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miR-29a and miR-30c negatively regulate DNMT3a in cardiac ischemic tissues: implications for cardiac remodelling.

Abstract
Recent evidences indicate that epigenetic changes play an important role in the transcriptional reprogramming of gene expression that characterizes cardiac hypertrophy and failure and may dictate response to therapy. Several data demonstrate that microRNAs (miRNAs) play critical roles both in normal cardiac function and under pathological conditions. Here we assessed, in *in vivo* rat models of myocardial infarction (MI) and ischemia-reperfusion (IR), the relationship between two miRNAs (miR-29a and miR-30c) and *de novo* methyltransferase (DNMT3a) which, altering the chromatin accessibility for transcription factors, deeply impacts gene expression. We showed that the levels of members of miR-29 and miR-30 families were down regulated in ischemic tissues whilst the protein levels of DNMT3a were increased, such a relation was not present in healthy tissues. Furthermore, by an *in vitro* assay, we demonstrated that both miRNAs are able to down regulate DNMT3a by directly interacting with DNMT3a 3'UTR and that miR-29a or miR-30c overexpression in the cardiac HL1 cell line causes decrease of DNMT3a enzyme both at the mRNA and protein levels. Our data, besides confirming the down regulation of the miR-29a and miR-30c in infarcted tissues, envisage a cross-talk between microRNAs and chromatin modifying enzymes suggesting a new mechanism that might generate the alterations of DNA methylation often observed in myocardial pathophysiology.

Keywords
microRNAs • DNMT3a • DNA methylation • myocardial infarction

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1. Introduction

In response to acute and chronic stresses, heart frequently undergoes a remodelling process consisting in the reprogramming of cardiac gene expression and the activation of "fetal" cardiac genes, which encode proteins involved in contraction, calcium handling, and metabolism [1-4]. The activation of this pathological gene program weakens cardiac performance [1-4]. Although gene expression changes can be explained by the assembling of cardiac transcription factors at gene promoters during myocardial stress, recent data indicate that epigenomic changes could play an important role in the reprogramming of cardiac gene expression. Epigenomes are comprised, in part, of all genome-wide chromatin modifications including DNA methylation and histone modifications which do not change the underlying genetic sequences but regulate how the underlying genes are expressed. Chromatin modifications are particularly labile, providing a mechanism for organisms to respond and adapt to environmental cues.

DNA methylation in mammals occurs almost exclusively within 5'-cytosine-guanine-3' dinucleotides (CpGs), although CpNpG methylation has also been detected [5]. It is typically associated with gene silencing by affecting the binding of methylation-sensitive DNA binding proteins and/or by interacting with various modifications of histone proteins that alter DNA accessibility to promoters [6]. Patterns of DNA methylation are established by the *de novo* methyltransferases DNMT3a and DNMT3b during development and they are maintained through mitosis primarily by DNMT1 enzyme which, associated with Proliferating Cell Nuclear Antigen (PCNA) and the replication foci, acts on hemi-methylated DNA following DNA replication [7]. The perpetuation of the DNA methylation state in the new formed cells generated the conventional view that DNA methylation is a stable and reliable epigenetic mark.

More recently, the discovery of an active DNA demethylation mechanism [8,9] and the observation that dynamic changes in DNA methylation status can also occur in differentiated, non dividing cells and that variation in DNA methylation may be caused by diet, drugs, chemical aging and other stochastic events [10-13], suggested that DNA methylation changes could be an adaptive response of cells to extracellular changes.

By definition, the most reliable indicators of adaptive changes running in cells, tissues and organs are microRNAs (miRNAs). MicroRNAs are short (~20 bp) RNA molecules that act as negative

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regulators of gene expression at a post-transcriptional level. Numerous studies have revealed that alterations in the spectrum of miRNAs are correlated with various cardiovascular conditions, such as myocardial infarction, hypertrophy, cardiomyopathy and arrhythmias [14-16]. Members of miR-29 and miR-30 families have been reported as highly down–regulated in experimental rodent models and human heart failure [17]. Moreover, miR-29 was already shown to target DNMT3a and 3b in different types of tumors [18-20]. In this work we exploited whether miR-29 and miR-30, identified by *in silico* analysis as putatively targeting DNMT3a, and the DNMT3a are associated in cardiac ischemic tissues. We discovered that, i) the expression of miR-29 and miR-30 is inversely correlated with the expression of DNMT3a in different cardiac regions of rat models of both, myocardial infarction (MI) and ischemia/reperfusion (I/R); ii) in cardiac cells, DNMT3a is target of miR-29a and miR-30c.

The possibility that the upregulation of miR-29 and miR-30 with the consequent downregulation of DNMT3a could be a novel mechanism through which cardiac cells adapt themselves to environmental changes will be discussed.

2. Results

2.1 MiR-29 and miR-30 expression is reduced in infarcted tissues

We used several different alghorithms to look for miRNAs both modulated in infarcted tissues and targeting DNMTs. We identified several members of miR-29 family, already shown to target DNMT3a and 3b in different kind of tumors [18-20] and members of miR-30 family (Figure 1A). MicroRNAs members of both families are known to be strongly down-regulated in infarcted tissues [16,17]. To test whether the expression of miR-29 and miR-30 is associated to that of DNA methyltransferase in infarcted tissues, we induced myocardial infarction (MI) and ischemia-reperfusion (IR) by, respectively, stable and temporary occlusion of the left anterior descendant (LAD) coronary artery of rat (Figure 1B). First of all we quantified by Q-RT PCR, miRNA expression in the LAD and non-LAD territories of MI rat model and, for comparison, in the corresponding cardiac areas of sham-operated animals. Our data showed a strong down-regulation of both miR-29a and miR-30c in IZ and BZ areas 3 days and 14 days after MI (Figure 2A). We broadened our analysis to cardiac tissues of IR rats: although in IRZ areas miR-29a and miR-30c seemed less expressed on average compared to the sham animals, the extent of down-regulation did not reach any significant level, with the exception of miR-29a 3 days after operation (Figure 2B). The expression of miR-29a and miR-30c did not appear significantly changed in remote zone (RZ) of both MI and IR rat models. The only exception was miR-30c expression that, two weeks after operation, resulted significantly higher in RZ of rats with permanent LAD occlusion compared with sham-operated animals (Figure 2A).

