

miR-145 is differentially regulated by TGF- β ₁ and ischaemia and targets Disabled-2 expression and wnt/ β -catenin activity

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Abstract

The effect of wnt/ β -catenin signalling in the response to acute myocardial infarction (AMI) remains controversial. The membrane receptor adaptor protein Disabled-2 (Dab2) is a tumour suppressor protein and has a critical role in stem cell specification. We recently demonstrated that down-regulation of Dab2 regulates cardiac protein expression and wnt/ β -catenin activity in mesenchymal stem cells (MSC) in response to transforming growth factor- β ₁ (TGF- β ₁). Although Dab2 expression has been shown to have effects in stem cells and tumour suppression, the molecular mechanisms regulating this expression are still undefined. We identified putative binding sites for miR-145 in the 3'-UTR of Dab2. In MSC in culture, we observed that TGF- β ₁ treatment led to rapid and sustained up-regulation of pri-miR-145. Through gain and loss of function studies we demonstrate that miR-145 up-regulation was required for the down-regulation of Dab2 and increased β -catenin activity in response to TGF- β ₁. To begin to define how Dab2 might regulate wnt/ β -catenin in the heart following AMI, we quantified myocardial Dab2 as a function of time after left anterior descending ligation. There was no significant Dab2 expression in sham-operated myocardium. Following AMI, Dab2 levels were rapidly up-regulated in cardiac myocytes in the infarct border zone. The increase in cardiac myocyte Dab2 expression correlated with the rapid and sustained down-regulation of myocardial pri-miR-145 expression following AMI. Our data demonstrate a novel and critical role for miR-145 expression as a regulator of Dab2 expression and β -catenin activity in response to TGF- β ₁ and hypoxia.

Keywords: myocardial infarction • growth factors/cytokines • gene expression

Introduction

There is conflicting data regarding the effects of wnt/ β -catenin following acute myocardial infarction (AMI). Several studies have suggested that inhibition of wnt/ β -catenin signalling through the overexpression of antagonists [1, 2] or the delivery of small molecule inhibitors [3] leads to altered cardiac stem cell proliferation, decreased cardiac myocyte death and improved left ventricular remodelling. Conversely, studies have demonstrated that the haematopoietic stem cells [4] or myocardium transduced [5] to overexpress β -catenin have been shown to improve myocardial

healing and decrease infarct size. Wnt/ β -catenin signalling involves multiple down-stream targets. To define the relevance of this pathway and potential targets for modifying myocardial healing, the goal of our study was to begin to define the up-stream regulators that regulate activation of the wnt/ β -catenin signalling following AMI.

Disabled-2 (Dab2) is a TGF- β receptor adaptor protein, which is a tumour suppressor protein that is down-regulated in multiple different tumours [6–9]. Dab2 levels have been shown to regulate the TGF- β tumour suppressor/oncogenic effects [10, 11]. We recently showed that the down-regulation of dab2 in mesenchymal stem cells (MSC) in response to TGF- β is critical for the up-regulation of cardiac protein expression, as well as the improved functional response observed following the myocardial engraftment of TGF- β pre-treated MSC [12].

Dab2 directly interacts with two components of the β -catenin destruction complex, Dvl-3 and axin, and participates in regulation

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of the Wnt signalling pathway [13]. Dab2-mediated stabilization of axin leads to inhibition of canonical wnt/ β -catenin-mediated signalling [14, 15]. Several studies have demonstrated that up-regulation of β -catenin signalling can lead to a decrease in infarct size in models of AMI [16]. Thus, defining myocardial Dab2 expression at baseline and in response to acute ischaemia, as well as the molecular mechanisms responsible for modulation of Dab2 in the heart could begin to define the up-stream regulators of Dab2 expression in the heart as well as define novel determinants of infarct size.

There are conflicting studies as to whether the down-regulation of Dab2 in tumours is due to methylation of the Dab2 promoter [17–19]. To date, the molecular mechanisms associated with the modulation of Dab2 in response to TGF- β and myocardial ischaemia have not been defined. In this study, we demonstrate that miR-145 is up-regulated in response to TGF- β in MSC and that this up-regulation is required for TGF- β -mediated modulation of Dab2 expression. We further demonstrate that miR-145 is down-regulated in the myocardium following AMI and the down-regulation of myocardial miR-145 is associated with an increase in Dab2 expression in cardiac myocytes in the infarct border zone.

Material and methods

Animals

All animals were housed in the AAALAC animal facility of the Cleveland Clinic Foundation and maintained under standard conditions. This investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH publication No. 85-23, Revised 1996) and was approved by the IACUC of the Cleveland Clinic Foundation.

MSC preparation

MSC were isolated from adult Lewis rat bone marrow as we have described previously [12, 20]. As we have done in previous studies, we validated the MSC phenotype and stability of the MSC phenotype through *in vitro* differentiation of the MSC preparation into adipocytes, chondrocytes or osteoblasts [12].

Antibodies

In addition to those described earlier, additional antibodies used in our studies for analysis were: anti-TGF- β ₁ (5 ng/ml; Biovision, Mountain View, CA, USA), anti-cardiac myosin (Chemicon International, Burlington, MA, USA), monoclonal antibody to Dab2/p96 was purchased from Transduction Laboratories (Lexington, KY, USA). Rabbit polyclonal anti-Dab2 (1:1000; BD Transduction Laboratories, Lexington, KY, USA), anti-Glucose-6-phosphate-dehydrogenase (GAPDH; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Primary antibody detection was carried out using Alexa Fluor-conjugated secondary antibodies (Gibco, Grand Island, NY, USA) or near infrared dye-conjugated secondary antibodies for Odyssey Western blot system.

