate cellular uptake, was kindly provided by Prof. L. de Windt (University of Maastricht, the Netherlands) and was administered as previously described (el Azzouzi et al. 2013). At day 7 post-MI surgery the formation of an infarct was confirmed using echocardiography. Intraperitoneal injections began at day 7 post MI surgery, with either antagomiR-214 (20 mg/kg/day), or vehicle (100 µl PBS) for two consecutive days. A total of 15 mice were used in this part of the study, including six MI mice that were injected with vehicle and nine MI mice injected with antagomiR-214.

All experiments performed on animals complied with the Guide for Care and Use of Laboratory Animals of the National Institutes of Health (NIH Publication no. 86-23, revised 1996) and were approved by the Institutional Animal Care and Use Committees of VU University Medical Center Amsterdam.

Quantitative PCR and miRNA analysis  Total RNA was isolated from LV tissue of sham, post-MI, hypo- and hyperthyroid mice using mirVana PARIS kit for miRNA (Ambion, Foster City, CA, USA) or TriPure for mRNA (Roche, Basel, Switzerland) and treated with DNasel (Qiagen, Venlo, The Netherlands) to remove remnants of genomic DNA, followed by reverse transreaction with either Cloned AMV First Strand synthesis kit (Invitrogen, Carlsbad, CA, USA) or with miRCURY LNA Universal cDNA synthesis kit II (Exiqon, Vedbæk, Denmark). Expression levels of atrial natriuretic factor (Anf), MHCα (Myh6), MHCβ (Myh7), and Dio3 mRNA were determined by qPCR using specific primers (Myh6 sense primer: 5'- GACCAGCCAATTGATCCG -3'; Myh6 anti-sense primer: 5'- GCCTAGCCAACCCTCCCTTCC -3'; Myh7 sense primer: 5'- CGCTACCAGCCGACCACTCCTAC -3'; Myh7 anti-sense primer: 5'- GTCCACCCCTCCGGAGAC -3'; Anf sense primer: 5'- CGAAGATCCACGTCCGTCCGG -3'; Anf anti-sense primer: 5'- TTCGGTACCAGAGCTCTTGGT -3'; Dio3 sense primer: 5'- CGCTCTCTGTCTGCTCCTAC -3'; Dio3 anti-sense primer: TCTCTCGCTTTACACTTGA -3') and standard cycle parameters on an Applied Biosystems model 7500 (Applied Biosystems, Foster City, CA, USA) with hypoxanthine-guanine-phosphoribosyl-transferase (Hprt sense primer: 5'- TCCCTGCTTGGAGGTTC -3'; Hprt anti-sense primer: 5'- CAGAGGTCCTTTACACTTGA -3') as correction factor. Expression levels of miR-214-3p were analyzed using miRCURY LNA microRNA primers and normalized against U6 snRNA (Exiqon, Vedbæk, Denmark).

In situ hybridization  Before tissue sectioning, all equipment was heat-treated or cleaned with RNAzap (Sigma-Aldrich, St. Louis, MO, USA) to eliminate RNase contamination. Paraformaldehyde-fixed and paraffin-embedded sections (6 µm) were deparaffinized according to the manufacturer’s instructions for in situ hybridization (Exiqon, Vedbæk, Denmark) (Jørgensen et al. 2010). Sections were incubated with 5 µg/ml proteinase-K for 30 min at 37 °C, washed in PBS and immediately dehydrated using solutions with increasing concentration of ethanol. When dry, slides were placed in a specially designed hybridization chamber. To prepare the double-Digoxigenin (DIG) miRCURY LNA microRNA Detection probe (Exiqon, Vedbæk, Denmark), an aliquot was heated to 90 °C for 4 min and diluted to 40 nM in ISH buffer (Exiqon, Vedbæk, Denmark). Approximately 50 µl was applied directly on the dried tissue section. The sealed hybridization chamber was placed in an oven at an optimized temperature of 57 °C for 1 hour. Slides were then placed in a glass jar containing 5xSSC at room temperature. Stringent washes were performed in pre-heated SSC buffers (hybridization temperature, 57 °C) of 5 minutes each: once in 5xSSC, twice in 1xSSC, twice in 0.2xSSC. Next, the slides were placed in 0.2xSSC at room temperature and transferred to a glass jar containing PBS containing 0.1% Tween-20. To prevent nonspecific binding of anti-DIG, sections were blocked using PBS containing 0.1% Tween-20 and 2% goat serum for 15 min at room temperature. Alkaline-phosphatase (AP) conjugated anti-DIG (Roche, Basel, Switzerland) was diluted 1:500 in blocking buffer and incubated for 2 hours.
at room temperature. Slides were washed 3 times with PBS containing 0.1% Tween-20. Alkaline phosphatase substrate (3.4 µl NBT, 3.5 µl BCIP, 2.4 µl 1.25 mM Levamisol, 5 µl 10% Tween-20 in 1 ml substrate solution: 0.1 M Tris pH 9.5, 0.1 M NaCl, 50 mM MgCl₂ (Obernosterer, Martinez, and Alenius 2007)) was applied at room temperature and replaced with fresh solution after 1 hour and incubated overnight. Placing the slides two times in PBS for 5 min stopped the enzymatic reaction. For counterstaining the sections, we applied 100 µl Nuclear Fast Red (Vector Labs, Burlingame, CA, USA) on each section for 1 min and subsequently rinsed the slides for 10 min with running tap water. Before mounting the slides with Entellan™ (Merck Darmstadt, Germany), slides were dehydrated using an increasing gradient of ethanol solutions and twice in xylene for 5 min.