Other members of these two miRNA families (miR-29b and c and miR-30e) were quantified in MI and IR rat models and showed the same trend in the modulation (not shown).

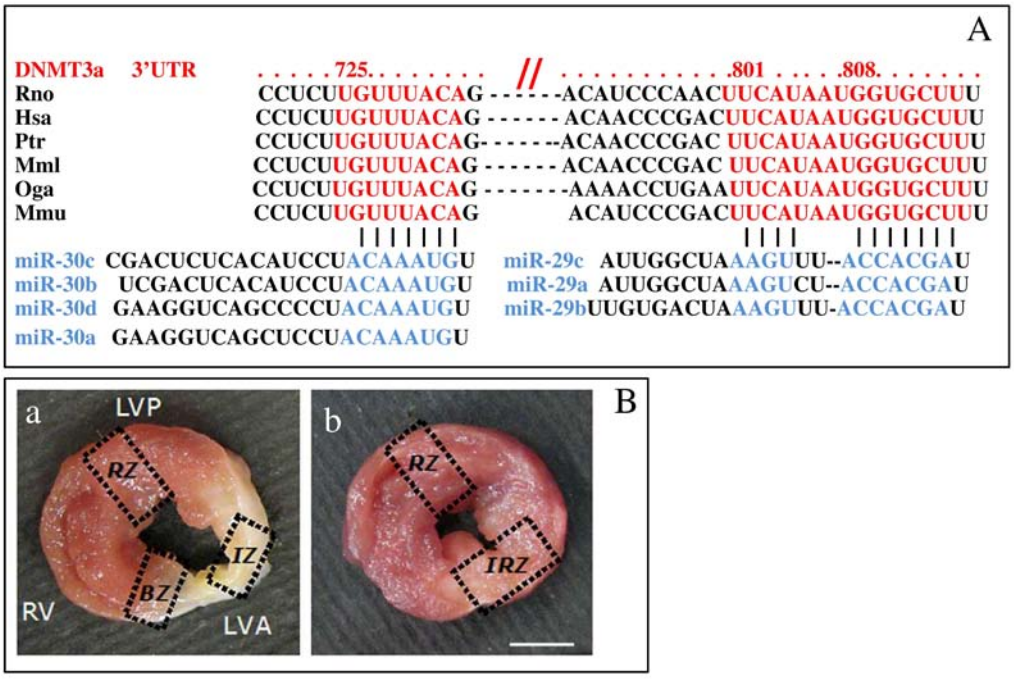

Figure 1. DNMT3a is a putative target of microRNAs members of miR-29 and miR-30 families. A, DNMT3a 3'UTR from different species showing the conservation of seed matches for members of miR-29 and miR-30 families. B, transverse sections of representative hearts from rats 14 days after permanent (a) or transient (b) occlusion of the LAD. IZ infarcted zone; BZ, border zone; RZ, remote zone; IRZ, ischemic reperfused region; RV, right ventricle; IR, ischemia-reperfusion zone. LVP, Left Ventricle Posterior; LVA, Left Ventricle Anterior Scale bar: 3 mm.