Immunohistochemistry

Animals were sacrificed at the indicated times after MI. Tissues were fixed in formalin and embedded in paraffin blocks according to established protocols. Antigen was retrieved using 10 mM sodium citrate buffer (pH 6.0) and heat at 95°C for 5 min. The buffer was replaced with fresh buffer and reheated for an additional 5 min., and then cooled for 20 min. After washing with deionized water, specimens were incubated with 1% normal blocking serum in PBS for 60 min. to suppress non-specific binding of IgG. Slides were incubated for 1 hr at room temperature or overnight with the indicated primary antibodies. Optimal antibody concentration was determined by titration. Slides were then washed with PBS and incubated for 45 min., with secondary antibodies (Alexa Fluor, Grand Island, NY, USA) diluted to 1.5 mg = ml in PBS with 10% serum in a dark chamber. After washing extensively with PBS, cover slips were mounted with aqueous mounting medium (Vectashield Mounting Medium with DAPI for nuclei detection, H-1200; Vector Laboratories, Burlingame, CA, USA).

Western blot analysis

After the treatments, the cells were scraped from the culture dishes, pelleted and gently washed with ice-cold PBS. Cells were lysed by adding pre-warmed (95°C) 125 mM Tris and 1% sodium dodecyl sulfate (SDS; pH 6.8) buffer to the cell pellets. Cell lysates were then centrifuged and the supernatant was used as whole protein cell lysate. After the proteins were electrophoretically separated in 10% SDS polyacrylamide gels and electrotransferred to blotting PVDF membranes, the unspecific bonds were blocked with 5% skimmed milk in 1 × TBST [25mM Tris (pH 8.0), 125 mM NaCl and 1% Tween 20] for 1 hr at room temperature and then probed with primary antibodies over night at 48°C. After incubation with near-infrared-dye-conjugated secondary antibodies (IRDye; Licor Biotech, CA, USA; 1:20,000, 1 hr, room temperature). Antibody recognition was detected with an Odyssey scanner manufacturer's instructions (Licor Biotech).

Left anterior descending ligation

Ligation of the left anterior descending (LAD) artery in Lewis rat was performed as previously described [12, 20]. Briefly, animals were anaesthetized with intraperitoneal ketamine and xylazine, intubated and ventilated with room air at 80 breaths/min. using a pressure-cycled rodent ventilator (RSP1002; Kent Scientific Corporation, Torrington, CT, USA). Anterior wall MI was induced by direct ligation of the LAD artery with the aid of a surgical microscope (M500; Leica Microsystems, Buffalo Grove, IL, USA).

Luciferase assay

Mesenchymal cells were plated in triplicate wells of a 24-well plates and transfected by electroporation using the Amaxa system (Amaxa, La Jolla, CA, USA) with TOPFLASH-LIF-luciferase reporter construct (were purchased from Upstate, Inc., Billerica, MA, USA) and/or miR-145 or miR-145 inhibitor. Transfection efficiency was corrected by a renilla luciferase vector (pRL-CMV; Promega, Madison, WI, USA). The cells were harvested for luciferase assays 24 hrs after transfection. The Dual-Luciferase Reporter Assay System (Promega) was used to measure the reporter activity according the manufacturer's protocol.

A
Alignment of putative conserved miR-145 binding sites in 3'-UTR of Dab2

	Predicted consequential pairing of target region (top) and miRNA (bottom)	Seed Match
Position 1213-1219 of Dab2 3'-UTR	5' ...AGGCCUUUAUCAAGCAACUGGAA... 	8mer
mo-miR-145	UCCCUAAGGACCCUUUUGACCUG	
Position 1693-1699 of Dab2 3'-UTR	5' ...CCUAAAGAUGUGCUAACUGGAG... 	7-8mer
mo-miR-145	UCCCUAAGGACCCUUUUGACCUG	
Position 1832-1838 of Dab2 3'-UTR	5' ...UUUCUCAUUCAAUAACUGGAA... 	8mer
mo-miR-145	UCCCUAAGGACCCUUUUGACCUG	

B
Alignment of 3'-UTR of Dab2 across multiple species, grey predicts putative miR-145 binding site at the positions 1213-1219.

1188-----1213---1220-----1241

Rno	AUGUCAUGCCAGGCCU---UUAUCAAGCAACUGGAAACUUU-CUUGA-AGCCA
Hsa	GGGUCAUGCUAGGCCU---UUAUCAAGUAACUGGAAACUUUUCUUGC-AGCCA
Ptr	GGGUCAUGCUAGGCCU---UAA-CAAGUAACUGGAAACUUU-CUUGC-AGCCA
Mml	GGGUCGUGCUAGGCCU---UUAACAAGUAACUGGAAACUUU-CUUGC-AGCCA
Mmu	AUG---ACUAGGCCU---UUAUCAAGUAACUGGAAACUUU-CUUGA-AGCCA
Cpo	AAGUCAUGCCGACC---UCAUCAAGUAACUGGAAACUUU-CCUGA-GGCCA
Ocu	GGGUCAUGUAGACC---UUAUCAAGUAACUGGAAACUUU-CUUGA-AGCCA
Sar	AGGUUAUGCUAGACC---UUAUUAAGUAACUGGAAACUUU-CUGGA-AGCCA
Eeu	GAGUCACGCGAGGCC---UUCUUGAACACUGGAAACUUU-GUGGA-AGCCA
Cfa	G---UCAUGCUAGACC---UUAUCAAGUAACUGGAAACUUU-CUUGA-AGCCA
Fca	GGGUCAUGCUAGACC---UUAUCAAGUAACUGGAAACUUU-CUUGA-AGCCA
Eca	GGGUCAUACUAGACC---UUAUCAAGUAACUGGAAACUUU-CUUGA-AGCCA
Bta	GGGUCAUGCCAGACC---UUAUCAAGUAACUGGAAAGCUUU-CUUGA-UGCCA
Dno	GGGUCAUAUAGGCC---UUGUCAAGUACUGGAAACUUU-CUUA-AGCCA
Laf	GGGUCAUGCUAGGCC---UUAUUAAGUAACUGGAAACUUU-CUUGA-AGCCA
Ete	-----AGGUCUAAUUUACCCAGUAACUGGAAACUUU-CUCGA-AGUCU
Mdo	UGGUUACAUUGGCC---UU-UGAAGUACCUGGUAACUUU-CUUC--AACAA
Oan	GGUUUUUGGUGCUC---CUGAUACUAACU-----UUU-CUUUAC----

