Mutual antagonism between IP₃RII and miRNA-133a regulates calcium signals and cardiac hypertrophy

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Introduction

Left ventricular hypertrophy is a maladaptive response to cardiac insults such as hypertension, damage, or aging (Dorn, 2007), and predisposes to heart failure and sudden death (Haider et al., 1998). Initially, the heart is enlarged through hypertrophy and concomitant pathological remodeling. Using a combination of in vivo and in vitro approaches, we demonstrated that IP₃-induced calcium release (IICR) initiated the hypertrophy-associated decrease in miR-133a. In this manner, hypertrophic stimuli that engage IICR set a feedforward mechanism in motion whereby IICR decreased miR-133a expression, further augmenting IP₃RII levels and therefore pro-hypertrophic calcium release. Consequently, IICR can be considered as both an initiating event and a driving force for pathological remodeling.

I nostitol 1,4,5'-triphosphate receptor II (IP₃RII) calcium channel expression is increased in both hypertrophic failing human myocardium and experimentally induced models of the disease. The ectopic calcium released from these receptors induces pro-hypertrophic gene expression and may promote arrhythmias. Here, we show that IP₃RII expression was constitutively restrained by the muscle-specific miRNA, miR-133a. During the hypertrophic response to pressure overload or neurohormonal stimuli, miR-133a down-regulation permitted IP₃RII levels to increase, instigating pro-hypertrophic calcium signaling of individual cardiomyocytes and extensive fibrosis within the ventricular wall. As the condition evolves, myocyte death is common and promotes the progression to heart failure. Studies in both animals and humans have indicated that remediation of the hypertrophic phenotype at the early stages of the disease improves outcome without compromising cardiac function (Hill et al., 2000; Kjeldsen et al., 2002). Hence, gaining a greater understanding of the mechanisms responsible for left ventricular hypertrophy may suggest novel therapeutic strategies.

Structural and molecular remodeling instigates pathological growth of the heart. Many of these maladaptive changes develop as a result of altered cardiomyocyte transcriptional

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Supplemental Material can be found at: /content/suppl/2012/11/15/jcb.201111095.DC1.html
profiles, induced through the coordinated action of a network of transcription factors activated by neurohormonal stimuli and/or stretch (Wu et al., 2006; Higazi et al., 2009). This produces genome-wide changes in gene expression and permits expression of the proteins responsible for pathological myocardial growth. Signaling pathways regulated by changes in intracellular calcium are central to the transduction of pro-hypertrophic stimuli, governing the activation of transcriptional regulatory mechanisms including calcineurin-nuclear factor of activated T cells (Cn-NFAT; Higazi et al., 2009; Nakayama et al., 2010; Rinne and Blatter, 2010) and CaM-dependent kinase II–histone deacetylase 4–myocyte enhancer factor 2 (CaMKII–HDAC4–MEF2; Wu et al., 2006). During hypertrophy and heart failure, activity of these regulators is enhanced as a consequence of the increased amplitude and altered localization of their upstream stimulatory calcium signals. For example, adult ventricular myocytes express low levels of the inositol 1,4,5′-triphosphate II (IP₃RII) calcium channel within the membranes of the sarcoplasmic and perinuclear calcium stores at baseline (Lipp et al., 2000; Guatimosim et al., 2008; Luo et al., 2008; Higazi et al., 2009). However, in failing human myocardium and cardiomyocytes from genetic and experimentally induced animal models of hypertrophy, IP₃RII levels are increased (Harzheim et al., 2009, 2010; Nakayama et al., 2010). Under these conditions, IP₃₁-induced calcium release (IICR) from IP₃RII has deleterious effects on cellular function. Ectopic release of calcium from the sarcoplasmic reticulum has pro-arrhythmic properties (Harzheim et al., 2009), while IICR in the perinuclear region engages the activity of calcium-dependent transcriptional regulators, leading to the induction of pro-hypertrophic patterns of gene expression (Wu et al., 2006; Higazi et al., 2009; Guo et al., 2012). Intriguingly, we have previously reported that increased protein expression of IP₃RII during hypertrophy is not accompanied by an increase at the mRNA level (Harzheim et al., 2009), suggesting that receptor expression is up-regulated post-transcriptionally.

Notably, post-transcriptional regulatory mechanisms are known to fulfill a critical role in both cardiac development and disease (Small and Olson, 2011) and commonly involve the activity of micro-RNAs (miRNAs). miRNAs are small noncoding RNAs that influence gene expression by binding to sequences that include the 3′ untranslated region (3′UTR) of newly synthesized mRNA transcripts. As a result, protein expression from these transcripts is inhibited by mRNA degradation or translational repression (Bartel, 2004). The power of miRNA-mediated regulation of gene expression is illustrated by the observation that a single miRNA can target hundreds of mRNAs (Lim et al., 2005; Matkovich et al., 2011), providing a mechanism whereby a network of genes can be subject to coordinated and simultaneous regulation. Consequently, a change in the pattern of miRNAs expressed by a cardiomyocyte could generate genome-wide remodeling of gene expression and hence induce hypertrophy. Consistent with this hypothesis, miRNA expression is altered in a diagnostic manner in left ventricular samples taken from humans suffering from a variety of cardiac pathologies (Ikeda et al., 2007) and in mouse models of hypertrophic remodeling (van Rooij et al., 2006). More specifically, miRNAs including miR-1 (Sayed et al., 2007; Li et al., 2010b), miR-133a (Carè et al., 2007), and miR-208a (van Rooij et al., 2007) show altered expression in hypertrophy and have been directly implicated in induction of the hypertrophic response. These disease-associated profiles of miRNA expression are likely to be generated in response to hypertrophic stimuli by modification of miRNA transcription or processing mechanisms, elicited by the activity of intracellular signaling pathways. Defining precisely how miRNA expression and pro-hypertrophic pathways are interrelated is likely to provide important insight into the complexity of cardiomyocyte gene regulatory networks.

Here, we investigate the mechanisms that produce up-regulation of IP₃RII during hypertrophic cardiomyocyte remodeling, showing that decreased expression of miR-133a allows IP₃RII expression to increase, thereby promoting hypertrophy. Furthermore, by identifying that calcium released from IP₃RII suppresses miR-133a levels, we delineate a pro-hypertrophic positive feedback loop initiated and perpetuated by IICR.