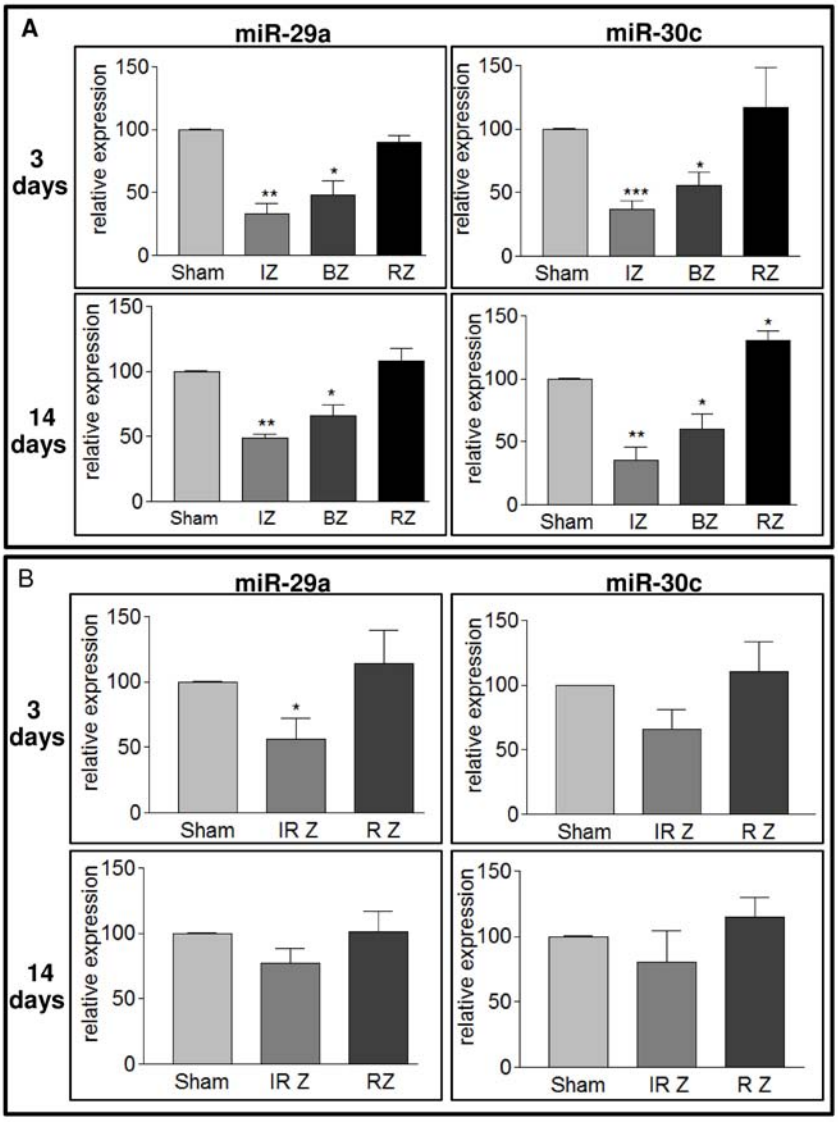

*Figure 2. MiR-29a and miR-30c are down-regulated in response to MI and IR. Q-RT PCR analysis of miR-29a and miR-30c relative expression in cardiac tissues of rats after myocardial infarction (A) or ischemia-reperfusion (B) compared with sham operated animals. IZ (MI infarcted zone); BZ (MI border zone); RZ (MI or IR remote zone); IRZ (IR ischemic zone). Q-RT PCR data are normalized against U1. *, p<0.05; **, p<0.001; ***,p<0.0005 (Student's t-test) compared with the expression of sham animals.*

2.2 The up regulation of DNMT3A in ischemic tissues is associated with the reduction of miR-29a and miR-30c

Next, we determined the expression of DNMT3a and miR-29a and miR-30c in myocardial tissues from LAD territory of the three different groups, 3 days after intervention: MI, IR, and sham operated animals. A statistically significant inverse correlation was detected between DNMT3a mRNA levels and miR-29a (p=0.0004) and miR-30c (p=0.005) levels (Figure 3A,B). Since the shift from a-MHC to "fetal" b-MHC expression is universally considered a marker of cardiac reprogramming, we determined also the expression levels of both a- and b-MHC. The findings that in stressed cardiac tissue we found a direct correlation between DNMT3a mRNA levels and b/aMHC ratio (Figure 3C) strengths the link between DNMT3a expression and ischemic induced myocardial remodelling process.

Then, we quantified DNMT3a protein levels in the same cardiac tissues which were qRT-PCR assessed for miRNAs and mRNA expression. The results of MI group are shown in Figure 4. Despite an increase in DNMT3a protein level was observed in the infarct zone of MI rats compared to corresponding regions in sham operated animals (Figure 4B,C), no significant differences were achieved at both 3 (p=0.0513) and 14 days (p=0.18) post LAD occlusion, mainly because of the high inter- and intra-group variability. However, by an intra-group analysis, the DNMT3a protein level was significantly increased in both IZ and BZ at 3 days and in IZ at 14 days after operation, when compared to the remote zone (Figure 4D, E).

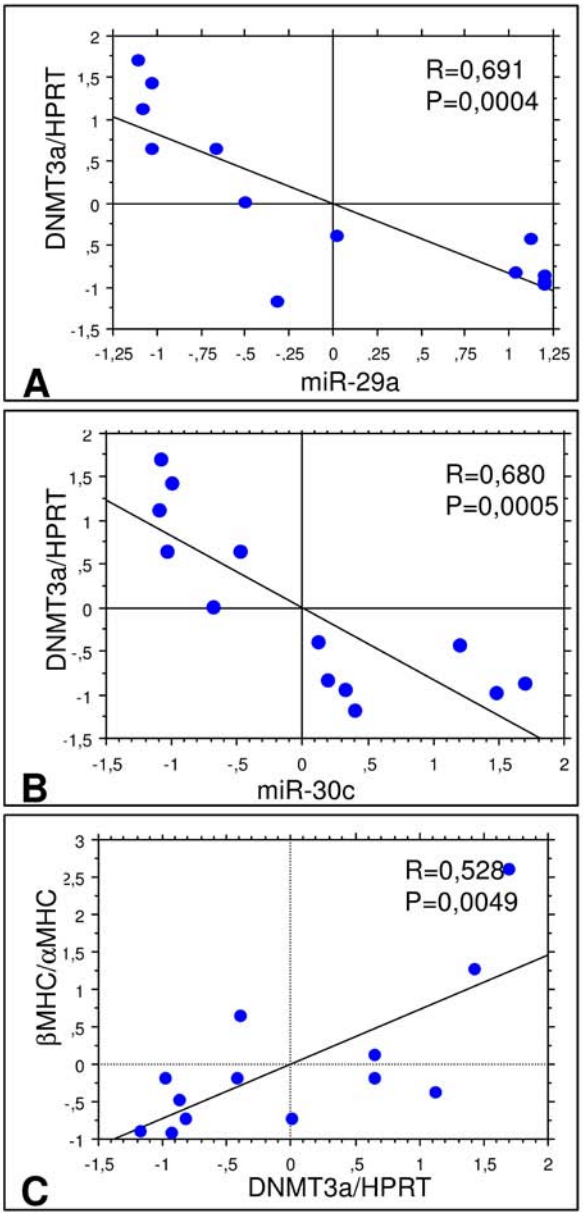

Figure 3. DNMT3A mRNA levels are inversely correlated with miR-29a and miR-30c and directly correlated with b-MHC/a-MHC ratio as remodelling marker. Correlations between endogenous mRNA levels of DNMT3A and endogenous levels of miR-29a, miR-30c, and b-MHC/a-MHC ratio determined by qRT-PCR in cardiac ischemic tissues Next, we determined the expression of DNMT3a and miR-29a and miR-30c in myocardial tissues from LAD territory of the three different groups, 3 days after intervention. R= regression coefficient. As internal standard U1 is used for miRNA quantification and HPRT is used for genes quantification.

The same analysis performed in the IR group showed a slight, but not significant increase of the DNMT3a protein in ischemic tissues (data not shown).

The present results suggest that the increase at both the mRNA and protein levels of DNMT3a is associated with the reduction of miR-29a and miR-30c in ischemic damaged tissues.