Rno - Rat	Ocu - Rabbit	Bta - Cow
Has - Human	Sar - Shrew	Dno - Armadillo
Ptr - Chimpanzee	Eeu - Hedgehog	Laf - Elephant
Mml - Rhesus	Cfa - Dog	Mdo - Opossum
Mmu - Mouse	Fca - Cat	Oan - Platypus
Cpo - Guinea Pig	Eca - Horse	

Fig. 1 (A) Alignment of miR-145 to three different regions of Dab2 3'UTR according to target scan. Notice the distance between target sites in a 3'UTR region of ~1.7 Kb. **(B)** Alignment of conservation of Dab2 miR-145 target site at position 1213 across multiple species.

Source: http://www.targetscan.org/cgi-bin/vert_50/view_gene.cgi?gs=DAB2&taxid=10116&members=&showcnc=1&shownc=0

MSC transfection

As previously described [12], MSC transfection was performed by electroporation with the Amaxa system according to the manufacturer's instructions for MSC (Amaxa, Gaithersburg, MD, USA). Cells (3×10^6) were transfected in each experiment with a transfection efficiency of 55–70% as assessed by GFP expression. The Dab2:cDNA was constructed with a cytomegalovirus promoter and poly A tail [12].

18S rRNA purchased from Applied Biosystems (Foster City, CA, USA). TaqMan One-step RT-PCR Master Mix Reagents, amplification and detection was performed using 7500HT Sequence Detection System (ABI), using 40 cycles of denaturation at 95°C (15 sec.) and annealing/extension at 60°C (60 sec.). This was preceded by reverse transcription at 50°C for 30 min. and denaturation at 95°C for 10 min. To quantitate mature miRNA, TaqMan MicroRNA Assays kits were purchased from ABI to specifically detect pri-miR145.

miRNA and mRNA detection

Total pri-miRNA was extracted with a mir-Vana miRNA Isolation Kit (Ambion, Austin, TX, USA) and subjected to reverse transcription with the reverse transcription kit-primers and probes specific for rat pri-miR145 and internal control

miR sequences

miR-145 mimic and miR-145 inhibitor with the following sequences were obtained from Applied Biosystems.

miRNA 145 mimic: GUCCAGUUUCCAGGAAUCCCU
miRNA 145 inhibitor: GUCCAGUUUCCAGGAAUCCCU

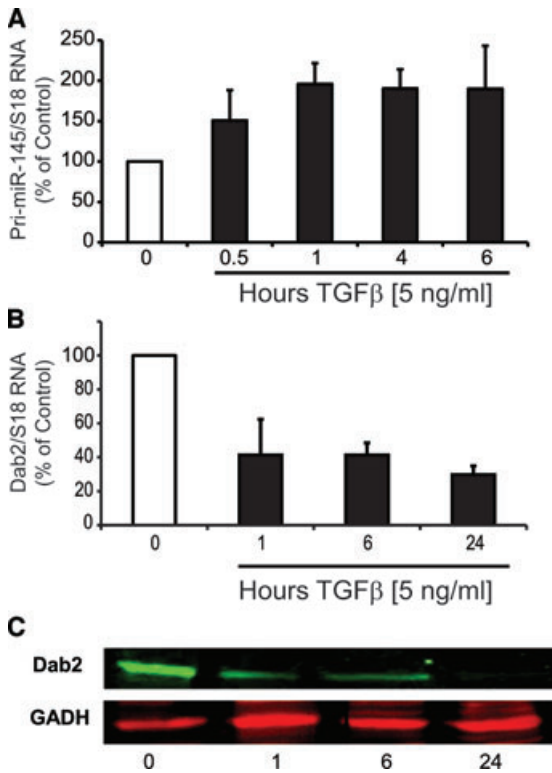


Fig. 2 Regulation of the expression of Dab2 and miR-145 in mesenchymal stem cells. MSC pri-miR-145 expression is increased in response to TGF- β_1 (5 ng/ml, black bars) (A). Dab2 expression is significantly reduced in response to TGF- β_1 (5 ng/ml) both at the mRNA (B) and protein level (C). qPCR experiments are $n = 3 \pm$ S.D.; Western blot is a representative experiment of four separate studies performed under the same conditions.