**Histochemistry**  Dio3 immunohistochemistry was performed as previously described (Pol et al. 2011). Briefly, paraformaldehyde-fixed and paraffin-embedded LV tissue sections (4 µm) were deparaffinized and rehydrated and exposed for 20 minutes to 0.02 M HCl. Epitope retrieval was performed by placing the sections at 93 °C for 10 minutes in 10 M citrate buffer (pH 6.0). Sections were incubated with antibody 718 at 1:50 dilution at 37°C for 1 hour and processed with Envision+ reagents (Dako, Glostrup, Denmark).

Polyclonal antibody 718 was raised in rabbit against the synthetic peptide KPEPEVELNSEGEEVP of the N-terminus of human DIO3 (amino acid residues 53-68). This antibody was kindly provided by Prof. D. Salvatore (University of Naples Federico II, Department of Molecular and Clinical Endocrinology and Oncology, Naples, Italy).

**Cell culture and western blotting**  Approximately 1.0x10⁵ COS-7 cells were seeded per well in 12-well plates in DMEM containing 10% FBS, 1% P/S. The next day, at 50-60% confluence, medium was refreshed. Cells were co-transfected with 100-400 ng full length human DIO3 (wtD3) pcDNA3 vector (Kuiper, Klootwijk, and Visser 2003). As a control a mutant DIO3 expression vector was used which results in the replacement of the selenocysteine residue in the active center of the enzyme with cysteine (CysD3). Mimic-214-3p or negative miRNA control #1 was co-transfected at a final concentration of 500 nM (Ambion, Foster City, CA, USA). To isolate RNA and protein, medium was removed after 24 hours, and cells were washed with PBS before being lysed with 50 µl lysis buffer (mirVana PARIS kit, Ambion, Foster City, CA, USA).

COS-7 cell lysates were separated on 15% SDS-PAGE gels and transferred to PVDF membrane by a semi-dry blotting system (Bio-Rad, Hercules, CA, USA). The membranes were cut in two at the 25 kDa weight marker and blocked with 5% (w/v) BSA in TBS containing 0.1% Tween (TBS-T) overnight at 4 °C. Subsequently, the part of the membrane containing proteins larger than 25 kDa was incubated with antibody 677 (1:300), and the part of the membrane containing proteins smaller than 25 kDa with antibody 675 (1:1000) in 5% BSA in TBS-T for 2 hours at room temperature. Membranes were washed 3 times for 10 min with TBS-T, followed by incubation with horseradish-peroxidase labeled goat anti-rabbit IgG in TBS-T (1:10000) for 1 hour at room temperature and 3 times for 10 min with TBS-T. Bands were visualized using chemiluminescence (Amersham, Little Chalfont, UK) and scanned by LAS-3000 (Fujifilm Life Science, Stamford, CT, USA). Band intensity was quantified using Aida 4.21 (Raytest, Straubenhardt, Germany) and gel loading normalized to α-actinin (Sigma-Aldrich, St. Louis, MO, USA).