2.3 DNMT3a is a direct target of both miR-29a and miR-30c.

To seek a direct link between altered miR-29a and miR-30c expression and DNMT3a expression, we cloned the 3'-UTR of the DNMT3a downstream of the luciferase firefly reporter construct pGLU [21] creating the pGLU-3A-3'UTR construct. HEK-293 cells were co-transfected with the pGLU-3A-3'UTR vector plus increasing concentrations of either si-miR-29a or si-miR-30c expressing the mature form of the two miRNAs. Increasing amounts of either miR-29a or miR-30c resulted in a dose-dependent decrease of luciferase activity (Figure 5A). Transfection with a mix of 40nM si-miR-29a and 40nM si-miR-30c caused as similar decrease of luciferase activity as the reduction generated by 80nM of each of the two si-miRNAs alone (Figure 5A). This result suggests that, although miR-29a and miR-30c target sites are localized rather close on the DNMT3a-3'UTR (Figure 1A), these two miRNAs are not able to synergism effect. In contrast, comparable miRNA amounts had no effect on pGLU-3A-3'UTRmut where a sequence encompassing the binding sites for miR-29a and miR-30c was deleted (Figure 5B). These data demonstrate that DNMT3a is a direct target of both, miR-29a and miR-30c.

2.4 MiR-29 and miR-30 dysregulation affects the expression of DNMT3a in cardiomyocyte cell line.

To validate the potential role of miR-29a and miR-30c as negative regulators of DNMT3a expression in myocardial environment, we used the HL1 cell line, a cardiac muscle cell line with a pattern of gene expression similar to that of adult atrial myocytes [22]. First of all we induced hypoxia in HL1 to verify whether they respond similarly to the cardiomyocytes after myocardial infarction or ischemia/reperfusion. We chemically mimic hypoxia by incubating HL1 cells over 24h in the presence of desferrioxamine (DFO) which inhibits Hypoxia-Inducible Factor-Propyl-4-Hydroxylase (HIF-P4H) and leads to the accumulation of HIF, a pivotal transcriptional mediator of cellular response to low oxygen exposure [23]. Increasing doses of DFO (from 0 to 200mM) in the culture incubation medium induced a significant and progressive increase of apelin transcript, a well estabilished marker of hypoxia in myocardium and in cultured cardiomyocytes [24] and a parallel decrease of both miR-29a and miR-30c (Figure 6A). In addition, 200mM of DFO caused a significant increase of DNMT3a protein level (Figure 6B). These data show that in HL1 cultures the effects of hypoxia–mimetic agents on miR-29a/miR-30c and DNMT3a changes are comparable to those occurring in cardiac tissues after myocardial infarction or ischemia/reperfusion. In addition, they also suggest that at least part of these changes are induced via HIF signalling pathway.

Next, to ascertain a direct link between the miRNAs of interest and DNMT3a, HL1 cells were transfected with miR-29a, miR-30c or a miR-Ct (a miRNA mimic with a scrambled sequence) at 80 nM concentration or, alternatively, co-transfected with miR-29a + miR-30c (40 nM each). Twenty-four hrs after transfection miRNA and DNMT3a (at both mRNA and protein level) concentration were quantified. A 20-fold increase

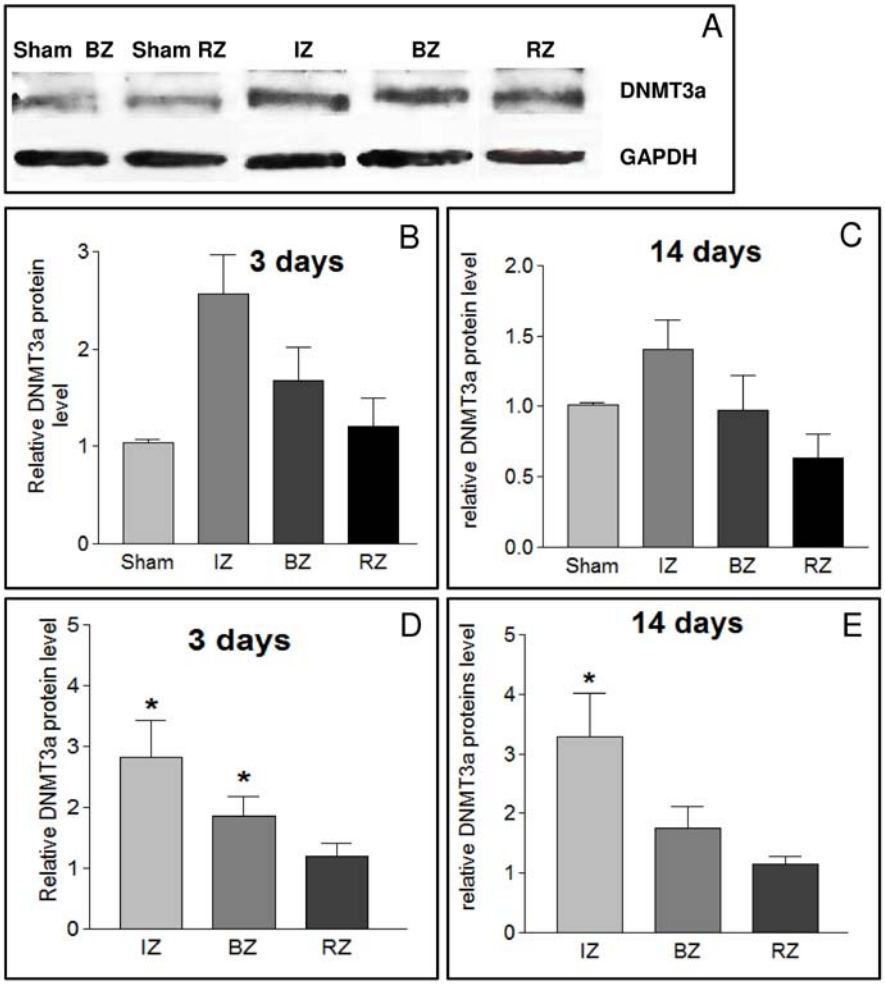

Figure 4. *DNMT3a is up-regulated in response to myocardial infarction. Western blot analysis of DNMT3a and GAPDH as internal control, performed in cardiac tissues of rats after MI. A, representative western blots for sham and MI groups. B, Densitometric quantification of western blot experiments: band intensity was normalized to that of GAPDH. DNMT3a values from different cardiac zones, 3 or 14 days after LAD occlusion, are expressed relative to the values of sham tissues (B and C, respectively) or relative to the zone remote to LAD territory (D and E, respectively) in MI group. IZ (infarcted zone); BZ (border zone); RZ (remote zone). *, p<0.05 (Student's t-test).*