**Results**

**IP₃RII protein expression increases in the cardiomyocyte during pressure overload-induced hypertrophy**

To examine the mechanisms responsible for increased IP₃₁-dependent signaling during pathological cardiac remodeling, we used an in vivo hypertrophy model. Aortic banding (AB) induces a pressure-overload hypertrophic response and eventual heart failure in adult rats, exemplified by an increase in the ratio of left ventricular weight to body weight (Fig. 1 a) and enhanced mRNA expression of the hypertrophic marker gene atrial natriuretic factor (ANF; Fig. 1 b). Echocardiography confirmed the presence of pathological left ventricular remodeling and contractile dysfunction (Fig. 1, c and d). Our previous observations have demonstrated an increase in IP₃RII protein expression that is restricted to the cardiomyocytes of the hypertrophic heart (Harzheim et al., 2009, 2010). This response is not recapitulated in the non-cardiomyocyte population, which expresses negligible amounts of IP₃RII protein under both control (Perez et al., 1997; Harzheim et al., 2010) and hypertrophic conditions (Harzheim et al., 2009, 2010). Consistent with these data, immunoblotting demonstrated that IP₃RII protein expression was increased in the AB samples (Fig. 1, e and f). Up-regulation of IP₃RII within the cardiomyocyte specifically was shown in immunofluorescence images of ventricular myocytes isolated from sham and AB rats (Fig. 1 g). Quantitation of the intensity and distribution of immunostained proteins by 2D Fourier analysis showed that expression of IP₃RII was increased in the AB samples, whereas RyR2 expression and distribution were unaltered (Fig. S1, a and b). Furthermore, the ratio of pixels positive for IP₃RII to RyR2 was increased in the hypertrophic myocytes (Fig. 1 h). Together, these data confirm that the increase in IP₃RII protein during hypertrophy is myocyte specific. Intriguingly, abundance of IP₃RII mRNA was unaltered (Fig. 1 i) and nascent IP₃RII RNA expression, a measure of transcriptional activity at the IP₃RII gene (Itrp2) locus, was decreased after AB (Fig. 1 j). Consequently, we considered a post-transcriptional regulatory mechanism to explain pro-hypertrophic IP₃RII up-regulation in the cardiomyocyte.
IP$_3$R11 expression is regulated by miR-133a. miRNAs are powerful mediators of post-transcriptional control of gene expression networks and have proven roles in cardiac development and disease (Small and Olson, 2011). To determine whether IP$_3$R11 expression is regulated in this manner, we visually inspected the 3’UTR of the Itpr2 gene for putative seed sequences for hypertrophy-related miRNAs. This analysis revealed a seed sequence for the anti-hypertrophic miRNA mno-miR-133a (Carè et al., 2007), which was conserved between rat, mouse, and human (Fig. 2 a). In view of this finding and previously published data that have demonstrated repression of miR-133a in hypertrophy (Carè et al., 2007), we measured miR-133a expression after AB. miR-133a levels were decreased in the AB samples (Fig. 2 b), thereby providing a potential mechanism for the increase in IP$_3$R11 expression. The hypertrophy-associated decrease in miR-133a expression was specific to the cardiomyocyte population, as after AB, miR-133a expression in noncardiomyocytes was unchanged (Fig. S1 c).

This result is in concordance with the observation that IP$_3$R11 protein expression is unaltered in noncardiomyocytes under hypertrophic conditions (Harzheim et al., 2010), suggesting that miR-133a and IP$_3$R11 expression levels may be inversely correlated. The genomic sequence encoding miR-133a is clustered bi-cistronically with the sequence for another muscle-specific miRNA, miR-1. Quantification of miR-1 levels in the AB samples demonstrated that expression of this miRNA is also decreased in hypertrophy (Fig. 2 c). However, as the Itpr2 3’UTR does not contain a seed sequence for miR-1, we chose to focus on regulation of IP$_3$R11 expression by miR-133a in our subsequent experiments.

Hence, to verify that IP$_3$R11 mRNA is a bona fide target for miR-133a, we analyzed the activity of a luciferase reporter that tests for miRNA regulation of potential target sequences. In this reporter, the 3’UTR of the Itpr2 was inserted downstream of constitutively expressed luciferase (Fig. 2 d). As a control, the putative miR-133a seed sequence within the Itpr2 3’UTR
miR-133a overexpression decreases IP3RII protein levels

To dissect the mechanisms underlying miR-133a regulation of the IP3RII, we conducted experiments using primary cultures of neonatal rat ventricular myocytes (NRVMs). First, we increased miR-133a expression using an adenoviral approach. miR-133a overexpression was confirmed by quantitative real-time PCR (qPCR; Fig. 3 a) and viral infection was monitored by the presence of coexpressed GFP (Fig. 3 b). In concordance with our hypothesis, miR-133a–overexpressing myocytes exhibited decreased IP3RII expression when compared with control myocytes (Fig. 3, c and d). These data indicate that increasing miR-133a down-regulates IP3RII.

Inhibition of miR-133a elicits IICR-dependent hypertrophic remodeling

To further establish the effect of miR-133a upon IP3RII expression, we inhibited miR-133a binding to its targets by antagonomir transfection. In the presence of the antagonomir, basal levels of the IP3RII were increased and the repressive effect of miR-133a overexpression was prevented (Fig. 3 e). This result indicates that under nonstimulated conditions in NRVMs, miR-133a