Polyclonal antibody 677 was raised in rabbit against the synthetic peptide RYDEQL-HGARPRRV of the C-terminus of human DIO3 (amino acid residues 265-278) (Kuiper, Klootwijk, and Visser 2003). Polyclonal antibody 675 was raised in rabbit against the synthetic peptide RRGKPEPEVELNS of the N-terminus of human DIO3 (amino acid residues 50-62). Both constructs and primary antibodies were kindly provided by...
Prof. T. Visser (Erasmus Medical Center, Rotterdam, The Netherlands). To correct for variations in transfection efficiency, DIO3 mRNA and protein levels were normalized to the mRNA level of the neomycin-resistance-gene, expressed from the DIO3 pcDNA3 vectors.

**Dio3 activity**  Dio3 activity in LV homogenates was determined as described previously (Kuiper, Klootwijk, and Visser 2003) with minor modifications. Reaction mixtures contained approximately 200,000 cpm of outer-ring-labeled T3 (3'I25I T3) and 0.25 mg protein in a final volume of 0.05 ml 0.1 M phosphate buffer (pH 7.2) containing 2 mM EDTA and 10 mM DTT. Mixtures were incubated for 60 min at 37 °C and the reaction was stopped by addition of 0.05 ml ice-cold ethanol. After centrifugation, 0.075 ml of the supernatant was mixed with an equal volume of 0.02 M ammonium acetate (pH 4.0), and 0.1 ml of the mixture was applied to a 250x4.6 mm Symmetry C18 column connected to an Alliance HPLC system (Waters Chromatography Division, Millipore Corp., Milford, MA, USA) and eluted with a 15-min linear gradient of acetonitrile (28-42%) in 0.02 M ammonium acetate (pH 4) at a flow rate of 1.2 ml/min. Radioactivity in the eluate was monitored online using a Radiomatic A-500 flow scintillation detector (Packard, Meriden, CT, USA) and product formation by the inner-ring deiodination activity, i.e., (3'I25I T2) was quantified.

**Statistics**  Data analysis was performed using Graphpad Prism version 6 (Graphpad, San Diego, CA, USA). Statistical significance was tested using a two-tailed Student t-test and accepted at p < 0.05.
In silico analyses: Dio3 is a bona fide target of miR-214

To identify potential miRNAs involved in regulating Dio3 expression in the remodeling LV, we performed in silico analysis using four different prediction databases. MiR-214 was identified as having a high potential to target the 3’UTR of Dio3. A site complementary to the seed region of miR-214, which is highly conserved among species, was found to be located in the selenocysteine insertion sequence (SECIS)-element, which is a secondary RNA structure [Fig. 1].

MiR-214 expression is increased in the remodeling LV of the post-MI heart

In a previous study we identified miR-214 as a differentially regulated miRNA in the remodeling LV of the post-MI mouse heart compared to the LV of sham operated mice, using TaqMan Megaplex array analysis of all 641 mouse miRNAs known at the time. Here we validated the increased expression level of miR-214 in the post-MI heart using quantitative RT PCR analysis with U6 snRNA as correction factor, showing a significant threefold increase in miR-214 expression in the LV tissue 7 days post-MI surgery compared to sham operated mice [Fig. 2]. Previous studies conducted by our group already established a ~6 fold increased expression of Dio3 mRNA using the same model (Pol et al. 2011; Janssen et al. 2013).

Validation of Dio3 as a target of miR-214

To investigate whether miR-214 targets Dio3, we performed in vitro luciferase reporter experiments. A DNA-construct which contained the mouse Dio3 3’UTR inserted downstream of a luciferase reporter gene was transiently transfected into HEK293 cells. The results show a 30% reduction in Renilla luciferase activity when co-transfecting the reporter construct for 24 hours with Pre-miR-214, relative to the ‘no-miR’. Compared to the condition with a control miRNA, this reduction was 22%. The addition of Anti-miR-214 to the transfection mix containing miR-214 abolished the repression [Fig. 3]. These luciferase reporter experiments support the in silico prediction that binding of miR-214 to the Dio3 3’UTR results in repression of Dio3 protein expression.

Analysis of the role of miR-214 in the translation of DIO3 in transfected COS cells and the involvement of the SECIS element

Analysis of the 3’UTR of Dio3 showed the presence of a highly conserved miR-214 target site in the SECIS element [Fig. 1], which is present in both mouse and human DIO3. Since the SECIS element is essential for the incorporation of selenocysteine in DIO3, it led us to the question whether miR-214 might also interfere with DIO3 translation at this level. The kind gift by prof. T. Visser of a DNA construct containing the full length human DIO3 enabled us to investigate this possible interference in transfection experiments using cultured COS-7 cells.