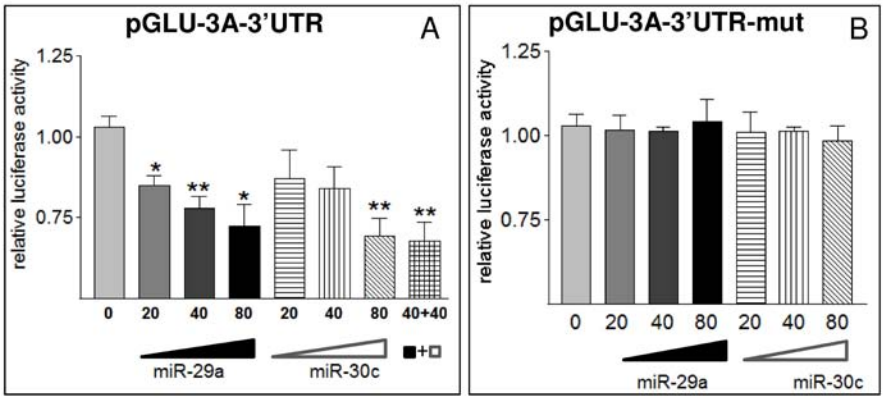

Figure 5. *DNMT3a is a direct target of miR-29a and miR-30c. HEK-293 cells were transiently transfected with luciferase reporters (100 ng) linked to the WT(A) or mutated (B) 3'UTR sequences of DNMT3A gene. Increasing amounts of the miR-29a or miR-30c (20–40-80 ng) or the mix of both miRNAs (40nM+40 nM, last column of figure A) were co-transfected with the luciferase reporter constructs together with the Renilla luciferase pRL–TK vector (100 ng) as internal standard. In each transfection the total amount of the transfected miRNA was kept constant by adding a scrumbled miRNA (miR-Ct) to the specific miRNA to obtain 80 ng. *p<0.05 ; **p<0.01 compared with luciferase reporter co-transfected with 80 ng of the miR-Ct.*

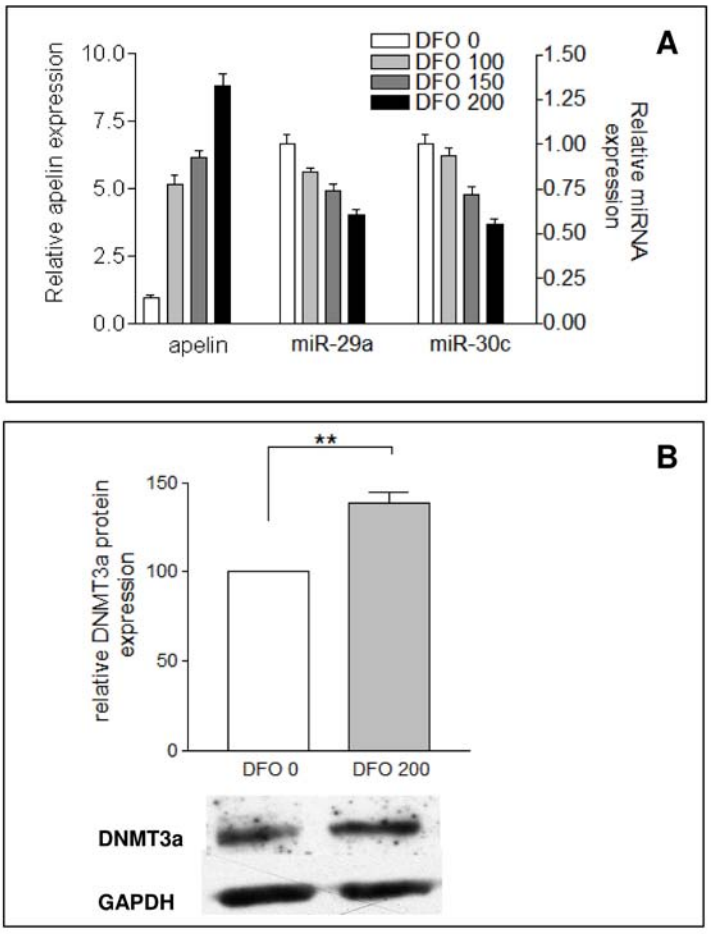

Figure 6. *The hypoxia mimetic DFO induces miR-29a/miR-30c decrease and DNMT3a increase in HL1 cells. HL1 cells were exposed to 0, 100, 150 and 200 mM DFO for 24 hrs. After the incubation period, cells were collected and processed for total RNA or protein extraction. A, Q-RT PCR analysis of miR-29a/miR-30c and transcript of apelin; U1 and HPRT were used as internal standard for miRNAs and apelin quantification, respectively. B, densitometric quantification of western blot experiments: band intensity was normalized to that of GAPDH. At the bottom of figure B an example of the western blot in HL1 cells is reported. **,p<0.001 compared with HL1 incubated in absence of DFO.*

of miR-29a (Figure 6A) almost halves DNMT3a (Figure 7B,C at mRNA and protein level, respectively). A similar increase of miR-30c (Figure 7A) negatively acted on DNMT3a protein level as well as miR-29a (Figure 7B), without affecting, however, its mRNA level (Figure 7C). A reduction of both DNMT3a mRNA and protein levels was also obtained by co-transfection of cells with miR-29a and miR-30c (40+40 nM, Figure 7B,C), showing an additive role of the two miRNAs in DNMT3a regulation but not a synergism effect. Taken together, these data strongly suggest that in the cardiac framework both miR-29a and miR30c are able to modulate the expression of DNMT3a.