was mutated to prevent miR-133a association. Co-transfection of the luciferase reporters and a miR-133a expression plasmid into HEK293 cells revealed that luciferase activity was decreased by the wild-type Ipr2 3' UTR but not the mutant Ipr2 3' UTR (Fig. 2 e). Therefore, the Ipr2 3' UTR contains a functional miR-133a binding site. To substantiate these results, we analyzed IP3RII protein and mRNA expression in ventricular samples from miR133a-1, miR133a-2 double-knockout mice (Fig. 2, f–h; Liu et al., 2008). In the absence of miR-133a, IP3RII protein levels were increased (Fig. 2, f and g) while IP3RII mRNA expression was diminished (Fig. 2 h). Together, these data confirm that miR-133a regulates IP3RII post-transcriptionally. Although the 3' UTRs of Ipr1 and Ipr3 do not contain seed sequences for miR-133a, the abundance of their protein products was also examined. Expression of IP3RI and IP3RIII were unaltered in the hearts of knockout animals (Fig. 2 g), confirming that these receptor isoforms are not a target for miR-133a and that up-regulation of IP3 receptors is not a general consequence of the miR-133a knockout phenotype. Together, these results imply that lack of miR-133a–mediated repression during hypertrophy augments IP3RII protein expression.
ANF mRNA expression and cell surface area were increased in NRVMs transfected with the miR-133a antagomir (Fig. 3, h and i). Notably, in the presence of GFP-5P, antagomir transfection did not elicit an increase ANF mRNA expression (Fig. 3 h) or cell surface area (Fig. 3 i), indicating that IICR is required for the pro-hypertrophic effect of antagomir transfection. More significantly, these results suggest that when miR-133a activity is decreased, as is observed during hypertrophy in vivo (Fig. 1), IP3RII expression increases and can initiate IICR-dependent cellular remodeling.

Basal IP3RII expression is restricted by miR-133a

As miRNAs can target multiple different mRNAs (Bartel and Chen, 2004), a direct link between miR-133a, IP3RII, and hypertrophy was lacking. To establish such a link, we transfected
a target protector (TP) oligonucleotide designed to specifically protect the \textit{Itrp2} 3′ UTR from miR-133a without affecting other miR-133a targets (Choi et al., 2007). TP transfection resulted in increased IP3RII abundance compared with control TP (Fig. 4 a), whereas expression of another miR-133a target, serum response factor (SRF; Liu et al., 2008), was unaltered. The \textit{Itrp2} miR-133a site-specific TP-induced increase in IP3RII expression was also observed in experiments in which oligonucleotides targeting regions of the \textit{Itrp2} 3′ UTR proximal to but not overlapping with the miR-133a seed sequence were used instead of the manufacturer’s control for transfection. This indicates that the effect of the TP was due to inhibition of miR-133a binding to the \textit{Itrp2} 3′ UTR rather than disruption of the \textit{Itrp2} 3′ UTR structure (Fig. S2). Moreover, in cells transfected with these control TPs, SRF expression was unaltered (Fig. S2). Thus, by preventing miR-133a binding, the TP, IP3RII TP specifically induces IP3RII up-regulation, in the absence of changes in the expression of other targets of miR-133a. Using this approach allows the specific role of miR-133a-mediated regulation of IP3RII expression in the cardiomyocyte to be interrogated. Therefore, we examined whether preventing miR-133a-mediated IP3RII repression by TP transfection is sufficient to induce hypertrophic remodeling. Indeed, baseline expression of the hypertrophic marker ANF and cell surface area were increased in NRVMs transfected with the IP3RII TP (Fig. 4, b and c), confirming that the cardiomyocyte hypertrophic response is restricted by the inhibitory effect of miR-133a on the IP3RII. Therefore, when miR-133a-mediated IP3RII repression is removed, a hypertrophic phenotype develops.

\textbf{miR-133a-mediated regulation of IP3RII controls IICR and hypertrophic gene expression}

We extended our studies regarding the role of miR-133a-mediated suppression of IP3RII to the adult rat ventricular myocyte (ARVM), giving particular consideration to calcium homeostasis and hypertrophic remodeling. At baseline, ARVMs express a low level of IP3RII. Under conditions of ryanodine-receptor inhibition, these receptors produce calcium puffs when stimulated with cell-permeant IP3-ester (Tovey et al., 2001; Berridge et al., 2003; Harzheim et al., 2009). We aimed to repress IP3RII expression and consequently IICR by increasing miR-133a activity using adenovirus. miR-133a overexpression and the associated down-regulation of IP3RII were confirmed by qPCR (Fig. S3 a) and immunoblotting (Fig. S3 b), respectively. We then assessed the effect of increased miR-133a on IP3RII function in control and miR-133a-overexpressing ARVMs. Fluo-4–loaded cells were acutely treated with tetracaine to block the ryanodine receptor, while the IP3RII was stimulated with IP3-ester. When miR-133a was overexpressed, IP3-ester elicited significantly fewer calcium release events (Fig. 4 d), an effect that correlates with reduced IP3RII expression.

As IP3-ester-dependent calcium signals promote hypertrophic gene expression (Wu et al., 2006; Higazi et al., 2009; Nakayama et al., 2010), we next examined whether miR-133a overexpression is sufficient to modulate the hypertrophic phenotype both in vitro and in vivo. First, we used the spontaneously hypertensive rat (SHR) model of cardiac hypertrophy. These animals have a disease progression that closely mirrors that observed in human patients (Dillmann, 2008), as they develop hypertension, cardiac hypertrophy, and heart failure with increasing age. As observed in hearts from AB rats, isolated hypertrophic adult SHR myocytes exhibited an increase in IP3RII protein expression, unaltered IP3RII RNA levels (Fig. 4 e), and decreased expression of miR-133a (Fig. 4 f) when compared with the Wistar Kyoto (WKY) control. Befitting their hypertrophic status, ANF expression was increased in SHR myocytes (Fig. 4 g). Upon miR-133a overexpression, ANF expression returned to the control level (Fig. 4 g), indicating regression of the hypertrophic phenotype.