Several studies showed that manipulation of the SECIS element results in increased levels of truncated DIO3. Therefore, COS7-cells transfected with the plasmid containing the full length human DIO3 (wtD3) together with mimic-214 or negative control miR-1 were analyzed for full length (36 kDa) and truncated (18 kDa) DIO3 protein levels using western blotting. Expression levels were corrected using neomycin mRNA, transcribed from the same plasmid, as a control for transfection efficiency.

The specificity of Ab 677 and Ab 675 for the full length and the truncated DIO3, respectively, was validated by transfecting COS7 cells with increasing amounts of wtD3 plasmid DNA [Fig. 4a]. COS-7 cells transfected with expression vector encoding CysD3 (which exclusively yields full length protein) confirmed the antibody specificity of Ab 675 against truncated DIO3 [Fig. 4b]. Co-transfecting the wtD3 plasmid with mimic miR-214 caused a ~13% reduction in mRNA expression when compared to cells co-transfected with negative control miR-1 [Fig. 4c]. Western blot analysis showed besides significantly reduced full-length DIO3 protein levels (~40%) [Fig. 4c], reduced truncated DIO3 protein

Results
Figure 1  Conserved miR-214 target site in the 3’UTR of Dio3. In silico analysis predicts a target sequence for miR-214 in the stem of SECIS element of the 3’UTR of Dio3 in mice, which is highly conserved among species. (Source: Targetscan and microRNA.org).

Figure 2  Increased miR-214 expression in the post-MI LV. Mir-214 expression levels were determined by quantitative RT PCR analysis in remote LV tissue from sham and MI animals isolated at day 7 post surgery. U6 snRNA expression levels were used as correction factor. Values are means ± SEM, n=6, normalized to the sham level, * p < 0.05.

Figure 3  Dio3 is targeted by miR-214. A dual luciferase construct containing the complete Dio3 3’UTR downstream of the Renilla luciferase gene was used to analyze the interaction between miR-214 and the 3’UTR of Dio3. Renilla luciferase activity (RLuc) is expressed relative to Firefly luciferase activity (FLuc), correcting for differences in transfection efficiency. Four independent transfections were performed in duplicate. No-miR: control without co-transfection; ctrl-miR: + negative control/scrambled miR-1; P214: + Pre-miR-214; PA214: + Pre-miR-214 and Anti-miR-214. Values are means ± SEM, n=4, normalized to the RLuc/Fluc ratio in the no-miR group, * p < 0.05.
Figure 4 Western blot analysis of DIO3 expression in vitro. [a] Transfection of COS-7 cells with increasing amounts of wtD3 plasmid confirmed the specificity of Ab 677 for full length DIO3 (36 kDa) and of Ab 675 for truncated DIO3 protein. Equal amounts of protein were loaded. [b] COS-7 cells transfected with the CysD3 plasmid confirmed the expression of the truncated isoform of DIO3 in COS-7 cells transfected with the wtD3 plasmid, which was detected with Ab 675 (18 kDa) (lower blot; the upper blot is stained using Ab 677). [c] COS-7 cells were transfected with 100 ng of the wtD3 plasmid containing full length human DIO3 (together with either mimic-214 (DIO3+214) or Negative control miR-1 (DIO3-nc#1) at a final concentration of 500 nM. Addition of the negative control miR-1 did not significantly affect transfection efficiency (results not shown). Expression levels of the neomycin resistance gene were analyzed using qPCR and used to normalize transfection efficiency. Values are means ± SEM, normalized to the value of the parameter in the DIO3-nc#1 group, * p < 0.05.
levels (~50%) [Fig. 4c]. However, the ratio of 36 over 18 kDa proteins remained unchanged [Supplemental Figure S1]. This suggests that binding of miR-214 to the DIO3 3’UTR does not interfere with selenocysteine incorporation. The results indicate that binding of miR-214 results in a decreased DIO3 mRNA stability, and although the reduction of DIO3 protein levels is larger than that of DIO3 mRNA, it cannot be inferred from these data that DIO3 translation efficiency is also affected by miR-214.

