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MicroRNA-140 Provides Robustness to the Regulation of Hypertrophic Chondrocyte Differentiation by the PTHrP-HDAC4 Pathway

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Abstract

Growth plate chondrocytes go through multiple differentiation steps and eventually become hypertrophic chondrocytes. The parathyroid hormone (PTH)-related peptide (PTHrP) signaling pathway plays a central role in regulation of hypertrophic differentiation, at least in part, through enhancing activity of histone deacetylase 4 (HDAC4), a negative regulator of MEF2 transcription factors that drive hypertrophy. We have previously shown that loss of the chondrocyte-specific microRNA (miRNA), miR-140, alters chondrocyte differentiation including mild acceleration of hypertrophic differentiation. Here, we provide evidence that miR-140 interacts with the PTHrP-HDAC4 pathway to control chondrocyte differentiation. Heterozygosity of PTHrP or HDAC4 substantially impaired animal growth in miR-140 deficiency, whereas these mutations had no effect in the presence of miR-140. miR-140–deficient chondrocytes showed increased MEF2C expression with normal levels of total and phosphorylated HDAC4, indicating that the miR-140 pathway merges with the PTHrP-HDAC4 pathway at the level of MEF2C. miR-140 negatively regulated p38 mitogen-activated protein kinase (MAPK) signaling, and inhibition of p38 MAPK signaling reduced MEF2C expression. These results demonstrate that miR-140 ensures the robustness of the PTHrP/HDAC4 regulatory system by suppressing MEF2C-inducing stimuli.

Keywords

GROWTH PLATE; GENETIC ANIMAL MODELS; PTHRP; CELL/TISSUE SIGNALING; PARACRINE PATHWAYS; MOLECULAR PATHWAYS; DEVELOPMENT; BONE MODELING AND REMODELING; EPIGENETICS

Introduction

The growth plate is the primary driver of longitudinal bone growth. In order to achieve normal bone growth, differentiation and proliferation of chondrocytes, cellular components

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Disclosures

All authors state that they have no conflicts of interest.

of the growth plate, need to be tightly regulated.[1] Growth plate chondrocytes go through multiple steps of differentiation. Resting chondrocytes (also called periarticular chondrocytes in embryonic stages), located at the most epiphyseal end, differentiate into vigorously proliferating columnar chondrocytes, and further differentiate into postmitotic hypertrophic chondrocytes. These processes are achieved by precisely controlled expression of genes that regulate chondrocyte differentiation.

Small regulatory microRNAs (miRNAs) modulate gene expression mainly at the posttranscriptional level. miRNAs are generally thought to serve as "genetic buffers" by suppressing expression of undesired genes to ensure the gene expression pattern unique to specific cell types.[2] Observations that manipulation of a single miRNA generally has a relatively modest effect support the idea of miRNAs as a secondary, "safeguard" regulatory system of gene expression.[3,4] This notion also implies that a miRNA may play critical roles when the primary regulatory system is impaired.

miRNA-140 (miR-140), generated from the *Mir140* gene, is abundantly and relatively specifically expressed in chondrocytes. We and others have shown that loss of the *Mir140* gene causes a mild skeletal growth defect in mice.[5,6] miR-140 deficiency alters chondrocyte differentiation at multiple steps; differentiation of resting zone chondrocytes into columnar chondrocytes is inhibited, whereas differentiation of proliferating chondrocytes into hypertrophic chondrocytes is accelerated. Because of the accelerated hypertrophic differentiation, miR-140–null bones show advanced mineralization; nevertheless, the effect of miR-140 loss in chondrocyte differentiation is relatively mild.

The signaling molecules, Indian hedgehog (Ihh) and parathyroid hormone (PTH)-related peptide (PTHrP) (*Pthlh*, Mouse Genome Informatics; The Jackson Laboratory, Bar Harbor, ME, USA; http://www.informatics.jax.org), play a central role in regulation of chondrocyte differentiation. Ihh regulates chondrocyte differentiation and proliferation as well as expression of *PTHrP* in resting/periarticular chondrocytes. PTHrP negatively regulates differentiation of Ihh-expressing hypertrophic chondrocytes through the cAMP/PKA signaling pathway. Thus, Ihh and PTHrP form an autoregulatory system that controls chondrocyte differentiation.[1]

MEF2 transcription factors, particularly MEF2C and MEF2D, play a critical role in regulation of hypertrophic chondrocyte differentiation.[7] MEF2 activity directly controls hypertrophic differentiation; a reduction in MEF2 function through genetic ablation or overexpression of a repressive mutant MEF2 protein inhibits hypertrophic differentiation, whereas it is stimulated by MEF2 overactivity. Thus, signals that control hypertrophic differentiation appear to ultimately converge on regulation of MEF2 transcription factors. MEF2 activity is modulated by physiological regulators; class IIa histone deacetylases (HDACs) are some of the critical regulators of MEF2 transcription factors. Class IIa HDACs directly bind to MEF2 transcription factors to inhibit their function.[8,9] In chondrocytes, HDAC4 plays a particularly important role; *Hdac4*-null mice show acceleration of hypertrophic differentiation, a phenotype similar to that of PTHrP-null mice[10]; this phenotype is rescued by heterozygous loss of *Mef2c*.[7]

Nuclear translocation primarily determines HDAC4's activity. HDAC4 nuclear translocation is negatively regulated by direct binding of the chaperon protein, 14–3-3.[11] Dephosphorylation of the 14–3-3 binding site of HDAC4 dissociates HDAC4 from 14–3-3 and facilitates its nuclear translocation. The phosphorylation status of HDAC4 is determined by the balance between kinase and phosphatase activities. Salt-inducible kinases (SIKs) phosphorylate the 14–3-3 binding site of class II HDACs to suppress their nuclear localization. In chondrocytes, SIK3-deficiency increases nuclear HDAC4 and delays hypertrophic differentiation.[12] In contrast, protein phosphatase 2A (PP2A) dephosphorylates HDAC4 and facilitates its nuclear translocalization.[13,14]

Based upon the finding that PTHrP activates PP2A to dephosphorylate HDAC4 and increases its nuclear localization, it was proposed that PTHrP suppresses the function of MEF2 transcription factors by enhancing HDAC4's inhibitory action on MEF2 transcription factors.[13]

Because *Mir140*-null mice show mild acceleration of hypertrophy, we hypothesized that the miR-140 pathway might interact with the PTHrP/HDAC4 pathway to reinforce the regulatory system controlling hypertrophic chondrocyte differentiation. In this article, we present evidence that miR-140 modulates the PTHrP/HDAC4 pathway by suppressing MEF2C expression.