To establish a link between miR-133a-mediated regulation of IP3RII and hypertrophic remodeling in vivo, we analyzed mice infused via an osmotic mini-pump for 1 mo with saline or a miR-133a–specific antagonim oligo (Carè et al., 2007). It has been previously reported that inhibition of the activity of miR-133a by antagonim infusion elicits a hypertrophic response (Carè et al., 2007). This was exemplified by increased diastolic left ventricular posterior wall dimension (LVPWd) in the antagonim-treated mice (Fig. 4 h). Therefore, in the absence of miR-133a, hypertrophy is induced. Consistent with its role in hypertrophic remodeling, IP3RII protein expression was enhanced under antagonim-treated conditions (Fig. 4, i and j). Comparison of IP3RII protein levels in control mice and mice infused with a scrambled miR-133a antagonim revealed that IP3RII expression was unaffected by the scrambled oligonucleotide (Fig. S4, a and b). Hence, up-regulation of IP3RII is a specific effect of the miR-133a antagonim. Taken with the data arising from miR-133a inhibition/overexpression in neonatal and adult cardiomyocytes in vitro (Fig. 4, b, c, and g), these results demonstrate that miR-133a inhibits IP3RII expression in ventricular myocytes, restraining pro-hypertrophic IICR and preventing hypertrophic remodeling of the heart.
levels were significantly decreased upon ET-1 stimulation (Fig. 5 d), showing that reduced transcription rather than altered processing mediates the hypertrophy-associated decrease in miR-133a.

As pro-hypertrophic transcriptional activity is regulated by IICR (Wu et al., 2006; Higazi et al., 2009; Nakayama et al., 2010), we examined whether this signaling pathway participates in ANF in response to ET-1 was also prevented by miR-133a overexpression (Fig. 5 c). These data are consistent with the results from the in vivo models of hypertrophy, validating the use of ET-1–treated NRVMs to investigate miR-133a repression. Next, we tested whether the ET-1–induced reduction in miR-133a has a transcriptional mechanism of origin by quantifying expression of primary miR-133a (primiR-133a). Interestingly, primiR-133a expression was reduced upon ET-1 stimulation (Fig. 5 d), showing that reduced transcription rather than altered processing mediates the hypertrophy-associated decrease in miR-133a.

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IICR and spontaneous mild hypertrophy at 3 mo of age (Nakayama et al., 2010). We also investigated the effect of thoracic aortic constriction (TAC) on these mice. In control mice expressing the tetracycline transactivator (tTA) alone, TAC induced a decrease in miR-133a expression (Fig. 6 b) and an increase in IP3RII protein expression (Fig. 6 c). At baseline, miR-133a expression was lower in IP3RII-TG mice, with no further decrease produced upon TAC (Fig. 6 b). These data support the hypothesis that IICR is a key initiating signal, which suppresses miR-133a and induces hypertrophy. Interestingly, IP3RII overexpression did not elicit a similar decrease in miR-1 expression (Fig. S5 a), suggesting that the IICR-mediated signal has a specific effect on miR-133a.

Next, we performed the converse experiment, examining the effect of inhibition of IICR upon miR-133a and IP3RII levels in vivo. To this end, IICR was inhibited in NRVMs by GFP-SP overexpression (Fig. 3 f), and the effect of this manipulation on the ET-1—stimulated decrease in miR-133a quantitated. GFP-SP prevented both the ET-1—stimulated decrease in miR-133a (Fig. 5 e) and increase in IP3RII protein expression (Fig. 5 f). These data imply that IICR is required for ET-1—induced repression of miR-133a expression and the resulting up-regulation of IP3RII. Therefore, by diminishing miR-133a expression, IICR enhances expression of its source calcium channel. We further substantiated this hypothesis by using the Ipr2 3′ UTR reporter construct (Fig. 2 d) to more directly measure the repressive action of miR-133a on the Ipr2 3′ UTR. Activity of this reporter was increased in NRVMs treated with ET-1 (Fig. 5 g), reflecting the ET-1—induced decrease in miR-133a. In cells overexpressing GFP-SP, ET-1 did not elicit a change in reporter activity, supporting a mechanism whereby IICR diminishes miR-133a levels. Together, these data suggest an intriguing hypothesis, whereby increases in IP3RII expression would create a positive feedback loop to further increase IICR, driving miR-133a down-regulation and the hypertrophic response. Having demonstrated in NRVMs that IICR controls IP3RII expression through modulation of miR-133a levels, we next determined whether this mechanism is conserved in vivo. Consequently, we examined miR-133a expression in IP3RII-transgenic mice (Fig. 6 a), which exhibit enhanced IICR and spontaneous mild hypertrophy at 3 mo of age (Nakayama et al., 2010). We also investigated the effect of thoracic aortic constriction (TAC) on these mice. In control mice expressing the tetracycline transactivator (tTA) alone, TAC induced a decrease in miR-133a expression (Fig. 6 b) and an increase in IP3RII protein expression (Fig. 6 c). At baseline, miR-133a expression was lower in IP3RII-TG mice, with no further decrease produced upon TAC (Fig. 6 b). These data support the hypothesis that IICR is a key initiating signal, which suppresses miR-133a and induces hypertrophy. Interestingly, IP3RII overexpression did not elicit a similar decrease in miR-1 expression (Fig. S5 a), suggesting that the IICR-mediated signal has a specific effect on miR-133a.

Next, we performed the converse experiment, examining the effect of inhibition of IICR upon miR-133a and IP3RII levels in vivo. To this end, we used mice engineered to inducibly express the IP3-sponge, a fragment of IP3RII spanning its ligand-binding domain that binds free IP3 with very high affinity (Uchiyama et al., 2002; Nakayama et al., 2010). These mice show blunted hypertrophic responses to systemic infusion with the hypertrophic agonists angiotensin II and isoproterenol (Nakayama et al., 2010). IP3-sponge mice demonstrated elevated baseline miR-133a expression when compared with tTA mice (Fig. 6 d) and no significant change in miR-1 expression (Fig. S5 b). There was no difference in IP3RII protein expression.
stimulation. To examine the relationship between miR-133a and IICR using an alternative strategy, we analyzed samples from IP3RII knockout mice (Fig. 6 g; Futatsugi et al., 2005). miR-133a expression was significantly increased when the IP3RII was absent (Fig. 6 h), whereas miR-1 levels were unaltered (Fig. S5 c), mirroring the results observed in IP3-sponge mice. Taken together, the in vitro and in vivo data indicate that IICR specifically controls expression of miR-133a. Enhanced IICR decreases miR-133a expression, whereas inhibition of IICR increases miR-133a.