Dio3 and miR-214 are co-expressed in adult cardiomyocytes  In a previous study, a heterogeneous, cardiomyocyte specific expression pattern of Dio3 was observed in the post-MI LV (Pol et al. 2011). Based on the finding that miR-214 targets Dio3, we expected cardiomyocytes expressing a high level of Dio3 protein to have a low expression level of miR-214, and vice versa. Therefore, we performed miR-214 in situ hybridization and Dio3 immunohistochemistry on serial sections of post-MI LV tissue. A heterogeneous Dio3 expression pattern was again observed [Fig. 5]. Surprisingly, miR-214 in situ hybridization revealed a similar heterogeneous pattern, with the majority of miR-214 positive cardiomyocytes co-expressing Dio3 [Fig. 5]. This suggests either that Dio3 and 214-miRNA are coordinately, but independently upregulated, or that the expression of both is mechanistically linked.

Dio3 expression precedes miR-214 expression in the post-MI LV  Previous work conducted by our group revealed a time-dependent increase in both Dio3 expression and activity in the post-MI heart compared to sham animals (Pol et al. 2011). To further explore the possible interaction between Dio3 and miR-214 expression, we analyzed miR-214 expression at 3, 5, 7, 14, 28 and 56 days post-MI surgery in the same set of LV samples that was previously used for analysis of Dio3 mRNA expression and Dio3 activity (Pol et al. 2011). Figure 5 shows that miR-214 expression levels did not increase significantly until day 5 following MI compared to sham operated mice, and reached a plateau at post-MI day 7, which was maintained for at least 56 days without significant changes. Time-course analysis showed that Dio3 mRNA expression and Dio3 activity were already elevated at day 3 post-MI. Both Dio3 mRNA and Dio3 activity subsequently decrease towards a steady elevated level 56 days post-MI compared to sham operated mice (Pol et al. 2011) [Fig. 6a and b, respectively]. These data indicate that the increase in Dio3 mRNA expression and Dio3 activity precede miR-214 induction, suggesting that any mechanistic link between the two involves an effect of Dio3 activity on the expression of miR-214.

T3 decreases cardiac miR-214 expression  The suggested regulation of miR-214 expression by increased Dio3 expression would most likely be mediated by a local reduction in T3 levels induced by Dio3. In a previous study, we demonstrated that reduced T3 levels and reduced transcriptional activity of T3 accompany the Dio3 induction in the post-MI heart. Therefore, we examined the effect of T3 on miR-214 expression in LV tissue of hypothyroid (-T3) and hypothyroid mice treated with T3 for three days (+T3) mice. Exposure of the heart to high systemic levels of T3 resulted in a 50% reduction of miR-214 expression in the LV compared to the LV of hypothyroid mice [Fig. 7]. These findings suggest that reduced T3 levels may play a role in a negative feedback mechanism regulating Dio3 expression that involves miR-214.

Effects of antagomiR-214 in the post-MI heart  Since miR-214 was shown to be able to target mouse Dio3 in vitro, we investigated this interaction in vivo in a vehicle controlled pilot study using antagomiR-214 [Fig. 8a]. Although the antagomiR treatment resulted in a 98% reduction in miR-214 levels at 14 days after MI surgery [Fig. 8b], Dio3 mRNA levels remained unchanged after antagomiR-214 treatment when compared to untreated MI mice [Fig. 8c]. Furthermore, Dio3 activity was not changed upon antagomiR-214
treatment [Fig. 8d]. Expression analysis of the marker for cardiac hypertrophy \textit{Anf} and the TH sensitive genes \textit{Myh6}, and \textit{Myh7} also showed no differences between the MI-mice treated with antagomiR-214 and the MI-mice treated with vehicle. We additionally analyzed the expression levels of \textit{Ncx1}, a known target of miR-214 in the ischemic mouse heart (Aurora et al. 2012), but no significant difference was observed after antagomiR-214 treatment [Fig. 8c].