3. Discussion

The impact of epigenetic mechanisms on disease development and progression, for long considered a characteristic of tumors, is now emerging as an important player also in cardiovascular pathophysiology. There are several epigenetic mechanisms in mammalian cells which have already been linked to cardiomyopathy or heart failure. These mechanisms include ATP-dependent chromatin remodelers important for organ development and adaptation [25], post translational histone modifiers whose alterations may result in cardiac arrhythmias, heart failure or cardiac hypertrophy [26,27], DNA methylation associated with human dilated cadiomyopathy [28] and atherosclerotic lesions [29] and miRNAs, whose importance as novel biomarkers and/or therapeutic targets for vascular disease is increasing rapidly [17,30]. The present study demonstrates that two of these epigenetic mechanisms of gene expression control, miRNA expression and DNA methylation, can be strictly related. Using rat models of myocardial damage by coronary artery occlusion we demonstrated that the levels of these two miRNAs are downregulated in ischemic cardiac regions whilst the DNMT3a protein level is increased, an effect not observed in healthy tissue. Then, we verified that the *in vitro* model of cardiomyocyte-like cells (HL1) could respond similarly to the *in vivo* cardiomyocyte cells when exposed to hypoxia. Finally, by *in vitro* assays we showed that miR-29 and miR-30 directly interact with the chromatin modifying enzyme DNMT3a. MiR-29 has already been recognized as a direct regulator of different

isoforms of DNMT in tumors. In lung cancer, aberrant DNA methylation, frequently associated with poor prognosis in this kind of tumors, was reverted by enforced expression of miR-29 family [20]. Down-regulation of miR29 family has also been associated with hypermethylation of tumor related genes in cutaneous melanoma [19] and hepatocarcinoma cells [31]. However miR-29-mediated DNMT modulation is not only a pathological response but also a physiological mechanism to induce differentiation: the high expression of the three isoforms of miR-29 in adult pancreatic β-cells allows specific silencing of the MCT1 transporter thus preventing inappropriate insulin secretion [32]. Results presented in our paper show for the first time that miR-29 family is capable of modulating DNMT expression also in cardiac tissue. Up to now the importance of miR-29 in cardiovascular disease was highlighted by data showing that this microRNA family directly targets many extracellular matrix genes. Strong antifibrotic effects of miR-29s have been demonstrated in heart, kidney, and other organs [33-35]. The downregulation of miR-29 in ischemic tissues, as a consequence of TGF-β induction, promotes the increase of genes such as collagens, fibrillin and elastin contributing to the fibrotic response [35].

MiR-30 also is strongly downregulated in ischemic tissues and actively contributes to the fibrotic process by directly targeting the Connective Tissue Growth Factor (CTGF), a key molecule in the process of fibrosis [36]. There are no data showing a role of this large miRNA family in DNMT modulation. Our data indicate that miR-29 and miR-30, both preferentially expressed in fibroblasts although also detectable in cardiomyocytes, might impact ischemic cardiac tissue reprogramming by modifying post ischemic heart remodelling epigenome. Data obtained from experiments in cell cultures (Figure 6) and *in vitro* assays (Figure 5) allowed us to study separately the impact of the two miRNAs on DNMT3a and indicated comparable inhibition of DNMT3a by increasing levels of the two miRNAs. *De novo* methylation activity is present mainly in embryonic stem (ES) cells and embryonic carcinoma cells, early post implantation embryos, and developing germ cells, whereas it is largely suppressed in differentiated somatic cells. Therefore the increase of DNMT3a, as a consequence of miR-29/30 decrease, represents the activation of another element of the "fetal reprogramming" which characterizes the responses to cardiac acute and chronic stresses [1,15]. DNMT modifications entail alterations of DNA methylation. Few recent data support the idea that changes of DNA methylation may represent a novel mechanism in the pathogenesis of heart failure and dilated cardiomyopathy (DCM) [28]. However, unlike carcinogenesis where global DNA hypomethylation has been widely described [37], in cardiovascular diseases, contradicting results suggest that the effects of DNA methylation could be genome site specific. Indeed, lower methylation of LINE-1 was detected by Baccarelli et al. in patients affected by cardiovascular disease (CVD) compared to healthy subjects [38], while other authors correlated an higher level of methylation of repetitive elements such as Alu and Sat2, with the prevalence or predisposition