Reduced SRF activity contributes to IICR-dependent miR-133a suppression

Although our results demonstrate a role for IICR in regulation of miR-133a, a remaining question concerns the mechanism(s)
by which IICR signals can repress primiR-133a transcription. It is known that the transcriptional regulator SRF targets the miR-133a locus (Zhao et al., 2005) and it has also been reported to inhibit synthesis of the primary miRNA transcript (Zhang et al., 2011). Thus, we tested whether SRF was involved in IICR-mediated regulation of miR-133a expression. Using a SRF-sensitive luciferase reporter, we determined that ET-1 stimulates SRF transcriptional activity in an IICR-dependent manner in NRVMs (Fig. 7 a). Furthermore, ET-1 also up-regulated SRF protein levels (Fig. 7, b and c), which is unsurprising given that SRF is a known miR-133a target (Liu et al., 2008). To address whether SRF has a role in regulation of miR-133a expression, we used an adenovirus to overexpress SRF and siRNA to reduce SRF expression (Fig. 7, d and e). SRF overexpression increased miR-133a (Fig. 7 f), decreased IP3RII protein (Fig. 7, g and h), and was without effect on IP3RII mRNA/nascent RNA expression (Fig. 7 i), indicating a lack of effect of SRF on IICR transcription. Conversely, SRF knockdown increased IP3RII protein expression (Fig. 7, g and h). These results are consistent with a mechanism whereby SRF positively regulates miR-133a expression. However, SRF expression and activity are increased under hypertrophic stimulation, when miR-133a is down-regulated, suggesting a more complex interplay between miR-133a and SRF. This was further illustrated through the use of adenovirus to overexpress a dominant-negative (DN) form of SRF. DN-SRF lacks part of the transcription transactivation domain of SRF but retains the DNA-binding activity of the full-length protein (Belaguli et al., 1999). By binding to SRF target sequences while being unable to activate transcription, overexpression of the mutant has a dominant-negative effect on the activity of endogenous SRF. When DN-SRF was overexpressed, baseline miR-133a expression decreased (Fig. 7 j). This result supports the observation that under nonstimulated conditions, SRF activity drives miR-133a expression. DN-SRF prevented a further decrease in miR-133a expression after ET-1 treatment, implying that the repressive effect of pro-hypertrophic stimulation is mediated through inhibition of SRF transcriptional activity at the miR-133a loci.

The ability of SRF to regulate transcription in either an activating or repressive manner is determined by which of its many interacting proteins it is associated with (Posern and Treisman, 2006). We suggest that IICR promotes miR-133a suppression by altering the interaction and/or activity of one or more of these cofactors with SRF, leading to repression of SRF activity. One such protein is the homeodomain-only protein (HOP), which negatively regulates SRF-dependent transcriptional responses by recruiting class 1 HDACs (Kook et al., 2003). Hence, we next tested whether overexpression of HOP by adenoviral infection could mimic the effects of ET-1. miR-133a levels were diminished in HOP-overexpressing NRVMs, with ET-1 failing to elicit a further decrease (Fig. 7 k). In agreement with the reduced level of miR-133a, HOP overexpression increased baseline cell surface area (Fig. 7 l), indicative of hypertrophy. These findings support the notion that the inhibitory effect of ET-1 on miR-133a expression is mediated via a repressive transcription factor such as HOP, thus promoting development of a hypertrophic response. Further strengthening these observations, we demonstrate that HDAC activity is required for the ET-1–stimulated decrease in miR-133a. HDAC inhibition with trichostatin A (TsA) increased basal levels of miR-133a (Fig. 7 m) and prevented the inhibitory effect of ET-1 on miR-133a expression. As has been previously reported (Cao et al., 2011), TsA also prevented the ET-1–induced increased in ANF expression, confirming the role of HDACs in regulation of hypertrophic gene expression (Fig. 7 n).

Discussion

This study delineates an anti-hypertrophic role for miR133a that is intrinsic to the ventricular myocyte (Fig. 8). We show that in cardiomyocytes, the inhibitory action of miR-133a maintains low basal IP3RII expression. Consequently, miR-133a activity holds IICR in check and the pro-hypertrophic effect of this pathway is restrained. This maintains an appropriate level of myocyte growth until the balance between miR-133a and IP3RII is perturbed by pathological stimuli that elicit IICR. When IICR is engaged in this manner, miR-133a is suppressed via changes in SRF transcriptional activity/targeting. Hence, IP3RII expression increases. A deleterious positive feedback loop is generated because enhanced IICR promotes sustained repression of miR-133a, providing a powerful driving force for pathological remodeling. Consequently, by inhibiting IP3RII expression and IICR, miR-133a would be expected to prevent IICR-dependent pro-hypertrophic transcriptional responses.