Materials and Methods

Mice

Mir140,[6] *Hdac4*,[10] *PTHrP*,[15] and *Ihh*[16] knockout mice, *Col2-PTHrP*,[17] and *Col2-caPPR*[18] transgenic mice have been described. Mice were of a mixed genetic background. Comparisons were made between littermates. The animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) and performed in accordance with the regulations and guidelines.

Skeletal preparation and histology

Alizarin red and Alcian blue staining was performed using a modified McLeod's method.[19] For histological analysis, mice were dissected, fixed in 10% formalin, decalcified in 10% EDTA, paraffin-processed, cut, and subjected to hematoxylin-eosin staining.

Primary chondrocyte isolation and culture

Isolation and culture of primary rib chondrocytes were performed as described.[6] After overnight culture, cells were trypsinized and replated at the concentration of 5×10^5/mL in DMEM containing 10% FCS. For assessment of p38 mitogen-activated protein kinase (MAPK) signaling, upon reaching confluence, primary chondrocytes were serum-starved for 3 hours before stimulating with 10% FCS. Cells were lysed at indicated time points and subjected to Western blot analysis.

Retrovirus production and infection

The RalA overexpression (pBabe-Puro-hRalA-wt) virus has been described.[20] The virus expressing an active from of Rac1 (caRac1)[21] was constructed by cloning the coding sequence into the pMSCV-neo vector (Clontech, Mountain View, CA, USA). The retrovirus for GFP has been described.[22] The *Dnpep*-expressing virus was constructed by cloning a PCR-amplified mouse Dnpep cDNA using primers, Dnpep5, 5′-CAGAATTCGCCAGTGAAGTCCTCGGAAGC-3′ and Dnpep3, 5′-CTCTCGAGTGAAAATCTACTT TAATAACCAACA-3′ into the pMSCV-neo vector at the *EcoR* I and *Xho* I site (underlines are *EcoR* I and *Xho* I recognition sequences). The *Creb3l1*-expressing virus was constructed by cloning a PCR-amplified mouse Creb3l1 cDNA using primers, Creb3l1 × 5, 5′-TTCTCGAGTGGAACCCGGCGCGATGGA-3′ and Creb3l1R3, 5′-TTGAATTCCTGGGTCTTGGAGTGGCCTA-3′ into the modified pMSCV vector[22] at the and *Xho* I and *EcoR* I site (underlines are *Xho* I and *EcoR* I recognition sequences). To generate the miR-140 expression construct, first a fragment of *Mir140* genomic sequence was PCR-amplified using primers, Pre-mir140–5 5′-CAAGGTTCCTGCCAGTGGTTTTACCCTATG-3′ and Pre-mir140–3, 5′-CAAGGTTCCTGTCCGTGGTTCTACCCTGTG-3′ and subcloned into the pTarget vector (Promega Madison, WI, USA). The *Sal* I/*EcoR* I fragment including the *Mir140* sequence was further cloned into the *Xho* I/*EcoR* I site of the modified pMSCV vector, pMSCV-GFP.[22] These Moloney murine leukemia virus (MMLV)-based viruses were packaged using the EcoPack packaging plasmid (Clontech, Mountain View, CA, USA).

Transfection and luciferase assay

DNA containing 0.18 μg of a Mef2 luciferase reporter vector[23] and 0.02 μg of a *Renilla* luciferase control vector was transfected into primary rib chondrocytes using the Attractene transfection reagent (Qiagen Valencia, CA, USA). Luciferase and *Renilla* activities were measured 24 hours after transfection using the Dual-Luciferase Reporter Assay System (Promega).

Quantitative RT-PCR

cDNA synthesis was performed using random hexamers using the DyNAmo cDNA Synthesis Kit (Finnzymes, Waltham, MA, USA). Quantitative PCR was performed using the StepOnePlus Real-Time PCR System (Applied Biosystems,Grand Island, NY, USA) and the Eva Green qRT-PCR mix(Solis Bio Dyne,Tartu, Estonia). Primer sequences were as follows : Actb-L, 5′-GCACTGTGTTGGCATAGAGG-3′ and Actb-R, 5′-GTTCCGATGCCCTGAGGCTCTT-3′; GAPDH-L, 5′-CACAATTTCCATCCCAGACC-3′ and GAPDH-R, 5′-GTGGGTGCAGCGAACTTTAT-3′; Mef2c-L, 5′-CCCTTCGAGATACCCACAAC-3′ and Mef2c-R, 5′-ATGCGCTTGACTGAAGGACT-3′; Sox9-L, 5′-AGGAAGCTGGCAGACCAGTA-3′ and Sox9-R, 5′-CGTTCTTCACCGACTTCCTC-3′; RalA-L, 5′-GATGGAGTCCTTCGCAGCTA-3′ and RalA-R, 5′-ACGTTCCACTGGTCAGCTCT-3′; hRala-L, 5′-AAGTCATCATGGTGGGCAGT-3′ and hRala-R, 5′-AGCTGTCTGCTTTGGTAGGC-3′; Ihh-L, 5′-GAGCTCACCCCCAACTACAA-3′ and Ihh-R, 5′-TGACAGAGATGGCCAGTGAG-3′; Pthr1-L, 5′-CGCAGACGATGTCTTTACCA-3′ and

Pthr1-R, 5′-TCCACCCTTTGTCTGACTCC-3′; Creb3l1-L, 5′-TCTTTGATGACCCTGTGCTG-3′and Creb3l1-R, 5′-TGGTGTCCTCCATCTTGACA-3′;hRalA-L,5′-AAGTCATCATGGTGGGCAGT-3′ and hRalA-R, 5′-AGCTGTCTGCTTTGGTAGGC-3′; and Rac1-L, 5′-TATGGGACACAGCTGGACAA-3′ and Rac1-R, 5′-ACAGTGGTGTCGCACTTCAG-3′.

Expression of miR-140 was determined using the mirVana qRT-PCR miRNA Detection Kit (Ambion, Waltham, MA, USA).