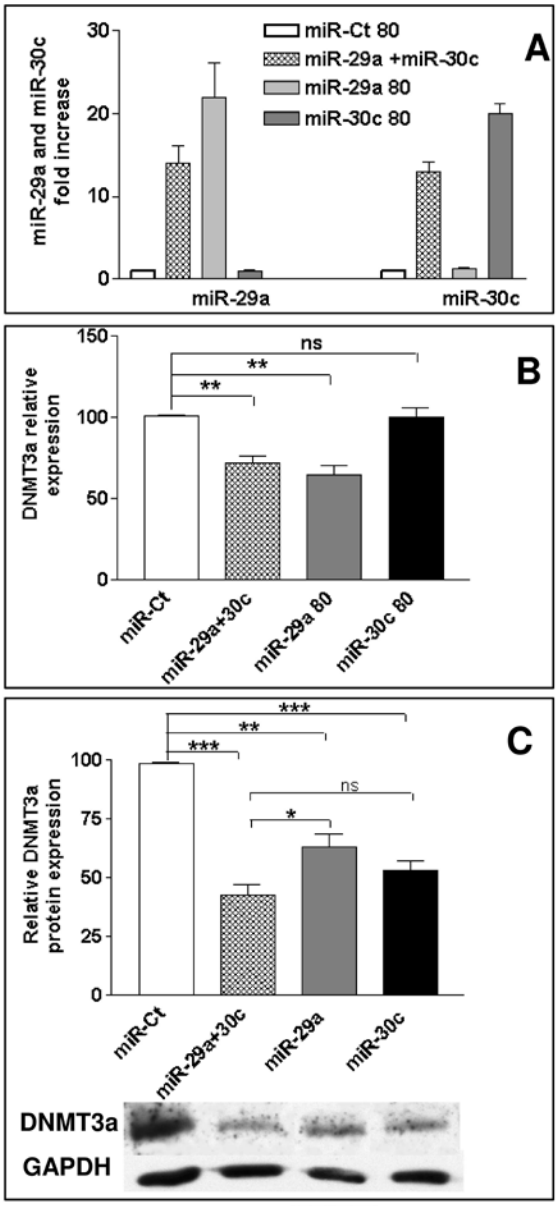

*Figure 7. MiR-29a and miR-30c overexpression in HL1 downregulates DNMT3a expression. HL1 cells were transfected with 80 nM miR-29a or miR30c and with a mix of the two miRNAs (40 nM each). As control 80 nM of a control miRNA, miR-Ct, was transfected. 24 hrs after transfection cells were collected. Each transfection was split in two for total RNA and protein extractions. A,B Q-RT PCR analysis of miR-29a/miR-30c (A) and transcript of DNMT3a (B). U1 and HPRT were used as internal standard for miRNAs and DNMT3a quantification respectively. C, densitometric quantification of western blot experiments: band intensity was normalized to that of GAPDH. At the bottom of figure C an example of the Western blot in HL1 cells is reported. *, p<0.05; **, p<0.001; ***,p<0.0005.*

of CVD [39]. Genome wide DNA methylation analysis showed that promoter CG islands were more hypomethylated and intragenic CGIs were more hypermethylated in human end-stage cardiomyopathy compared to healthy subjects [40]. More recently, alterations in cardiac DNA methylation were detected

in DCM patients [28]. Interestingly these authors, carrying out a clustering approach on genes with known abundant expression in the human heart, demonstrated that about one-third showed increased methylation in DCM patients whereas approximately two–thirds were significantly less methylated. The complexity of these analysisis further increased by the recent studydemonstrating that mammalian DNMTs (DNMT1, DNMT3a and 3b) can function as Ca^{2+} and redox state-dependent DNA demethylases [8]. Since intracellular Ca^{2+} overload can occur in cardiomyocytes as a consequence of ischemic injury or other stresses [41,42], and ROS overproduction is a consequence of heart damage and ischemia [43], we might hypothesize that the catalytic functions of the DNMTs might switch between methylation and demethylation according to the specific cardiac pathological conditions or local concentration of Ca2+, the presence of SAM and the redox state of DNMTs [8]. Keeping in mind all these considerations it is difficult to predict which might be the consequence of the DNMT3a increase we observed in our model in terms of methylation levels.

In conclusions our data show that modulations of two miRNAs are able to modify the expression of DNMT3a and that these two events are correlated to tissue reprogramming after ischemic myocardial damage. Our results suggest a new mechanism that might generate the alterations in DNA methylation often observed in cardiac pathophysiology.

These data, besides indicating a pivotal role of miRNAs in epigenetic reprogramming of ischemic cardiac tissues, highlight once more the importance of miRNAs as potential therapeutic targets also in cardiovascular diseases.

4. Materials and methods

4.1 Rat model of myocardial ischemic damage

Experimental protocols conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and was approved by the Animal Care Committee of the Italian Ministry of Health (Endorsement n.135/2008-B).

Male Wistar rats 12-15 weeks old and weighing 353 ± 11g were used in the study. Rats were anesthetized using Zoletil® + xylazine (50 and 3 mg/kg respectively). A standard limb D1-D3 electrocardiogram (ECG) was monitored with subcutaneous stainless steel electrodes. Rats were connected to a respirator through a oropharingeal cannula and the heart was rapidly exposed through a left lateral thoracotomy and pericardial incision. A 6-0 silk snare was passed through the epicardium layer around the origin of the left artery about 2-3 mm from its origin. The left anterior descendent (LAD) coronary artery was tied according two alternative protocols: 1) *ischemia-reperfusion (I/R)*, LAD was occluded for 30 min (period of ischemia) by tying a loop, then the knot around the vessel was opened and unrestrained reperfusion allowed (n=10); 2) *infarction (MI)*, LAD was permanently occluded (n=10).

In all cases ischemia was confirmed by ST segment elevation at ECG and visually assessed regional cardiac cyanosis. The heart was returned to its normal position and the thorax closed. A control group of rats underwent all surgical procedures except for the occlusion of the LAD (sham-operated group n=5).

Post-operatively, all rats were hydrated with physiological saline and given buprenorphin 0.05 mg/kg s.c. for analgesia (Temgesic®, Schering-Plough, Brussels, Belgium).

Under deep anesthesia rats were killed by a lethal KCl injection 3 or 14 days after surgery. Hearts were cut below the plane of LAD occlusion and left ventricular tissue samples were obtained from a) the free wall remote to LAD region (RZ) (protocol 1 and 2), b) the core of either the ischemic reperfused region (IRZ, protocol 1) or the infarcted area (IZ, protocol 2), c) border region to LAD area (BZ, protocol 2) (Figure 1). In sham-operated animals we used the same terms as in IR hearts, with IRZ and RZ correspondent to the territories pertinent or remote to the LAD perfusion, respectively.

Samples were snap frozen in liquid nitrogen and stored at -80°C until assayed.

4.2 DNMT3a-3'UTR plasmid

A 1500 bp fragment of the 3'-UTR of DNMT 3a was obtained by PCR amplifying genomic DNA with the following primers: F(5'-ACCGGAAGCGGGATGAGT-3'), R(5'-ACTGCAATCACCTTGGCTTT-3').The amplified fragment was cloned in the pGEM T-easy vector (Promega) after a tailing reaction. After sequencing the fragment was subcloned in the pGL3 basic vector in the 3'-UTR of luciferase's gene, using SmaI and SacI restriction sites. The 3'-UTR-DNMT3a-mut plasmid was prepared as described by Fabbri et al [20].