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\caption{\textbf{Fig. 5} Dio3 protein and miR-214 are co-expressed. Representative images of immunohistochemical staining for Dio3 [a] and miR-214 \textit{in situ} hybridization [b] in sequential sections of LV tissue 7 days post-MI. The indicated area shows a group of cardiomyocytes expressing both Dio3 protein and miR-214, with adjoining cells negative for both. Bar = 100 \mu M.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure6.png}
\caption{\textbf{Fig. 6} Time course of miR-214 expression, Dio3 mRNA and Dio3 activity after MI. For the sake of clarity, the miR-214 data are shown in separate panels in combination with Dio3 mRNA expression [a] and Dio3 activity [b]. The different parameters are expressed relative to their basal expression level determined in sham-operated mice at day 7. Dio3 mRNA and Dio3 activity data from this set of samples were published in part before (Pol et al. 2011). Values are means ± SEM, miR-214 (n = 5), Dio3 mRNA (n\textsubscript{3d} = 7, n\textsubscript{5d} = 6, n\textsubscript{16d} = 16, n\textsubscript{28d} = 10, n\textsubscript{56d} = 16) and Dio3 activity (n\textsubscript{3d} = 8, n\textsubscript{5d} = 9, n\textsubscript{16d} = 16, n\textsubscript{28d} = 6, n\textsubscript{56d} = 6).}
\end{figure}
Figure 7 Reduced miR-214 levels due to T3 treatment. Total RNA isolated from the LV of hypothyroid (-T3) and hypothyroid mice treated with T3 for three days (+T3) mice was analyzed by qPCR [a] using U6 snRNA as correction factor for miR-214, and [b] Hprt as correction factor for Dio3. Values are means ± SEM, n=6, normalized to the −T3 values, * p < 0.05.

Figure 8 In vivo silencing of miR-214 in a mouse model of post-MI. [a] Design of the vehicle controlled antagomiR-214 treatment pilot. One week post-MI surgery, the extent of the infarct was established by echocardiography before mice were injected IP with either 20 mg/kg/day antagomiR-214 or vehicle on two consecutive days. At 14 days after MI-surgery (6 days after the second antagomiR-214 injection), LV tissue from both groups was collected. [b] To verify the silencing efficiency of antagomiR-214, qPCR was performed. Values are means ± SEM, normalized to the MI value, MI: n=6, MI-A214: n=7, * p< 0.05 vs sham. [c] AntagomiR-214 treatment did not affect the mRNA expression levels of Dio3, Anf, Myh6 or Myh7 in the post-MI heart. Also the mRNA expression level of Ncx1, which has been validated as a positive target for miR-214, was unaltered. Values are means ± SEM, normalized to the expression levels measured in mouse LV tissue 14 days post-sham surgery. Sham: n=5, MI: n=6, MI-A214: n=7, * p< 0.05 vs sham. [d] AntagomiR-214 treatment did not affect Dio3 activity in the post-MI heart. Values are means ± SEM, normalized to the expression levels measured in mouse LV tissue 14 days post-sham surgery. Sham: n=6, MI: n=6, MI-A214: n=7, * p< 0.05 vs sham.
Several lines of evidence support the involvement of reduced TH-signaling in LV remodeling (Danzi and Klein 2002; Klein and Qjamaa 2001; Dillmann 2010). The increased expression of the T3-degrading enzyme Dio3 in the failing heart provides the most likely explanation for the reduced TH-signaling and has emerged as a potential common denominator in the development of heart failure (Pol et al. 2011; Wassen et al. 2002; Simonides et al. 2008; Trivieri et al. 2006; Olivares et al. 2007). However, the mechanism underlying the regulation of Dio3 expression in the heart remains largely unclear. Since Dio3 expression during development is known to be very precisely regulated in a spatiotemporal manner, miRNAs may be involved, as these regulators are known to play a role in fine-tuning of gene expression. The aim of the present study was to explore the possible involvement of miRNAs in the regulation of Dio3 in the remodeling LV of the mouse after myocardial infarction. We show that Dio3 mRNA is a target of miR-214, and that this miRNA may play a role in a negative feedback mechanism regulating Dio3 expression in the post-MI heart, thereby limiting the decrease of cardiac T3 levels.

In silico analysis revealed that of all miRNAs that were shown to be differentially regulated in the post-MI mouse heart (Janssen et al. 2013), miR-214 was predicted to have the highest potential to target Dio3. This miRNA is suggested to play a role in the process of hypertrophy and heart failure in various models (el Azzouzi et al. 2013; van Rooij et al. 2006; Aurora et al. 2012). Sequence analysis of the mouse Dio3 3’UTR predicted the presence a miR-214 target site in the SECIS element, which was found to be highly conserved among species. Transfection experiments using a construct with a Renilla luciferase reporter gene containing the mouse Dio3 3’UTR demonstrated functional interaction of miR-214 with Dio3 mRNA. The observed 30% reduction of reporter expression is comparable to the in vitro effects of miR-199b and miR-214, targeting Dyrk1a and Ncx1 respectively, which have been shown to modify cardiac performance (da Costa Martins et al. 2010; Aurora et al. 2012).