Western blot analysis

Anti-MEF2C (#5030), anti-HDAC5 (#2082), anti-Histone 2A (#2578), and anti-Phospho-HDAC4 (Ser246)/HDAC5 (Ser259)/ HDAC7 (Ser155)(#3443) antibodies were purchased from Cell Signaling Technology, Denvers, MA, USA. Anti-MEF2D (BDB610774) was from BD Biosciences, San Jose, CA, USA, Anti-HDAC4 antibody (ab12172) was purchased from Abcam. AntiRalA antibody was from GeneTex, Irvine, CA, USA. Anti-Sox9 antibody (bs-4177R) was from Bioss, Woburn, MA, USA. Anti-Actin antibody (I–19) was purchased from Santa Cruz, Dallas, Texas, USA Biotechnology. Anti-GAPDH antibody (10R-2932) was purchased from Fitzgerald Industries International, Acton, MA, USA. Western blot analysis was performed according to the standard procedure.

Results

Skeletal phenotypes of *Mir140*-null mice can be partially rescued by overactivation of PTHrP signaling

Loss of *Mir140* causes a mild growth impairment and shortening of the longitudinal dimension of the skull.[6] Lengths of endochondral bones, including those in the basal skull, are reduced due to the decrease in number of columnar proliferating chondrocytes of the growth plate. The reduction in number of columnar chondrocytes is caused by an inhibition of differentiation of resting zone chondrocytes into columnar chondrocytes and an acceleration of hypertrophic differentiation of the columnar chondrocytes. Because hypertrophic differentiation is negatively regulated by PTHrP signaling, we hypothesized that *Mir140* loss might compromise PTHrP signaling. In order to test this hypothesis, we first attempted to rescue the skeletal phenotype of *Mir140*-null mice by overactivating PTHrP signaling. In order to overactivate PTHrP signaling, we crossed *Mir140*-null mice with transgenic mice overexpressing a constitutively active PTH/ PTHrP receptor (PPR; *Pthr1*; Mouse Genome Informatics) in chondrocytes (*Col2-caPPR*) or transgenic mice expressing PTHrP (*Pthlh*) in chondrocytes (*Col2-PTHrP*). We used *Col2-caPPR* mice for long bone analysis because *Col2-caPPR* mice show relatively normal long bones except for a delay in the initial hypertrophic differentiation during embryonic stages, whereas *Col2-PTHrP* mice show severe deformities in long bones. In E14.5 embryos, *Mir140* loss increased the size of the mineralized domain of the ulna and radius due to acceleration of chondrocyte hypertrophy,[6] whereas *caPPR* overexpression delayed mineralization (Fig. 1A). When *caPPR* was overex-pressed in *Mir140*-null ($Mir140^{-/-}$) mice, the compound mutant ($Mir140^{-/-}$ *Col2-caPPR*) mice showed delayed mineralization, a phenotype similar to that of *Col2-caPPR* mice (Fig. 1A). Another prominent skeletal defect of *Mir140*-null

mice was a longitudinal growth defect of the skull due to growth plate abnormalities of the basal skull.[6] This phenotype was partially corrected by simultaneous overexpression of PTHrP in chondrocytes (*Col2-PTHrP*), whereas PTHrP overexpression alone caused a mild global growth impairment but showed little effect on the skull shape, again demonstrating that overactivation of PTHrP signaling partially rescued skeletal defects of *Mir140*-null mice (Fig. 1B).

Mir140 deficiency and *PTHrP* heterozygosity synergistically impair skeletal growth

In order to test whether miR-140 interacts with the PTHrP signaling pathway, we crossed *Mir140*-null mice to *PTHrP* (Pthlh) heterozygous null ($PTHrP^{+/-}$) mice. Whereas $PTHrP^{+/-}$ mice showed normal growth, and *Mir140*-null mice showed only a mild growth impairment, compound mutant mice ($Mir140^{-/-}$ $PTHrP^{+/-}$) showed a significant growth defect compared with control or *Mir140*-null mice (Fig. 2A, B). Although the expansion of the resting zone was similarly present in the compound mutant mice, the columnar region of the growth plate was significantly shortened, suggesting premature hypertrophic differentiation (Fig. 2C, D).

Because Ihh is a critical upstream regulator of PTHrP, we also bred *Mir140*-null mice with *Ihh* heterozygotes ($Ihh^{+/-}$). *Ihh* heterozygosity did not cause detectable skeletal growth defects, and $Mir140^{-/-}$ $Ihh^{+/-}$ compound mutant mice also showed growth identical to that of $Mir140^{-/-}$ mice (Fig. 2E, F). This result may suggest that miR-140 functions at a level downstream of the PTHrP and PTHrP receptor in the Ihh-PTHrP system. Alternatively, the possible reduction of Ihh signaling in Ihh heterozygous knockout mice may not be sufficient enough to bring out the effect of miR-140-deficiency.

The miR-140 pathway and PTHrP pathway converges on MEF2 regulation

Using primary rib chondrocytes, we investigated the effect of miR-140 deficiency on components of the PTHrP/HDAC4 pathway. There were no overt changes in the protein level or phosphorylation status of HDAC4 or HDAC5 in *Mir140*-null chondrocytes (Fig. 3A). We also found that intracellular localization of HDAC4 was not altered (Supporting Fig. 1). However, we found that the level of MEF2C, but not MEF2D, was upregulated in *Mir140*-null mice (Fig. 3B). MEF2C upregulation was likely due to the increased *Mef2c* transcript abundance (Fig. 3C). We also found that the MEF2 transcriptional activity, assessed by a luciferase reporter assay, was mildly, but significantly, increased in PTH-treated *Mir140*-null chondrocytes (Fig. 3D). Conversely, overexpression of miR-140 in primary rib chondrocytes missing *Mir140* via retrovirus-mediated transduction decreased MEF2C expression level both at the protein and mRNA levels (Fig. 3E, F).