4.3 HEK-293 cells and Luciferase Assay

293T cells were seeded at a density of $1{,}5x10^5$ cells per well in 12 well dishes in Dulbecco's modified Eagle Medium (Gibco DMEM) with 10% FBS, 2µg/ml L-glutamine and 50µg/ml streptomycin. Cells were grown for 24 hours at 37°C in a humified atmosphere containing 5% CO_2.Twenty-four hours later, cells were transfected. Polyfect (Qiagen) was used as transfectant, according to the manufacturer's recommendations. In each transfection 100ng/µl of DNMT3a-3'UTR plasmid was cotrasfected with different concentrations of duplex si-miRNA 29a(5'-UAGCACCAUCUGAAAUCGGUUA-3'; 5'-UAACCGAUUUCAGAUG GUGAAAAA-3') or duplex si-miRNA 30c (5'-UGUAAACAUCCUACACUCUCA GCUU-3'; 5'-GCUGAGAGUGUAGGAUGUUUUAAUU-3') associated with different concentration of negative control (5'-UUCUCCGAACGUGUCACGUTT-3';5'-ACGUGACACGUUCGGAGAATT-3') in the presence of 100ng/µl of renilla expressing plasmid as internal standard. After 24 hours at 37°C cells were washed with PBS for two times. To extract the proteins in each dish was added 100µl of Passive Lysis Buffer 1x (Promega) and after five minutes at room temperature the plate was put at -80°C for ten minutes. The contents of each dish was collected and centrifuged for ten minutes at 4°C. The obtained supernatant was immediately assaied or stored at -80°C. Luciferase assay was performed using Dual luciferase

Reporter assay System. Firefly luciferase activity was normalized to renilla activity for each transfected dishes. Three independent experiments assayed were performed.

4.4 HL-1 cells experiments

HL-1 cells were seeded at a density of $2x10^5$cells per well in 6 well dishes on a layer of gelatin/fibronectin in Claycomb Medium (Sigma-Aldrich) with 10% FBS, 100μM norepinephrine, 2mM L-glutamine and penicillin/streptomycin.

Transfection assays. Cells were grown for 24 hours at 37°C in a humified atmosphere containing 5% CO_2. Twenty-four hours later, cells were transfected with 80nM duplex si-miRNA NC or duplex si-miR-29a or duplex si-miR-30c or with the mix of duplex si-miR-29a and duplex si-miR-30c (40nM+40nM). HiPerfect Tranfection Reagent (Qiagen) was used as transfectant, according to the manufacturer's recommendations. Twenty-four hours later the medium was aspirated and after washing with versene, cells were detached with trypsin 1x. Doses of miRNA mimics higher than 80/100 nM were never used due to the increasing cell toxicity of the transfection mixture (not shown).

Induction of hypoxia.To chemically mimic hypoxia responses, HL-1 cells were incubated in the presence of a HIF prolyl-4-hydrxylase (HIF-P4H) inhibitor, desferrioxamine (DFO; 0-200 mM, Sigma-Aldrich) for 24 hrs.

4.5 Western blot analysis

Cardiac tissue and transfected HL-1 cells were lysed in Lysis buffer [20mM Tris-HCl pH 8.0, 20mM Nacl, 10% glycerol, 1% NP40, 10mM EDTA, 2mM PMSF, 2.5 μg/ml leupeptin, 2.5 μg/ml pepstatin]. For cardiac tissues mechanical fragmentation by Tissuelyser (Qiagen) was also used. Protein (30μg per lane) were separated on 4-15% polyacrylamide gel (Mini-PROTEAN TGX Stain-Free Gels BIO RAD) and tranferred to Hybond-C Extra nitrocellulose membranes (Amersham Biosciences). Immunoblotting of the membranes was performed with the following primary antibodies: antibodies agaist DNMT 3a (1:250)[64B1446, IMGENEX] and GAPDH (1:80000)[14C10, Cell Signaling]. Signals were revealed after incubation with the recommended secondary antibody coupled to peroxidase using ECL or ECL Prime (GE Healthcare). Scanned images were quantified using Optiquant software.

4.6 Real-time PCR analysis

Total RNA was extracted from cardiac tissue and from 3x 10^5 HL-1 cells with the miRNeasy Mini kit (Qiagen) following the manufacturer's instructions.

0.5- 1μg of total RNA was retrotranscribed using the miScript II RT Kit (Qiagen) for miRNAs or the Quantitect Reverse Transcription Kit (Qiagen) for transcripts following the manufacturer's instructions.

Real-time PCR (qRT-PCR) was carried out with LightCycler 480 (Roche) to quantified mature miRNAs or with Rotor-Gene Q (Qiagen) to quantified transcripts: mature miRNAs and transcripts were detected with SybrGreen fluorescence.

Oligonucleotides used were as follows: for miR-29a, F(5'-TAGCACCATCTGAAA TCGGTTA-3'); for miR-30c, F(5'-TGTAAACATCCTACACTCTCAGC-3'); for DNMT 3a, F(5'-AACGGAAGCGGGATGAGT-3') and R(5'-TTCAGTCCTGTCCATAATCAGT CC-3'). for apelin, F(5'-CCGAGTTGCAGCATGAATC-3') and R(5'-GTTCCATCTG GAGGCAACAT-3').

Mature miRNA and transcript values were normalized respectively with those obtained from the amplification of snRNA-U1 and HPRT(internal standards) with the following primers: for U1F(5'-CGACTGCATAATTTGTGGTAG-3'), and for HPRT F(5'-CCCAGCGTCGTGATTAGTGATG-3'), R(5'TTCAGTCCTGTCCATAATCAG TCC-3').

All reactions were performed in triplicate and the relative expression ±SE of three independent experiments is shown.

4.7 Statistical analysis

Data were analyzed using GraphPad Prism (GraphPad Software, San Diego, CA).Statistical differences were determinated by unpaired *t*-test, with values of $p<0.05$ considered statistically significant. Each experimental point in the graph represents the mean ± SE of at least three independent experiments.

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