By specific suppression of miR-133a, calcium arising from IP3RII increases expression of its source channel, thereby amplifying the intensity of the pro-hypertrophic calcium signal. This creates an inherently unstable system whereby enhanced IICR serves to continually repress miR-133a and persistently increase IP3RII expression. Interestingly, other pro-hypertrophic positive feedback loops have been identified involving miRNAs and the signaling pathways that regulate their expression. These include miR–1-dependent modulation of insulin-like growth factor–1 signaling (Elia et al., 2009) and miR-199b–mediated regulation of the NFAT negative regulatory kinase dual-specificity tyrosine phosphorylation–regulated kinase 1a (da Costa Martins et al., 2010). When taken together, these results indicate that several reciprocal regulatory loops may be active within the myocardium under pro-hypertrophic conditions. The self-perpetuating nature of these loops could serve to produce a sufficiently powerful and prolonged stimulus to induce genome-wide remodeling of gene expression and hence hypertrophic growth, overcoming mechanisms of negative regulation. The significance of a control loop involving miR-133a within this network is increased when collective consideration is given to the postulated targets of this miRNA. We have demonstrated regulation of IP3RII, while other studies show repression of CnA (Dong et al., 2010) and NFATc4 (Li et al., 2010a). By targeting the IICR–CcN–NFAT pathway at multiple levels, miR-133a is an effective brake on pro-hypertrophic transcription. Consequently, when miR-133a levels decline in response to IICR, the source of activating calcium (IP3RII), transducing mechanism (CnA), and effector (NFATc4) of the pathway are all up-regulated and pathological gene expression would be strongly promoted.
Figure 7. IICR affects miR-133a expression through modulation of SRF activity. (a) Luciferase reporter assay of SRF transcriptional activity in GFP/GFP-5P overexpressing NRVMs cultured in the presence and absence of 100 nM ET-1 for 24 h. (b) Immunoblot for SRF in control and ET-1–treated NRVM lysates. (c) Quantification of immunoblots as in b; n = 6. (d) Immunoblot for SRF in control or SRF-overexpressing NRVMs. (e) Immunoblot for SRF in control or siSRF-transfected NRVMs. (f) qPCR analysis of miR-133a expression in GFP control, SRF overexpressing, or SRF knockdown NRVMs. (g) Immunoblot to show IP3RII expression in GFP-control, SRF-overexpressing, and SRF knockdown NRVMs. (h) Quantification of immunoblots as in g; n = 4. (i) IP3RII nascent RNA expression in SRF-overexpressing NRVMs measured using qPCR. (j) qPCR analysis of miR-133a expression in control and DN-SRF-overexpressing NRVMs treated with ET-1. (k) miR-133a expression in control or HOP-overexpressing NRVMs cultured in the presence and absence of 100 nM ET-1 for 24 h. (l) Cell surface area quantification in control/HOP-overexpressing NRVMs. More than 300 cells taken from 4 separate experiments were measured per condition. (m) qPCR analysis of miR-133a expression in NRVMs treated with ET-1 in the presence and absence of 0.4 µM trichostatin A (TsA). (n) qPCR analysis of ANF expression in NRVMs treated as in k. Data are mean ± SEM from at least three independent experiments. *, P < 0.05; **, P < 0.01.
The IICR-dependent transcriptional mechanism of origin for disease-associated repression of miR-133a is a significant finding. The murine genome encodes two copies of miR-133a: miR-133a-1 and miR-133a-2. These are located in the mouse in the vicinity of and cotranscribed with the sequences for miR1-2 and miR-1-1, respectively (Liu et al., 2007). Muscle-specific expression of the miR-133a, miR-1 bicistron is regulated by SRF and MEF2 (Zhao et al., 2005; Liu et al., 2007). Therefore, it is intriguing that miR-133a and miR-1 are repressed during hypertrophy, when global SRF activity is enhanced. However, different context-dependent modes of operation of SRF have been reported in other cellular systems, allowing a variable pattern of gene expression to be elicited without a change in bulk SRF activity (Wang et al., 2004). This is achieved through the association of SRF with diverse function-specific cofactors (Niu et al., 2007) including HOP, Nkx2.5, GATA4, Elk1, and myocardin. Regulation of these cofactors by intracellular signaling pathways allows positive or negative regulation of SRF in response to cellular stimulation. As calcium regulates multiple signal transducers including CaMKII, Cn-NFAT, and protein kinase C, there are a number of plausible mechanisms whereby the activity of SRF could be altered in response to IICR, preventing transcriptional activity at the miR-133a loci. We show that hypertrophy-associated decreases in miR-133a require HDAC activity and that overexpression of a dominant-negative SRF mutant or the negative transcriptional regulator HOP mimics the effect of ET-1 stimulation. This suggests that repression of SRF activity at the miR-133a loci may be a driving mechanism for the hypertrophy-associated decrease in miR-133a. Future work will aim to establish how IICR mediates decreased miR-133a expression via SRF, without similarly repressing expression of miR-1. The presence of additional transcription factor response elements between the miR-1 and miR-133a genes provides the potential for mechanisms of differential regulation (Thum et al., 2008).

Our data are consistent with previously published observations, which demonstrated that manipulation of miR-133a activity in vivo is sufficient to mediate pathological cardiac growth. In particular, infusion of miR-133a antagonir is sufficient to induce hypertrophic remodeling in adult mice (Carè et al., 2007). We have demonstrated that under these conditions, IP3,RII expression is increased, thus amplifying pro-hypertrophic IICR. miR-133a antagonir transfection generated a similar effect on myocyte phenotype eliciting both an increase in IP3,RII expression and hypertrophy. Notably, under these conditions we showed that IICR was required for the hypertrophic remodeling that arises as a consequence of a reduction in miR-133a activity. These data therefore show that as well as suppressing miR-133a abundance, IICR is the direct and required downstream mediator of hypertrophy induced by reduced miR-133a and further emphasizes the importance of miR-133a-mediated inhibition of IP3,RII for the restriction of pathological cellular growth. However, other studies have indicated that miR-133a maintains cardiac function via anti-apoptotic and anti-fibrotic effects within the adult myocardium (Matkovich et al., 2010). Some of the differences in the published data can be accounted for by variation in experimental model, the developmental stage from which miR-133a is expressed, the time-point at which observations are taken, and the extent to which adaptive mechanisms may be engaged in transgenic animals. Nevertheless, given the multitude of targets of any one miRNA and the complexity of regulation of myocyte gene expression networks, it is conceivable that more than one function exists for miR-133a in the maintenance of the healthy adult heart. Furthermore, the gross effect of a lack of miR-133a activity may change depending on the extent of disease progression, with different functions taking center stage over time. We believe that our data, by defining both a cause and consequence of dysregulated miR-133a centered on the IP3,RII, provide a new insight into how remodeling of calcium signaling and transcription promotes arrhythmias, left-ventricular hypertrophy, and eventually, heart failure.

Materials and methods

Animal procedures

All experiments involving animals were performed in accordance with local ethical and welfare guidelines. Specifically, for experiments in the UK, in accordance with the guidelines from the code of practice for humane killing under Schedule 1 of the Animals (Scientific Procedures) Act 1986 (UK); for experiments in Norway, 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); for experiments in the USA, The Institutional Animal Care and Use Committee (Cincinnati, OH); and for experiments in Japan, the Japanese Physiological Society.

Antibodies and other reagents

Antiserum against the IP3,RII C-terminal region (NH2-CLGSNTPH/VHMPPH-OH) was raised in rat (Harzheim et al., 2009) and used for immunoblotting (IB) at...
was triturated and the supernatant placed in myocyte plating medium.