Statistics

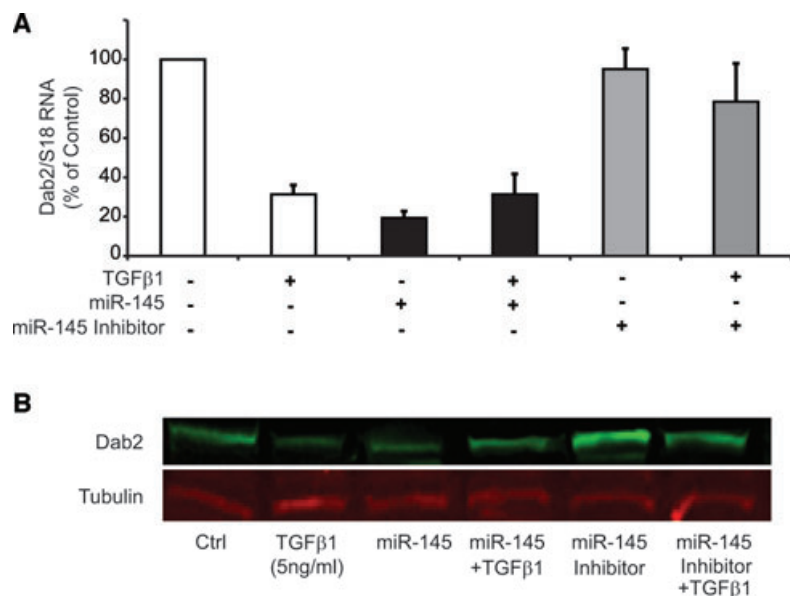
Data are presented as mean \pm standard deviation. Comparisons were made using Student's *t*-test with significance defined as $P < 0.05$.

Results

From our *in silico* analysis using mirBase (www.mirBase.org) and Targetscan (www.targetscan.org), we found the microRNA miR-145 binding to three different motifs of the 3'UTR mRNA Dab2 region (Fig. 1A). These three miR-145 target sites potentially show high binding energy at the miRNA seed regions of seven and eight nucleotides, fundamental characteristics for miRNAs to induce gene silencing [21]. Noticeably, the three miR-145 target sequences are highly (>98%) conserved amongst mammals (http://www.targetscan.org/cgi-bin/vert_50/view_gene.cgi?gs=DAB2&taxid=10116&members=&showcnc=1&shownc=0#miR-145). The alignment for the 8mer binding site at 1213–1220 is shown in Figure 1 across multiple species. These analyses led us to focus our studies on miR-145.

To determine if miR-145 has a role in regulating Dab2 expression following TGF- β_1 treatment, we performed a series of experiments treating MSC with TGF- β_1 (5 ng/ml) and quantified pri-miR-145 and Dab2 levels as a function of time. The data in Figure 2A demonstrate that pri-miR-145 levels are significantly increased following TGF- β_1 treatment. To correlate the changes of pri-miR-145 with Dab2 levels, we quantified Dab2 levels as a function of time after TGF- β_1 treatment by qPCR (Fig. 2B) and Western blot analysis (Fig. 2C). As shown in Figure 2A–C, Dab2 levels are

Fig. 3 Gain and loss of miR-145 function in MSC and the effect on Dab2 expression. Transfection of MSC with 50vpM miR-145 mimic (black bars) by electroporation resulted in a decrease of Dab2 expression at the mRNA and protein levels, as determined by qPCR (A) and Western blot (B), respectively. No difference relative to control in Dab2 expression was observed when MSC were transfected with miR-145 inhibitors (grey bars). The presence of miR-145 did not alter the MSC response to TGF- β_1 (24 hrs, 5 ng/ml). The presence of miR-145 inhibitor completely blocked the down-regulation of Dab2 by TGF- β_1 . qPCR experiments are $n = 3 \pm$ S.D.; Western blot is a representative experiment of three performed in the same conditions.