In order to further test whether MEF2C upregulation in *Mir140*-null mice contributes to premature hypertrophic differentiation, we crossed *Mir140*-null mice to *Hdac4* heterozygous null mice. *Hdac4* heterozygous mice showed a normal animal growth, whereas the compound mutant mice, $Mir140^{-/-}$ $Hdac4^{+/-}$, exhibited a significant growth impairment compared with *Mir140*-null or control mice (Fig. 4A, B). $Mir140^{-/-}$ $Hdac4^{+/-}$ mice showed shortening of the columnar proliferating chondrocytes, a histological finding similar to that of $Mir140^{-/-}$ $PTHrP^{+/-}$ mice (Fig. 4C, D). As expected, the Mef2 transcriptional activity in primary rib chondrocytes in $Mir140^{-/-}$ $Hdac4^{+/-}$ mice was significantly upregulated

compared with control (*Mir140*$^{+/-}$) (Fig. 4E). *Hdac4* heterozygosity alone showed no effect on Mef2 activity, suggesting that one allele of *Hdac4* is sufficient to suppress aberrant MEF2 activation.

Mir140-loss increases p38 MAPK signaling

Mef2c is not predicted to be a miR-140 target gene by publicly available computational prediction programs. In addition, our previous attempt to experimentally identify direct miR-140 targets failed to identify *Mef2c*.[6] Therefore, MEF2C upregulation in *Mir140*-null chondrocytes is likely caused indirectly by the deregulation of miR-140-target genes.

Previous studies identified several direct target genes of miR-140 in various contexts.[5,6,24–28] Among them, we confirmed that *Dnpep*,[6] *RalA*,[25] and *Creb3l1* (OASIS)[29] were upregulated in *Mir140*-null chondrocytes (Fig. 5A, B). In addition, we confirmed that *Rac1*, a predicted miR-140 target, was also upregulated in *Mir-140*–deficient chondrocytes (Fig. 5A). In a study using human mesenchymal stem cells (MSCs), miR-140 regulated Sox9 and RalA during chondrocyte differentiation.[25] However, we did not find alteration of Sox9 expression in primary mouse rib chondrocytes missing *Mir140* (Fig. 5B). In order to investigate whether upregulation of these miR-140 target genes play a causal role in *Mef2c* upregulation, we overexpressed *Dnpep*, *Creb3l1*, an active form of *Rac1*, or *RalA* in wild-type primary rib chondrocytes using a retrovirus expression system. Overexpression of none of these genes increased *Mef2c* expression, whereas expression of these genes was reasonably upregulated (Fig. 5C). MEF2C activity or expression can be regulated by multiple pathways. p38 MAPKs directly phosphorylate MEF2C and enhance its transcriptional activity to stimulate hypertrophic differentiation.[30] p38 MAPK overactivation in chondrocytes impairs endochondral bone growth in vivo.[31] We found that p38 MAPK signaling was upregulated in miR-140– deficient chondrocytes (Fig. 5D, E). Conversely, miR-140 overexpression in *Mir140*-null chondrocytes reduced p38 MAPK signaling (Fig. 5F). Inhibition of p38 MAPK signaling decreased MEF2C expression in a dose-dependent manner (Fig. 5G). These data suggest that the upregulation of p38 MAPK signaling contributes to the acceleration of hypertrophic differentiation of miR-140–deficient chondrocytes.

Discussion

miRNAs are considered to act as genetic buffers to suppress undesired gene expression to maintain the robustness of the gene expression pattern and phenotype of a cell. In this work, we found that the miR-140 pathway interacts with the PTHrP-HDAC4 pathway to control chondrocyte differentiation. PTHrP negatively regulates hypertrophic differentiation, at least in part, by enhancing HDAC4 function. In the presence of miR-140, PTHrP or HDAC4 produced from one allele of these genesis sufficient to maintain normal skeletal growth, and the absence of miR-140 alone causes a modest effect in endochondral growth. Therefore, our observation that *PTHrP* or *Hdac4* heterozygosity causes a substantial growth defectinmiR-140 deficiency is consistent with the idea that the miR-140 buffers the negative impact caused by a partial impairment of the primary regulatory system of chondrocyte differentiation. We previously showed that the mineralization of the talus of neonatal

Mir140-null mice is mildly advanced due to accelerated hypertrophic differentiation.[6] In this study, we found that compound mutant mice, *Mir140*$^{-/-}$ *PTHrP*$^{+/-}$ or *Mir140*$^{-/-}$ *Hdac4*$^{+/-}$, showed a further increase in mineralization compared with *Mir140*$^{-/-}$ mice, a result consistent with the data on skeletal growth, although this data needs to be interpreted cautiously because we found that both *PTHrP* and *Hdac4* heterozygosity showed advanced mineralization in this bone in the *Mir140* heterozygous background (Supporting Fig. 2A, B).

A previous study showed that PTHrP regulates MEF2C function by facilitating HDAC4 nuclear localization.[13] We found that miR-140 loss increased the expression and function of MEF2C without changing the abundance or phosphorylation of HDAC4. Thus, PTHrP and miR-140 appear to regulate hypertrophic differentiation through independent mechanisms, but nevertheless the PTHrP pathway and miR-140 pathway converge on MEF2C regulation. MEF2 transcription factors drive hypertrophic chondrocyte differentiation. *Mef2c* expression is upregulated in prehypertrophic chondrocytes.[7] This transcriptional upregulation of *Mef2c* upon chondrocyte differentiation likely creates a positive feedback loop that further accelerates hypertrophic differentiation.

Expression and function of MEF2 transcription factors is controlled by diverse upstream regulatory systems.[32] MAPKs, including p38 MAPKs, are major HDAC-independent regulators of MEF2C.p38 MAPKs phosphorylate MEF2C to augment its function in monocyte/macrophage-like cells,[33] B cells,[34] myoblasts,[35] cardiomyocytes,[36,37] and vascular endothelial cells.[38] In primary chondrocytes, p38 MAPK inhibition reduces MEF2C function and hypertrophic differentiation.[30] On the other hand, overactivation of p38 MAPK,via overexpression of a constitutive form of MKK6, in chondrocytes results in an endochondral bone growth defect and reduces the number of proliferating chondrocytes, suggesting that p38 MAPK signaling induces premature cell cycle exit and hypertrophic differentiation.[31] In light of these reports, it is likely that the upregulation of p38 MAPK signaling in miR-140–deficient chondrocytes, at least in part, contributes to the acceleration of the hypertrophic differentiation; p38 MAPK signaling enhances MEF2C function to promote the hypertrophic differentiation program that further increases MEF2C expression.