An interesting feature of the conserved miR-214 target site in the 3’UTR of Dio3 is that it is located within the SECIS element, which is a secondary, double stranded RNA structure. The SECIS element is responsible for the incorporation of selenocysteine (Sec) encoded by a UGA codon, which otherwise acts as a STOP codon (Low 1996). Impairment of the incorporation of Sec consequently results in a truncated form of DIO3 without deiodinase activity (Kuiper, Klootwijk, and Visser 2003; D Salvatore et al. 1995; Latrèche et al. 2009). We hypothesized that interference of miR-214 with the SECIS element of DIO3 would result in increased levels of the truncated protein, and this was tested by analyzing the protein expression of full length human DIO3 in COS-7 cells co-transfected with miR-214 using Ab 677 to detect the full length 36 kDa DIO3 protein and Ab 675 to detect the 18 kDa truncated form of DIO3. Although miR-214 significantly affected DIO3 protein expression, the levels of both full-length and truncated protein were equally reduced, indicating that miR-214 does not interfere with the insertion of selenocysteine. However, the substantially greater relative reduction of DIO3 protein expression compared to the effect on the DIO3 mRNA level does suggest an additional effect of miR-214 on translation efficiency. Further research is needed to elucidate the mechanism of interaction of miR-214 in regulating DIO3 expression.

Having confirmed the suppressive effect of miR-214 on Dio3 expression, 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; Janssen et al. 2013). To explore the possible interplay between miR-214 and Dio3, their expression in cardiomyocytes was studied in sections of LV tissue. Immunohistochemical analysis of the post-MI LV confirmed the previously found heterogeneous expression of Dio3 protein (Pol et al. 2011). Also miR-214 exhibited a heterogeneous expression pattern, and analysis of serial sections indicated that Dio3 and miR-214...
were co-expressed in cardiomyocytes, suggesting either that Dio3 and miR-214 are independently upregulated, or that the expression of miR-214 and Dio3 expression is mechanistically linked. The latter option was supported by comparison of the temporal changes in miR-214 expression in the remodeling LV from the onset of MI up to 56 days later, with those of Dio3 mRNA and Dio3 activity as previously determined in the same samples. This showed that the increase of Dio3 mRNA levels and Dio3 activity (data from (Pol et al. 2011)) preceded the increase in miR-214 expression. While Dio3 mRNA levels and Dio3 activity were already increased to high levels at day 3-5 post-MI, miR-214 expression was still only modestly elevated. Furthermore, the expression of miR-214 expression reached a maximum level at day 7 post-MI, by which time the Dio3 mRNA and Dio3 activity levels appeared to go down, but still remained elevated compared to sham. The expression of miR-214 remained however unchanged up to at least 56 days post-MI. The increase of miR-214 over time is in line with previous results in a mouse MI model (van Rooij et al. 2008), showing increasing miR-214 expression levels over time in remote myocardium (van Rooij et al. 2008).