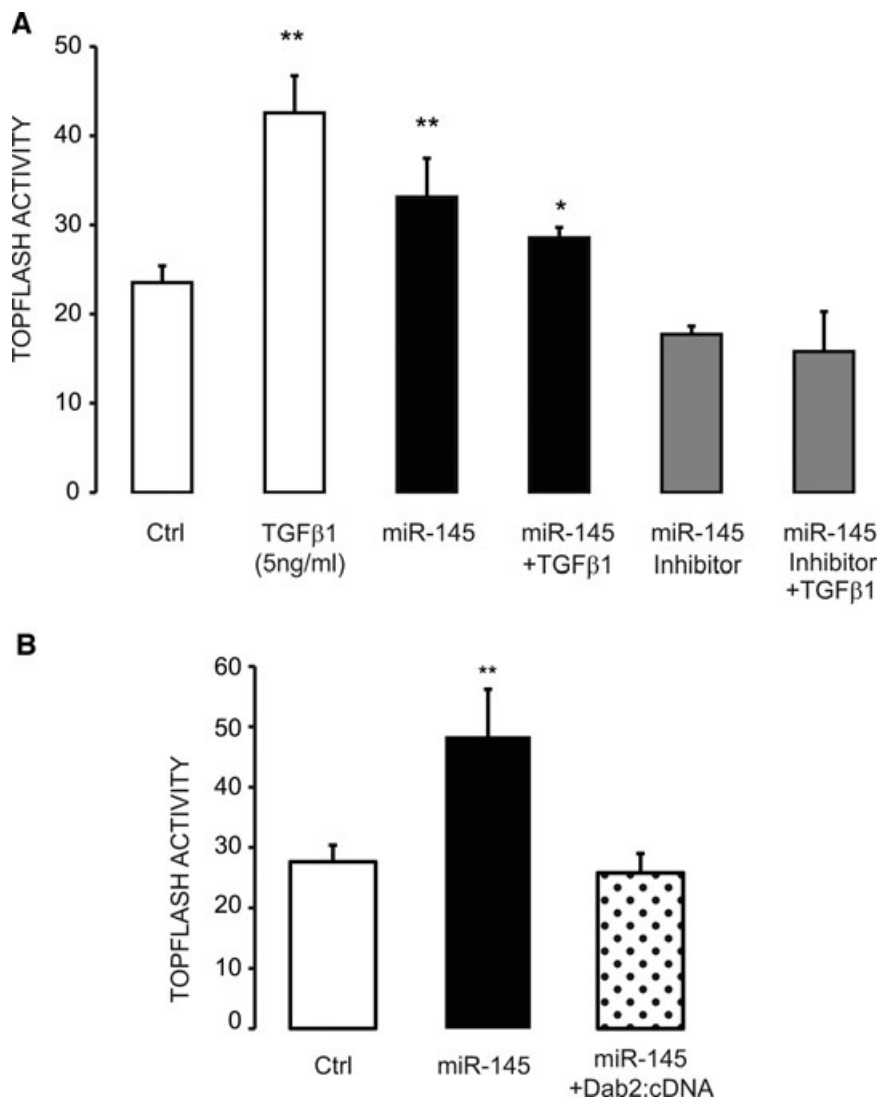


Fig. 4 miR-145 regulates wnt/ β -catenin activity in MSC. **(A)** TGF- β_1 treatment induced a two-fold increase in luciferase activity of the TOPFLASH LIF promoter. MSC transduced with miR-145 mimic (black bars) similarly exhibited increased luciferase activity, whereas the presence of the miR-145 inhibitor (grey bars) inhibited wnt/ β -catenin activity even in the presence of TGF- β_1 treatment. **(B)** The over-expression of Dab2 in the presence of miR-145 blocked the up-regulation of TOPFLASH LIF promoter. Data represent mean \pm S.D. of three experiments performed in triplicates (** $P < 0.01$ and * $P < 0.05$ relative to CTRL).

decreased as a function of time after TGF- β_1 treatment and this decrease correlates with an up-regulation of miR-145.

We wanted to determine if the up-regulation of miR-145 was sufficient to inhibit Dab2 expression, we transduced MSC with miR-145. As shown in Figure 3A and B, miR-145 mimic in MSC leads to a significant down-regulation of Dab2 mRNA 24 hrs later (Fig. 3A) and corresponding decrease in Dab2 protein (Fig. 3B). The down-regulation of Dab2 mRNA and protein by miR-145 was of a similar magnitude as seen in response to TGF- β_1 treatment.

To determine if the up-regulation of miR-145 was required for the down-regulation of Dab2, we performed loss of function studies with a miR-145 inhibitor. We transduced MSC with miR-145 inhibitors before treating cells with TGF- β . The presence of miR-145 inhibitors effectively blocked the down-regulation of Dab2 mRNA and protein in response to TGF- β (Fig. 3A). These

results confirm the critical role of miR-145 in regulating Dab2 expression in MSC.

Dab2 levels are known to be inversely related to wnt/ β -catenin signalling; therefore, we wanted to determine if modulation of Dab2 by miR-145 was sufficient to induce wnt/ β -catenin activity. For these studies, we transfected MSC with the TOPFLASH LIF-luciferase reporter construct. LIF is a downstream effector of the wnt/ β -catenin signalling pathway that is repressed in the presence of Dab2 thus giving low levels of luciferase activity as compared to control cells. We co-transfected MSC with the TOPFLASH reporter with miR-145 mimic or miR-145 inhibitor for 24 hrs before treating MSC with TGF- β_1 . At the end of this treatment we collected cell lysates and measured luciferase activity as previously described [22–24]. We observed a significant increase in luciferase activity in cells exposed to TGF- β_1 and miR-145 mimic,

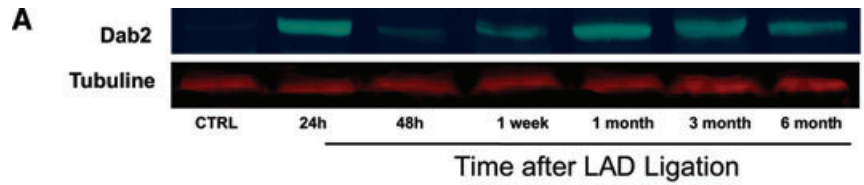


Fig. 5 Dab2 expression is increased in cardiomyocytes following myocardial infarction. **(A)** Western blot analysis of infarct and infarct-border zone from myocardial tissue at the specified times after LAD ligation. **(B)** Immunocytochemistry for nuclei (DAPI, blue), cardiac myosine (green), and Dab2 (red) on paraffin embedded cardiac tissue in sham (No MI) and 8, 24, and 48 hrs after myocardial infarction. Images demonstrate an increase in Dab2 expression in cardiomyocytes in the infarct border zone at 8 hrs post-AMI and maintained in surviving cells after 24 and 48 hrs.