The mechanism by which miR-140 deficiency enhances p38 MAPK signaling is currently unclear. Rac1, which was upregulated in miR-140–deficient chondrocytes, regulates chondrocyte differentiation[39–41] and activates p38 MAPK signaling in chondrocytes.[42] However, it is unlikely that Rac1 is responsible for the upregulated p38 MAPK signaling and the phenotype of *miR-140*–null mice because Rac1 overexpression did not change *Mef2c* expression.

Based on these findings, we propose a model in which miR-140 enhances the robustness of the PTHrP/HDAC4 axis in controlling hypertrophic differentiation of chondrocytes by suppressing MEF2C-inducing stimuli. miR-140 suppresses the positive feedback loop in which MEF2C upregulates itself by promoting hypertrophic differentiation (Fig. 5H); this effect is likely mediated, at least in part, by downregulating p38 MAPK signaling.

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Authors' roles: GP, FM, TSL, SN, MNW, and TK designed and performed the experiments. TK wrote the manuscript.

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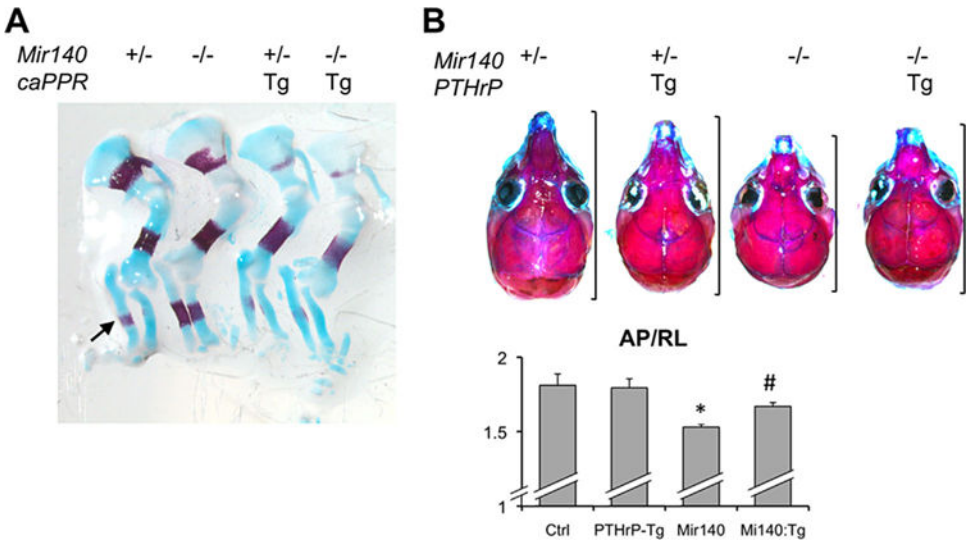

Fig. 1.

PTHrP overactivity rescues skeletal phenotypes of *Mir140*-null mice. (*A*) Alcian blue/Alizarin red-stained forelimbs of E14.5 embryos of indicated genotypes. *Mir140*-null mice show advanced bone mineralization in the radius and ulna compared with control *Mir140* heterozygotes (arrow), whereas transgenic expression of a constitutively active PTH/PTHrP receptor (caPPR) delays bone mineralization. Compound mutant mice, $Mir140^{-/-}$ *caPPR* show delayed mineralization similar to that of *caPPR* transgenic mice. This result was confirmed in 3 compound mutant mice. (*B*) Transgenic overexpression of PTHrP in chondrocytes ameliorates the shortening of the *Mr140*-deficient skull. Alizarin-red stained skulls of 3-week-old littermates of indicated genotypes. Brackets indicate the naso-occipital (anteroposterior, AP) length. The AP length relative to the skull width (RL) was calculated and compared among $Mir140^{-/-}$ (Ctrl), $Mir140^{-/-}$*:PTHrP-Tg* (PTHrP-Tg), $Mir140^{-/-}$ (Mir140), and $Mir140^{-/-}$*:PTHrP-Tg* (Mir140:Tg). *p < 0.01 versus Ctrl or PTHrP-Tg; #$p <$ 0.05 versus Mir140; $n \geq 3$.