Isolation and culture of ARVMs

Male SHR and WKY rats (Harlan) were anaesthetized by CO2 inhalation with 66% N2O, 27% O2, and 7% isoflurane. Vevo 2100 (Visual Sonics) with a 24 MHz Microscan linear array transducer was used to obtain echocardiographic examinations. Ventricular dimensions and Doppler signals were recorded in the parasternal long axis view. After echocardiography, rats were intubated and ventilated through endotracheal intubation. The chest was opened, followed by excision of the heart. The four heart chambers were rapidly dissected, weighed, and frozen in liquid nitrogen. Left ventricular myocardium was used for further molecular analysis.

Mouse models

Protocols for generation of iP, RII and iPS- transgenic mice and TAC of miR-133a have been described previously (Nakayama et al., 2006, 2010). Cardiac-specific expression of mouse iP, R II or flag-tagged iPS- protein was generated by placing the respective cDNAs under the control of the RA beta/myo (Gao et al., 2003). Transgenic mice overexpressing these proteins were crossed with driver tetracycline transactivator transgenic mice. iP, RII knockout mice are disrupted within the first exon of the Itpr2 gene (Futatsugi et al., 2005). Single-transgenic miR133a-1mice or miR133a-2mice knockout mice were generated by replacing 68 bp of the premiR133a-1 stem-loop or 108 bp of the premiR133a-2 stem-loop with a neomycin resistance cassette flanked by 2 FRT sites (Lu et al., 2008). Double heterozygote mice were generated crossing miR133a-1mice with miR133a-2mice and then intercrossed to obtain double knockout mice lacking both miR133a-1 and miR133a-2.

Methods for infusion of mice with saline or the antagonor oligo via cosmetic mini-pump are described in Caré et al. (2007). In brief, antagonors were dissolved in saline solution at doses of 80 mg/kg body weight and loaded in Alzet osmotic minipumps (model 1003D; Alza). To prime the pumps for continuous delivery of the antagonor, loaded minipumps were placed in a flask with saline solution and incubated for 4 h at 37°C. Acti

Isolation and culture of ARVMs

Male SHR and WKY rats (Harlan) were anaesthetized by CO2 inhalation and killed by cervical dislocation. After the thorax was opened, the heart was dissected, and the tissue triturated to release cells. Supernatant containing cells were collected by centrifugation (5 min, 390 g, room temperature). At the end of the digests, resuspended cells were preplated in a T175 flask and incubated for 45 min at 37°C to allow contaminating fibroblasts to adhere. Subsequently, myocytes were recovered by centrifugation (5 min, 390 g, room temperature). Cells were plated onto tissue culture vessels coated with 1% gelatin. Cultures were >95% pure. 48 h after plating, myocytes were washed into serum-free medium (DMEM/M199 4:1, 1 mM sodium pyruvate, 5.5 µg/ml transferin, 5 ng/ml sodium selenite, 1 x antioxidant-antimycin [Invitrogen], 5% fetal calf serum [Invitrogen], 1 mM sodium pyruvate [PAA], 1 x MEM non-essential amino acids [Invitrogen], 1 x antibiotic-antimycin [Invitrogen], and 2 µM cytosine (beta-arabinofuranoside). After every second digest, plat

Molecular biology and preparation of adenovirus

To construct the Itpr2 3’ UTR luciferase reporter, the Itpr2 3’ UTR was PCR amplified from rat liver genomic DNA and cloned into psiChk2 (Promega). Due to the presence of an internal XhoI site, PCR product was subcloned into psiChk2. Forward primer sequence: 5’-GGT ACCACGCGTACACTAGTGTGGGACCCTC-3’; reverse primer sequence: 5’-CAOTGGCCGCGCCCTTAATACA-AACAAATTTTAAT-3’. This inserted the 3’ UTR into psiChk2. Next, the 5’ end of the 3’ UTR was PCR amplified, digested with XhoI, and inserted into the XhoI site of psiChk2. This generated a 3’ UTR-luciferase reporter construct. To construct the miR-133a binding site within the Itpr2 3’ UTR reporter construct, the 3’ UTR-luciferase reporter construct was used to perform site-directed mutagenesis. This construct was subcloned into psiChk2. To allow generation of adenovirus, miR-133a was subcloned into the psiShuttle using the BglII and HindIII sites. C-myc–tagged miR-133a was amplified, digested with XhoI and NotI, and inserted into the psiChk2 to recreate the full-length adenoviral shuttle vector (pShuttle H1 miR-133a) was created by subcloning the H1 promoter and miR-133a from pRNAT H1.2 psiChk2. To allow generation of adenovirus, miR-133a target protector and custom target protectors C1, C2, and IP3RII were reagents), calnexin (IB 1:2000, C4731, Sigma-Aldrich), α-actinin (IB 1:500, A-57732, Sigma-Aldrich), and ANF (IB 1:500, T-4014, Bachem). Endothel

Adult rat thoracic aortic constriction and echocardiography

~170-g male Wistar rats were anaesthetized by a mixture of 67% N2O, 27% O2, and 4% isoflurane, and subsequently intubated and ventilated with a mixture of 67% N2O, 28% O2, and 2% isoflurane. The ascending aorta was dissected through a right-sided thoracotomy, and a silk suture (3–0) was tightened around the ascending aorta and a steel wire (0.85 mm) proximal to the brachiocephalic trunk as described previously (Bratell et al., 2007). Immediately after the ligation, the steel wire was removed. The chest was closed and the rats extubated. Sham-operated animals served as controls and underwent the same surgical procedure, except tightening the silk suture around the ascending aorta. Buprenorphine was given as postoperative analgesia.

6 wks after aortic banding, rats were anaesthetized by a mixture of 66% N2O, 27% O2, and 4% isoflurane, and subsequently mask ventilated with a mixture of 67% N2O, 28% O2, and 2% isoflurane. Vevo 2100 (Visual Sonics) with a 24 MHz Microscan linear array transducer was used to obtain echocardiographic examinations. Ventricular dimensions and Doppler signals were recorded in the parasternal long axis view. After echocardiography, rats were intubated and ventilated through endotracheal intubation. The chest was opened, followed by excision of the heart. The four heart chambers were rapidly dissected, weighed, and frozen in liquid nitrogen. Left ventricular myocardium was used for further molecular analysis.