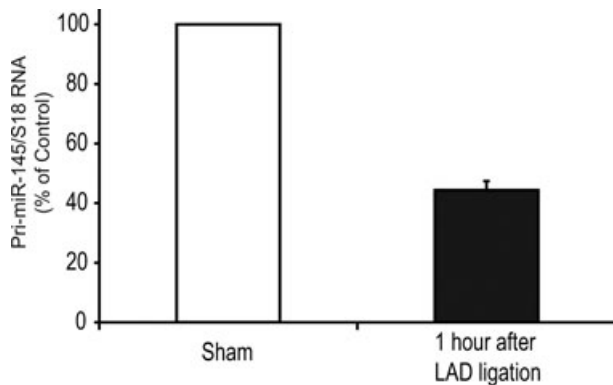
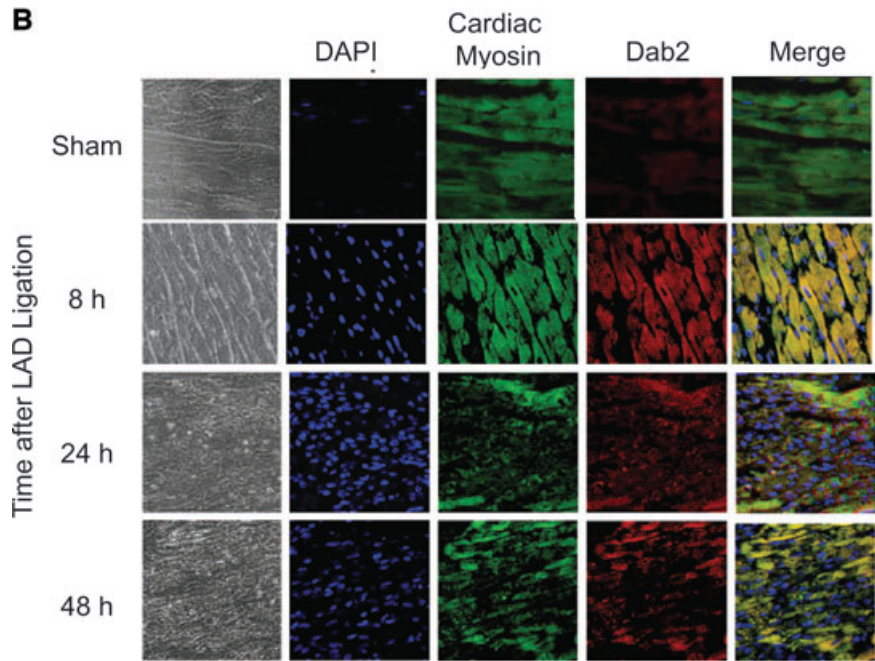


Fig. 6 miR-145 expression in infarcted rat myocardium. Total RNA was isolated from dissected infarct border zones from sham (No MI, open bar) and 1 hr (black bar) after AMI. The expression of miR-145 was analyzed by qPCR ($n = 5$ per group). A dramatic decrease in miR-145 expression was seen 1 hr after AMI. Results are presented as mean \pm S.D. (** $P < 0.01$, * $P < 0.01$ Student's t -test).

indicating that the down-regulation of Dab2 by miR-145 alone could be sufficient to induce wnt/ β -catenin signalling (Fig. 4A). Consistent with a link between the effects of miR-145 on Dab2

regulating wnt/ β -catenin activity is our observation that inhibition of miR-145 blocks the up-regulation of wnt/ β -catenin signalling in response to TGF- β (Fig. 4A).

To prove that Dab2 down-regulation is required for the up-regulation of wnt/ β -catenin by miR-145, we transfected MSC with both miR-145 and Dab2:cDNA. miR-145 cannot down-regulate the Dab2:cDNA because the 3'-UTR of cDNA was a poly A tail. The addition of Dab2:cDNA blocked the up-regulation of wnt/ β -catenin activity by miR-145; therefore, the down-regulation of Dab2 is required for the up-regulation of wnt/ β -catenin by miR-145. These data further demonstrate a critical role for Dab2 in regulating wnt/ β -catenin signalling.

To determine the *in vivo* relevance of Dab2 regulation following AMI, we quantified myocardial Dab2 levels as a function of time after LAD ligation. As shown in Figure 5A, there is no significant Dab2 expression in the myocardium at baseline; however, as early as 24 hrs after AMI, there is a significant increase in Dab2 protein in myocardial tissue and the Dab2 levels remain elevated for at least 6 months. To define the cell type in which Dab2 expression was up-regulated, we performed immunofluorescence studies with co-staining for cardiac myosin (Fig. 5B). These studies demonstrate that at baseline there is no Dab2 expression in cardiac myocytes but that as early as 8 hrs after AMI, there is

increased expression of Dab2 in surviving cardiac myocytes and that this expression persists for at least 48 hrs in cardiac myocytes in the infarct border zone.

To determine if miR-145 was responsible for the regulation of Dab2 in the heart in response to ischaemia, we quantified pri-miR-145 at 1 hr after LAD ligation. As shown in Figure 6, there is a significant level of pri-miR-145 in the heart at baseline that is rapidly and significantly decreased as early as 1 hr after AMI. These data are consistent with our findings *in vitro* with MSC and suggest that down-regulation of miR-145 in response to ischaemia results in an up-regulation of Dab2 protein in the heart.

Discussion

Numerous studies over the past decade have investigated the importance of Dab2 several cell functions since its original appearance as an oncogene in ovarian carcinoma [6]. Dab2 has been implicated in the regulation of cell positioning and trafficking by influencing cytoskeleton organization [23] induction of cell adhesion in immune cells [24–27] and intracellular cell signalling for mitogenic factors [22, 23, 28–30]. Dab2 has been shown to have a role in stem cell differentiation and is a key regulatory protein in the epithelial to mesenchymal transformation [22, 23]. Moreover, we recently demonstrated that Dab2 regulates the expression of cardiac proteins in adult MSC, impacting MSC behaviour and cardiac performance after MSC engraftment into infarcted heart tissue [12].

Dab2 lacks catalytic activity and apparently functions through physical interactions with other protein mediators [31, 32]. It has been traditionally considered as an adaptor protein that mediates intracellular signalling events from the activation of membrane receptors (*i.e.* TGF- β_1) to the triggering of gene transcription and protein expression [22, 23]. Also, Dab2 expression levels have been reported to be distinctly regulated depending on the cell type of interest. Its expression is increased in response to growth factor exposure in epithelial cells but decreases in MSC in response to the same stimuli. The mechanisms that regulate Dab2 expression in naïve cells, in activated cells and tumours are still largely unknown. In this regard, it has been recently demonstrated that Dab2 possess a TGF- β -activated translation element in its 3'UTR,

which specifically binds the heterogeneous nuclear riboprotein E1, resulting in repression of translation [33].