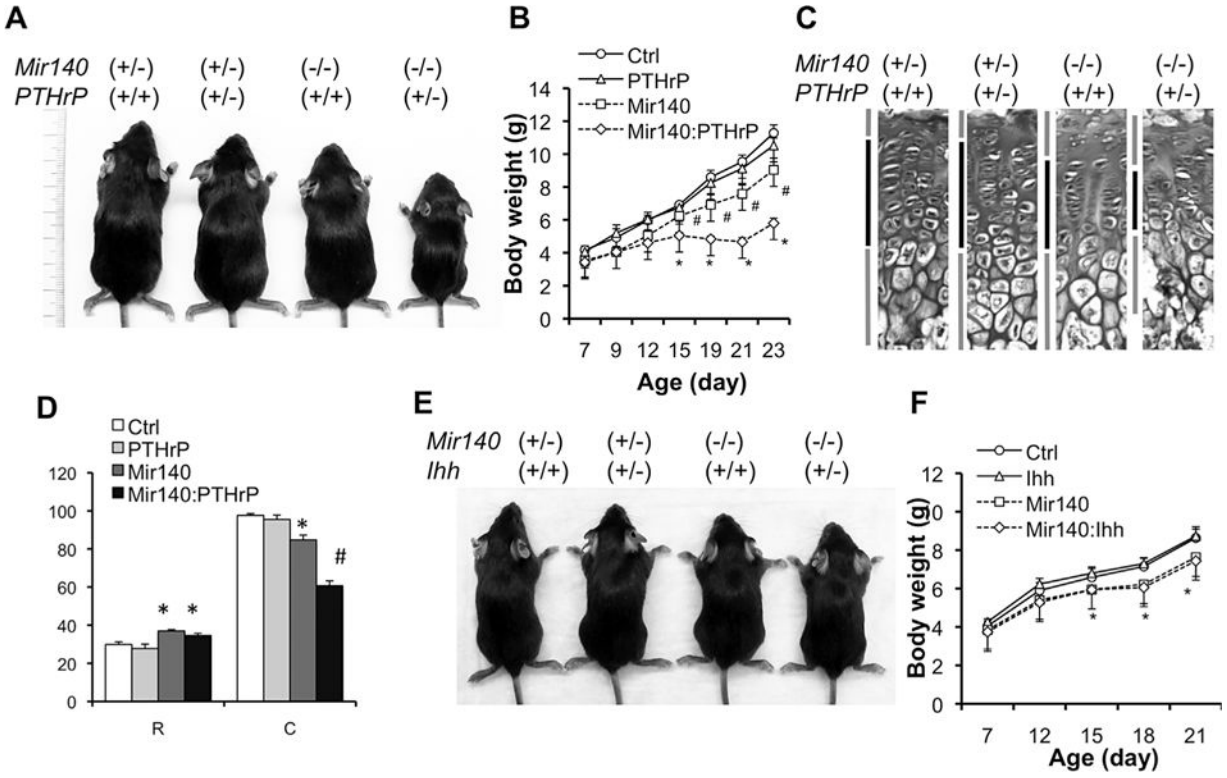

Fig. 2.

PTHrP heterozygosity exacerbates the growth defect of *Mir140*-null mice. (*A*) Pictures of 3-week-old littermates with indicated genotypes. (*B*) Growth curve of mice with indicated genotypes. Ctrl, $Mir140^{+/-}$ $PTHrP^{+/+}$ ($n = 6$); PTHrP, $Mir140^{+/-}$ $PTHrP^{+/-}$ ($n = 4$); Mir140, $Mir140^{-/-}$ $PTHrP^{+/+}$ ($n = 5$); Mir140:PTHrP, $Mir140^{-/-}$ $PTHrP^{+/-}$ ($n = 7$). #$p < 0.05$ versus Ctrl or PTHrP. *$p < 0.05$ versus Ctrl, PTHrP, or Mir140. (C) H&E-stained section of growth plates of the proximal tibia. Bars indicate the resting, columnar and hypertrophic regions. Doubly mutant mice show a significantly shorter columnar region than that of single mutants. (*D*) Quantification of growth plates of mice with indicated genotypes. Mice were euthanized at P23. The length of the resting zone (R) and the columnar proliferating zone (C) was measured. *$p < 0.05$ versus Ctrl or PTHrP; #$p < 0.05$ versus Ctrl, PTHrP, or Mir140; $n \geq 3$. (*E*) Pictures of 3-week-old littermates with indicated genotypes. (*F*) Ihh heterozygosity has no effect on growth of *Mir140*-null mice. Ctrl, $Mir140^{+/-}$ $Ihh^{+/+}$ ($n = 7$); Ihh, $Mir140^{+/-}$ $Ihh^{+/-}$ ($n = 7$); Mir140, $Mir140^{-/-}$ $Ihh^{+/+}$ ($n = 6$); Mir140:Ihh, $Mir140^{-/-}$ $Ihh^{+/-}$ ($n = 5$). *$p < 0.05$ Ctrl or Ihh versus Mir140 or Mir140:Ihh.

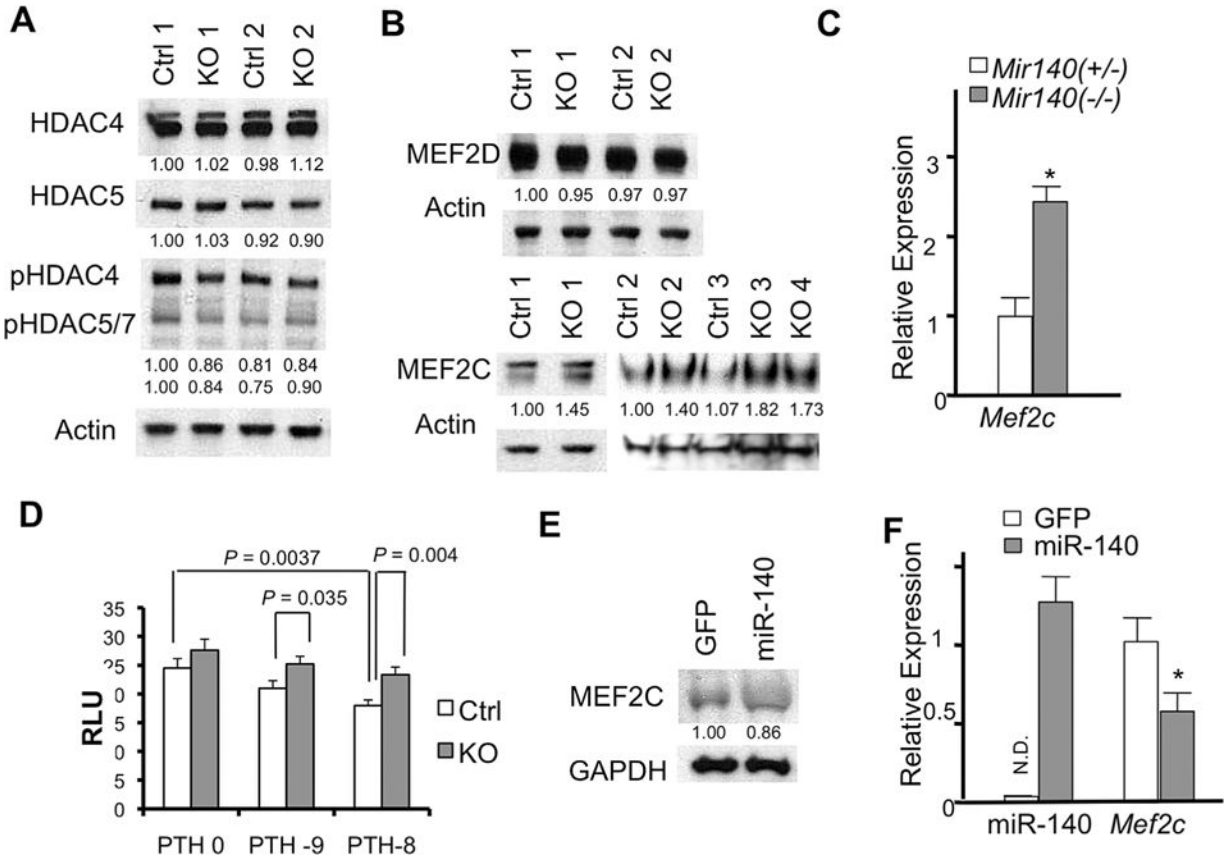

Fig. 3.