Published November 19, 2012
Genotype expression analysis

Total RNA was isolated from cardiac myocytes and tissue samples using the mirVana-miRNA isolation kit (Ambion) and cDNA was synthesized from 500 ng total RNA using the NCCode Vilo miRNA cDNA synthesis kit (Invitrogen). qPCR was performed using SYBR GreenER (Invitrogen) and primers specific for the sequence of interest. Reactions were performed using a Real-Time PCR Cycler (model CFX96; Bio-Rad Laboratories). For the analysis of mRNA expression, primers amplified targets spanning exon-exon boundaries. To analyze nascent RNA, expression primers amplified targets within the intron, allowing unspliced miR-112 RNA to be quantified. GAPDH, β-microglobulin, and tropinin T were used as internal normalization controls. Analysis was performed using the comparative ΔΔCt method (Vandesompele et al., 2002), whereby the geometric mean of the expression levels of three independent normalization controls is calculated and used as a normalization factor to compare the expression level of the gene of interest between samples from different experimental conditions.

Protein expression analysis

Tissue samples were placed in lysis buffer (10 mM Tris-HCl, pH 7.6, 1 mM EDTA, 1 mM EGTA, and protease and phosphatase inhibitor cocktails [Sigma-Aldrich]) and homogenized in a lysing matrix D tube (MP Bio) for 30 s at 40 m/s in a FastPrep machine (MPBio). Debris was pelleted by centrifugation for 1 min at 3,500 g and the remaining supernatant incubated on ice for 30 min. The membrane fraction was recovered from all sample types by centrifuging the lysate for 15 min at 100,000 g (4°C), and the resulting pellet solubilized in lysis buffer containing 140 mM NaCl and 1% Triton X-100. Protein concentration was determined using the BCA assay (Thermo Fisher Scientific). The expression level of the gene of interest between samples from different experimental conditions. miR-1 expression in control mice and mice overexpressing the IP 3-sponge was performed for 20 s every 30 s with the addition of 1 mM tetracaine in Hepes buffer and 1 mM tetracaine (a gift from S. Conway, University of Oxford, Oxford, UK) at the start of the second and third movies, respectively. A total of six movies was captured per cell. ImageJ was used to background subtract the data. Calcium release events were detected using SparkSpotter, an ImageJ plugin developed by Babraham Bioinformatics, which counted events with at least 100 intensity units difference above the mean value that lasted at least 4 frames.

Confocal imaging

WKY myocytes cultured on laminin-coated 16-mm coverslips were infected overnight with adenovirus to overexpress miR-133a or with empty vector control virus (both without fluorescent protein expression). The next morning, culture medium was removed and cells were loaded with 8 µM fluo-4 (Invitrogen) in Hepes buffer for 30 min at room temperature. After this incubation and one wash in Hepes buffer, cells were incubated in Hepes buffer for a further 30 min to deesterify the dye. Coverslips were then placed in a pacing chamber on the stage of a confocal imaging system (A1R; Nikon) and electrically paced at 0.33 Hz, 30 V at room temperature throughout the experiment. Only rod-shaped cells showing regular calcium transients in response to pacing were selected for further analysis. Movies were captured in NIS elements software at 120 frames/s with averaging every 2 frames, sector zoom 1.12, AA Plan Neofluar 10×/0.25 NA, using a 60×/1.24 NA ApoWorlimage objective (Nikon) and 488-nm laser to excite fluo-4 fluorescence. Imaging was performed for 20 s every 30 s with the addition of 1 mM tetracaine in Hepes buffer and 1 mM tetracaine + 10 µM IP3, ester (a gift from S. Conway, University of Oxford, Oxford, UK) at the start of the second and third movies, respectively. A total of six movies was captured per cell. ImageJ was used to background subtract the data. Calcium release events were detected using SparkSpotter, an ImageJ plugin developed by Babraham Bioinformatics, which counted events with at least 100 intensity units difference above the mean value that lasted at least 4 frames.

Statistical analysis

Results are presented as mean ± SEM. Statistical analyses were performed in GraphPad Prism using the one-sample t test for two groups and one-way ANOVA followed by Tukey analysis for two or more groups. P values <0.05 were considered significant.

Online supplemental material

Fig. S1 (a and b) shows 2D Fourier analysis of IP3R and RyR2 expression in ventricular myocytes from sham and AB rats. Fig. S1 c shows qPCR quantification of miR-133a expression in cardiomyocytes and noncardiac myocytes. Fig. S2 shows miR-133a expression in NRVMs transfected with control TPs or specific IP3R TP. Fig. S3 a shows qPCR quantification of miR-133a expression levels in ARVMs expressing GFP/GFP-miR-133a. Fig. S3 b shows IP3R protein expression in ARVMs expressing GFP/GFP-miR-133a. Fig. S4 a is an immunoblot showing IP3R expression in control and mice infused with scrambled miR-133a antagonist. Fig. S4 b is quantification of blots as in Fig. S4 a. Fig. S5 a is qPCR quantification of miR-1 expression in control and IP3Rtransgenic mice. Fig. S5 b shows miR-1 expression in control mice and mice expressing the IP3-αN.
protein. Fig. S5 c shows qPCR quantification of miR-1 expression in wild-type and IP3R1 knockout mice. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201111095/DC1.

We are grateful to S. Conway for IP3 ester; T. Lee for the SRF adenovirus; J.A. Epich for H2O2 plasma; V. Storchini for the β-c2 antibody; S. Walker and C. Seal (Imaging Facility, Babraham Institute, Cambridge, UK) for assistance with imaging; and the Roderick and Bootman laboratories for assistance throughout this work.

This work was supported by the British Heart Foundation (PG/07/040, PG/06/034), The Babraham Institute, The Royal Society (University Research Fellowship to H.L. Roderick), and the Biotechnology and Biological Sciences Research Council.

Submitted: 21 November 2011
Accepted: 25 October 2012

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