The 3'UTR region of Dab2 contains 1753 nucleotides and ranks as an average UTR in size. Our analysis suggested that the 3'UTR of Dab2 is could be targeted by a three miRNAs (miR-145, miR-203 and miR-17–5p) at five mRNA motifs. Three out of these five sites are targets for miR-145. A variety of functions have been described for miR-145 primarily in oncology [34–37] and there is increasing evidence of its role in controlling cell fate as well as cardiac development [38–40]. Our findings demonstrate that miR-145 has a critical role in the regulation of Dab2 in response to TGF- β_1 and that the miR-145 expression is altered in the myocardium in response to ischaemia. Our findings suggest a potentially important and novel role for miR-145 in regulating Dab2 levels in tumours. It is interesting to note that another target of miR-145 is the proto-oncogene c-myc [41]. Consistent with the multitude of targets for a specific miR, it is interesting that the up-regulation miR-145 decreases a tumour suppressor protein and increases oncogene simultaneously. With respect to the heart, our findings suggest a novel pathway that is involved in the regulation of wnt/ β -catenin signalling following AMI. It is interesting to note the miR-145 has been shown to be decreased in patients with coronary artery disease [42]. The down-regulation of miR-145 and up-regulation Dab2 following AMI suggests that wnt/ β -catenin signalling is down-regulated in cardiac myocytes following AMI. Future studies will need to target Dab2 or miR-145 in the myocardium to determine the extent to which this pathway can be manipulated to lead to improved myocardial healing following AMI.

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Conflict of interest

The authors confirm that there are no conflicts of interest.

References

1. **Barandon L, Couffignal T, Ezan J, et al.** Reduction of infarct size and prevention of cardiac rupture in transgenic mice overexpressing FrzA. *Circulation*. 2003; 108: 2282–9.
2. **Zelarayan LC, Noack C, Sekkali B, et al.** Beta-catenin downregulation attenuates ischaemic cardiac remodeling through enhanced resident precursor cell differentiation. *Proc Natl Acad Sci USA*. 2008; 105: 19762–7.
3. **Saraswati S, Alfaro MP, Thorne CA, et al.** Pyrvinium, a potent small molecule Wnt inhibitor, promotes wound repair and post-MI cardiac remodeling. *PLoS ONE*. 2010; 5: e15521.
4. **Templin C, Kotlarz D, Faulhaber J, et al.** *Ex vivo* expanded hematopoietic progenitor cells improve cardiac function after myocardial infarction: role of beta-catenin transduction and cell dose. *J Mol Cell Cardiol*. 2008; 45: 394–403.
5. **Hahn JY, Cho HJ, Bae JW, et al.** Beta-catenin overexpression reduces myocardial infarct size through differential effects on cardiomyocytes and cardiac fibroblasts. *J Biol Chem*. 2006; 281: 30979–89.