Mir140 loss upregulates MEF2C expression and function. (*A*) Expression or phosphorylation of class II HDACs in primary rib chondrocytes is not affected in *Mir140-null* mice (KO) compared with *Mir140* heterozygous mice (Ctrl). Protein extract isolated from overnight culture of rib chondrocytes (2 mice each genotype) was subjected to Western blot analysis. Signals were quantified by densitometric analysis, normalized by Actin, and expressed relative to Ctrl. (*B*) MEF2C, but not MEF2D, is upregulated in *Mir140*-null chondrocytes. Signals were quantified, normalized and expressed relative to Ctrl. (*C*) *Mef2c* RNA is upregulated in *Mir140*-null mice. qRT-PCR was performed using RNA isolated from rib chondrocytes. *$p < 0.05$, $n = 3$. (*D*) MEF2-luciferase assay using primary rib chondrocytes. Cell lysate was prepared for luciferase assay 24 hours after transfection of reporter constructs in the presence of PTH at concentration of 10^{-9}M (PTH-9) and 10^{-8}M (PTH-8). Values of p are indicated. *Mir140*-null chondrocytes show a modest, but significant, increased MEF2 activity in the presence of PTH. (*E*, *F*) Retrovirus-mediated miR-140 overexpression in primary chondrocytes reduces MEF2C expression at the protein (*E*) and mRNA (*F*) levels. Primary rib chondrocytes isolated from *Mir140*$^{-/-}$ mice were infected with viruses expressing miR-140 with GFP (miR-140) or GFP alone (control, GFP). Approximately 60% of cells were infected. Cells were harvested 2 days after infection. Protein expression was quantified, normalized, and expressed relative to control. miR-140 expression was normalized to U6, expressed relative to that of wild-type chondrocytes. ND = not detected. *$p < 0.05$, $n = 3$.

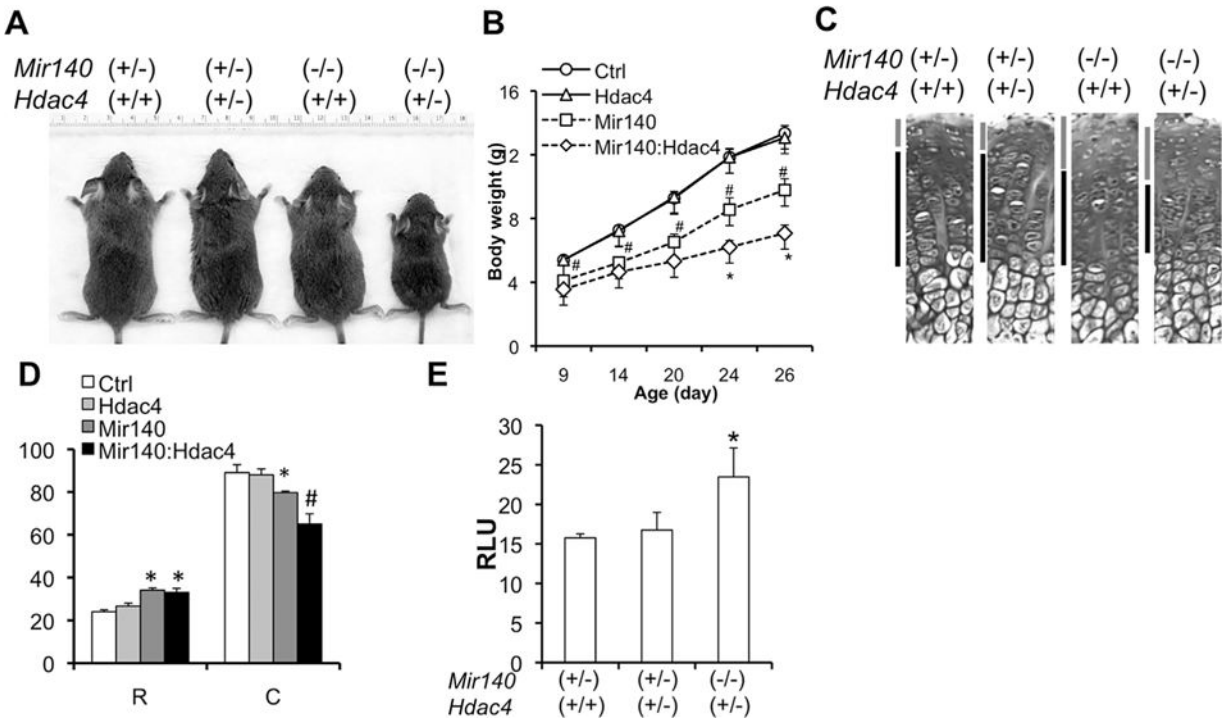

Fig. 4.

Hdac4 heterozygosity exacerbates the growth defect of *Mir140*-null mice. (*A*) Pictures of 3-week-old littermates with indicated genotypes. (*B*) Growth curve of mice with indicated genotypes. Ctrl, $Mir140^{+/-}$ $Hcac4^{+/+}$ ($n = 8$); HDAC4, $Mir140^{+/-}$ $Hdac4^{+/-}$ ($n = 4$); Mir140, $Mir140^{-/-}$ $Hdac4^{+/+}$ ($n = 7$); Mir140:Hdac4, $Mir140^{-/-}$ $Hdac4^{+/-}$ ($n = 10$). #$p < 0.05$ versus Ctrl or Hdac4; *$p < 0.05$ versus Mir140. (*C*) H&E stained section of growth plates of the proximal tibia. Bars indicate the resting and columnar regions. (*D*) Quantification of growth plates of mice with indicated genotypes. Mice were euthanized at P26. The length of the resting zone (R) and the columnar proliferating zone (C) was measured. *$p < 0.05$ versus Ctrl or HDAC4; #$p < 0.05$ versus Ctrl, HDAC4, or Mir140; $n \geq 3$. (*E*) Mef2-luciferase assay using primary rib chondrocytes. Mir140:Hdac4 compound mutant showed a significant increase in Mef2 activity, whereas *Hdac4* heterozygosity alone had no effect on Mef2 activity.