These data suggest a mechanistic link between miR-214 and Dio3 expression, which could involve the Dio3-mediated reduction of cardiac T3 levels. This implies that expression of miR-214 is negatively regulated by T3, and this was confirmed by the finding that treatment of hypothyroid mice with T3 markedly suppressed miR-214 expression levels in the LV. Dio3 mRNA expression showed a reciprocal response, being higher in the LV of T3-treated mice compared to the LV of hypothyroid mice. This is in line with previous findings of stimulation of Dio3 expression by high levels of T3 (Deng et al. 2014; Sabatino et al. 2015). These studies provided evidence for a T3-dependent 3-fold induction of Dio3 expression after 24 hours. Although suggestive of a direct transcriptional effect of T3, the mechanism of stimulation remained unclear. Our data suggest that in the post-MI heart, Dio3 expression may at least be additionally modulated by T3 via changes in miR-214 expression. Taken together, the result of the present study support the involvement of miR-214 in a negative feedback mechanism regulating Dio3 expression. The proposed sequence of events is depicted in Figure 9. In the post-MI heart, Dio3 expression is induced, possibly as part of the fetal gene program. This results in an increase in the local conversion of T3 to the inactive metabolite T2. We hypothesize that the documented local decrease of T3 levels stimulates the expression of miR-214, which dampens Dio3 expression. The relevance of this mechanism lies in the prevention of an excessive decrease of cardiac T3 levels and the associated changes in expression of genes that are critical for cardiac function (Klein and Ojamaa 2001). MiR-214 expression is likely to be regulated by other mechanisms associated with the remodeling process in addition to the decreased T3 levels. It was shown that Hif-1α, which is a key determinant of progression in the remodeling LV (S. H. Lee et al. 2000), activates the locus dinamin 3 opposite site (Dnm3os) harboring miR-214 (el Azzouzi et al. 2013). Upregulation of HIF-1α after MI is generally observed in the infarct or peri-infarct zone, but HIF-1α-dependent transcription activity was found in the remote LV at day 5 post-MI (C. Pol, unpublished results), which may contribute to miR-214 upregulation.

Recent studies using a miR-214 knockout mouse model showed that in the absence of miR-214, mortality after MI surgery was increased. In the same study, it was demonstrated that miR-214 is involved in preserving cardiac function after ischemic injury by repressing Ncx1, thereby controlling Ca²⁺ homeostasis and increasing survival (Aurora et al. 2012). In contrast, an unfavorable role of miR-214 was observed in a model of chronic cardiac overload induced by aortic constriction. Here, increased miR-214 levels repressed cardiac PPARδ expression and impaired mitochondrial fatty acid oxidation. AntagomiR-214 administration was shown to reverse cardiac hypertrophy and to improve cardiac function by releasing the suppression of miR-214 on PPARδ (el Azzouzi et al. 2013). Pilot data obtained using LV tissue from the latter study (kindly
provided by prof. dr. L. de Windt) showed that Dio3 expression was upregulated [not significant, see Supplemental Figure S2].

However, in contrast to the studies mentioned above, we did not observe changes in function of the post-MI heart in antagomiR-214 treated mice compared to untreated mice, in spite of the almost complete abolishment of miR-214 expression following antagomiR-214 treatment. Furthermore, antagomiR treatment did not result in changes in Dio3 mRNA expression, Dio3 activity, nor in the expression of TH-responsive genes Myh6 and Myh7. Also the expression of Ncx1, which was shown to be a miR-214 target by el Azzouzi et al, was not affected by antagomiR-214 administration in our study.

The absence of effects of antagomiR-214 treatment in the present study may result from differences in method used to abolish miR-214 (gene knockout versus treatment with antagomiR) and in the onset and duration of the miR-214 inhibition compared to other studies describing effects of miR-214 inhibition by antagomiR treatment. Aurora et al. (Aurora et al. 2012) used a knock-out model of miR-214 instead of antagomiR against miR-214. Similar to our study, Azzouzi et al. used an antagomiR protocol to inhibit miR-214 (el Azzouzi et al. 2013), but a difference compared to the present study is that antagomiR-214 was injected just after constricting the aorta and an additional injection was given 3 weeks later. These studies suggest that effects of miR-214 can only be blocked when miR-214 inhibition is applied in an early phase of MI-induced cardiac stress. In the present study, antagomiR-214 was injected one week post MI surgery, which may have been too late to inhibit the actions of miR-214 effectively. An almost complete abolishment of miR-214 levels was observed at 14 days after antagomiR treatment, but the time course of this reduction is unknown and the reduction of miR-214 may have occurred too late to modulate the expression of its targets at the time of analysis. Taken together, the results of this pilot study are not conclusive, and further experiments, in which the antagomiR is injected at earlier time points, are needed to evaluate the role of miR-214 in the regulation of Dio3 in vivo.

In conclusion, this study firstly shows that miR-214 is able to target both the human and the mouse 3’UTR of DIO3, affecting mRNA and protein expression. Secondly, the results indicate a novel negative feedback mechanism regulating Dio3 expression in the post-MI mouse heart, in which a Dio3-mediated decrease of T3 levels results in increased expression miR-214, which subsequently reduces Dio3 expression. This mechanism may therefore be aimed at restoring, or limiting the reduction of T3 levels in the post-MI heart (Fig. 9).

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