6. **Mok SC, Wong KK, Chan RK, et al.** Molecular cloning of differentially expressed genes in human epithelial ovarian cancer. *Gynecol Oncol.* 1994; 52: 247–52.
7. **Schwahn DJ, Medina D.** p96, a MAPK-related protein, is consistently downregulated during mouse mammary carcinogenesis. *Oncogene.* 1998; 17: 1173–8.
8. **Tseng CP, Ely BD, Li Y, et al.** Regulation of rat DOC-2 gene during castration-induced rat ventral prostate degeneration and its growth inhibitory function in human prostatic carcinoma cells. *Endocrinology.* 1998; 139: 3542–53.
9. **Huang Y, Friess H, Kleeff J, et al.** Doc-2/hDab2 expression is up-regulated in primary pancreatic cancer but reduced in metastasis. *Lab Invest.* 2001; 81: 863–73.
10. **Mok SC, Chan WY, Wong KK, et al.** DOC-2, a candidate tumour suppressor gene in human epithelial ovarian cancer. *Oncogene.* 1998; 16: 2381–7.
11. **Hocevar BA, Smine A, Xu XX, et al.** The adaptor molecule Disabled-2 links the transforming growth factor beta receptors to the Smad pathway. *EMBO J.* 2001; 20: 2789–801.
12. **Mayorga ME, Dong F, Sundararaman S, et al.** Central role for Disabled-2 in mesenchymal stem cardiac protein expression and functional consequences after engraftment in acute myocardial infarction. *Stem Cells Dev.* 2010; 20: 681–93.
13. **Cliffe A, Hamada F, Bienz M.** A role of dishevelled in relocating axin to the plasma membrane during wingless signaling. *Curr Biol.* 2003; 13: 960–6.
14. **Wong HC, Bourdelas A, Krauss A, et al.** Direct binding of the PDZ domain of dishevelled to a conserved internal sequence in the C-terminal region of frizzled. *Mol Cell.* 2003; 12: 1251–60.
15. **Hocevar BA, Mou F, Rennolds JL, et al.** Regulation of the Wnt signaling pathway by Disabled-2 (Dab2). *EMBO J.* 2003; 22: 3084–94.
16. **Tong H, Imahashi K, Steenbergen C, et al.** Phosphorylation of glycogen synthase kinase-3beta during preconditioning through a phosphatidylinositol-3-kinase-dependent pathway is cardioprotective. *Circ Res.* 2002; 90: 377–9.
17. **Barandon L, Dufourcq P, Costet P, et al.** Involvement of FrzA/sFRP-1 and the Wnt/frizzled pathway in ischaemic preconditioning. *Circ Res.* 2005; 96: 1299–306.
18. **Wong N, Hui AB, Fan B, et al.** Molecular cytogenetic characterization of nasopharyngeal carcinoma cell lines and xenografts by comparative genomic hybridization and spectral karyotyping. *Cancer Genet Cytogenet.* 2003; 140: 124–32.
19. **Lo KW, Teo PM, Hui AB, et al.** High resolution allelotype of microdissected primary nasopharyngeal carcinoma. *Cancer Res.* 2000; 60: 3348–53.
20. **Zhang M, Mal N, Kiedrowski M, et al.** SDF-1 expression by mesenchymal stem cells results in trophic support of cardiac myocytes after myocardial infarction. *FASEB J.* 2007; 21: 3197–207.
21. **Akiyama Y, Watkins N, Suzuki H, et al.** GATA-4 and GATA-5 transcription factor genes and potential downstream antitumour target genes are epigenetically silenced in colorectal and gastric cancer. *Mol Cell Biol.* 2003; 23: 8429–39.
22. **Jiang Y, Prunier C, Howe PH.** The inhibitory effects of Disabled-2 (Dab2) on Wnt signaling are mediated through Axin. *Oncogene.* 2008; 27: 1865–75.
23. **Sheng Z, Sun W, Smith E, et al.** Restoration of positioning control following Disabled-2 expression in ovarian and breast tumour cells. *Oncogene.* 2000; 19: 4847–54.
24. **Rosenbauer F, Kallies A, Scheller M, et al.** Disabled-2 is transcriptionally regulated by ICSBP and augments macrophage spreading and adhesion. *EMBO J.* 2002; 21: 211–20.
25. **Morris SM, Arden SD, Roberts RC, et al.** Myosin VI binds to and localises with Dab2, potentially linking receptor-mediated endocytosis and the actin cytoskeleton. *Traffic.* 2002; 3: 331–41.
26. **Morris SM, Tallquist MD, Rock CO, et al.** Dual roles for the Dab2 adaptor protein in embryonic development and kidney transport. *EMBO J.* 2002; 21: 1555–64.
27. **Inoue A, Sato O, Homma K, et al.** DOC-2/DAB2 is the binding partner of myosin VI. *Biochem Biophys Res Commun.* 2002; 292: 300–7.
28. **Smith ER, Capochichi CD, He J, et al.** Disabled-2 mediates c-Fos suppression and the cell growth regulatory activity of retinoic acid in embryonic carcinoma cells. *J Biol Chem.* 2001; 276: 47303–10.
29. **Zhou J, Hsieh JT.** The inhibitory role of DOC-2/DAB2 in growth factor receptor-mediated signal cascade. DOC-2/DAB2-mediated inhibition of ERK phosphorylation via binding to Grb2. *J Biol Chem.* 2001; 276: 27793–8.
30. **Prunier C, Howe PH.** Disabled-2 (Dab2) is required for transforming growth factor beta-induced epithelial to mesenchymal transition (EMT). *J Biol Chem.* 2005; 280: 17540–8.
31. **Xu XX, Yang W, Jackowski S, et al.** Cloning of a novel phosphoprotein regulated by colony-stimulating factor 1 shares a domain with the *Drosophila* disabled gene product. *J Biol Chem.* 1995; 270: 14184–91.
32. **Howell BW, Gertler FB, Cooper JA.** Mouse disabled (mDab1): a Src binding protein implicated in neuronal development. *EMBO J.* 1997; 16: 121–32.
33. **Chaudhury A, Hussey GS, Ray PS, et al.** TGF-beta-mediated phosphorylation of hnRNP E1 induces EMT via transcript-selective translational induction of Dab2 and ILE1. *Nat Cell Biol.* 2010; 12: 286–93.
34. **Kent OA, Chivukula RR, Mullendore M, et al.** Repression of the miR-143/145 cluster by oncogenic Ras initiates a tumour-promoting feed-forward pathway. *Genes Dev.* 2010; 24: 2754–9.
35. **Gao P, Wong, CC, Tung, EK, et al.** Deregulation of microRNA expression occurs early and accumulates in early stages of HBV-associated multistep hepatocarcinogenesis. *J Hepatol.* 2011; 54: 1177–84.
36. **Song T, Xia W, Shao N, et al.** Differential miRNA expression profiles in bladder urothelial carcinomas. *Asian Pac J Cancer Prev.* 2010; 11: 905–11.
37. **Chen Z, Zeng H, Guo Y, et al.** miRNA-145 inhibits non-small cell lung cancer cell proliferation by targeting c-Myc. *J Exp Clin Cancer Res.* 2010; 29: 151–60.
38. **Xu N, Papagiannakopoulos T, Pan G, et al.** MicroRNA-145 regulates OCT4, SOX2, and KLF4 and represses pluripotency in human embryonic stem cells. *Cell.* 2009; 137: 647–58.
39. **Chivukula RR, Mendell JT.** Abate and switch: miR-145 in stem cell differentiation. *Cell.* 2009; 137: 606–8.
40. **Cordes KR, Sheehy NT, White MP, et al.** miR-145 and miR-143 regulate smooth muscle cell fate and plasticity. *Nature.* 2009; 460: 705–10.
41. **Sachdeva M, Zhu S, Wu F, et al.** p53 represses c-Myc through induction of the tumour suppressor miR-145. *Proc Natl Acad Sci USA.* 2009; 106: 3207–12.
42. **Fichtlscherer S, De RS, Fox H, et al.** Circulating microRNAs in patients with coronary artery disease. *Circ Res.* 2010; 107: 677–84.