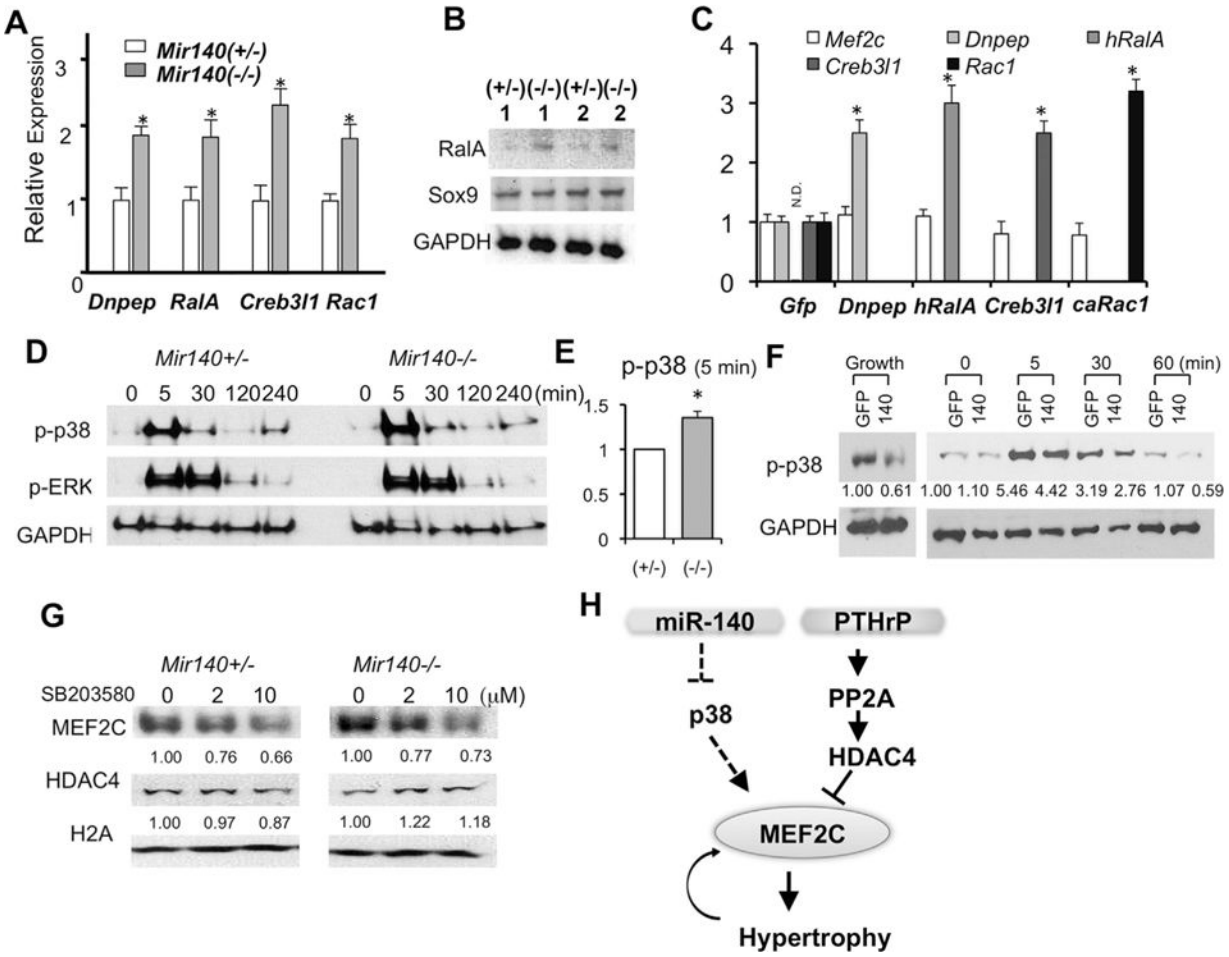

Fig. 5.

miR-140 regulate MEF2C expression via the p38 MAPK pathway. (*A*) *Mir140*-null chondrocytes show increased *Dnpep, RalA, Creb3l1, and Rac1* transcripts. qRT-PCR was performed on primary rib chondrocytes form $Mir140^{+/-}$ and $Mir140^{-/-}$ mice. (*B*) RalA protein expression is increased in *Mir140*-null chondrocytes, whereas Sox9 expression is not altered. (*C*) Overexpression of *Dnpep*, human *RalA* (*hRalA*), *Creb3l1*, or a constitutively active mutant of *Rac1* (*caRac1*) does not increase Mef2c expression. Gene expression was determined byqRT-PCR 3days after retrovirus infection to overexpress indicated genes. Infected viruses are indicated below the x-axis. Retrovirus-mediated gene transfer achieved twofold to threefold upregulation of indicated genes. Human *RalA* transcript was not detected in control (GFP) (ND). (*D*) p38 MAPK signaling is upregulated in *Mir140*-null chondrocytes. Phosphorylation of p38 MAPK (p-p38 MAPK) and ERK1/2 (p-ERK) was assessed by immunoblot analysis at indicated time points after serum stimulation. A representative result from three independent experiments using pairs of control and *Mir140*-null littermates is shown. (*E*) Quantification of the p-p38 level at the 5-min time point in *D*. Relative p-p38 levels in $Mir140^{-/-}$ (−/−) and $Mir140^{+/-}$ (+/−) were calculated in three sets of independent experiments. $*p < 0.05$, $n = 3$. (*F*) Retrovirus-mediated miR-140 overexpression reduces basal and stimulated levels of p-p38 MAPK. *Mir140*-null primary rib chondrocytes were infected with control (GFP) and miR-140-GFP (140) retroviruses and cultured for 2

days. p-p38 levels were determined in a similar fashion as in *D*. Cell lysates were also prepared from cells cultured in a growth medium without serum starvation (Growth). Signals were quantified, normalized, and expressed relative to control. (*G*) p38 MAPK inhibition reduces expression of MEF2C in a dose-dependent manner, whereas expression of HDAC4 and Histone 2 A (H2A) is not affected. Cells were cultured for 48 hours in the presence of indicated concentration of SB 203580. Signals were quantified, normalized, and expressed relative to vehicle control. (*H*) Proposed model. miR-140 enhances the PTHrP/HDAC4 pathway to control hypertrophic differentiation by suppressing premature MEF2C expression, at least in part, through dampening p38 MAPK signaling. ND = not detected.