HHS Public Access

Author manuscript

Nat Cell Biol. Author manuscript; available in PMC 2019 May 01.

Published in final edited form as:

Nat Cell Biol. 2018 May ; 20(5): 516–518. doi:10.1038/s41556-018-0077-4.

Dissecting the roles of miR-140 and its host gene.

Masafumi Inui[1,2,6,7], **Sho Mokuda**[3,6], **Tempei Sato**[1,4], **Moe Tamano**[1], **Shuji Takada**[1], and **Hiroshi Asahara**[1,3,4,5,7]

[1]Department of Systems BioMedicine, National Research Institute for Child Health and Development, Tokyo 157-8535, Japan

[2]Laboratory of Animal Regeneration Systemology, Department of Life Sciences, School of Agriculture, Meiji University, Kanagawa, 214-8571, Japan

[3]Department of Molecular Medicine, The Scripps Research Institute, La Jolla, CA 92037, USA

[4]Department of Systems BioMedicine, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, Tokyo 113-8510, Japan

[5]AMED-CREST, Japan Agency for Medical Research and Development (AMED), Tokyo 100-0004, Japan

[6]These authors contributed equally to this work.

[7]Correspondence should be addressed to M. I. (inui_m@meiji.ac.jp) or H. A. (asahara.syst@tmd.ac.jp)

To the editor

The functional coherence between microRNAs (miRNAs) and their host genes remains enigmatic. As host genes and its intronic miRNAs are transcribed as cistronic units, their functional coherence could be beneficial for the organism. Indeed, several intronic miRNAs are reported to act coherently to their host genes for the processes such as muscle fiber type selection or cholesterol metabolism[1,2], however, it is not clear how broad the coherence takes place in the organisms. Previously we and others have reported that the targeted deletion of a chondrocyte specific miRNA, *miR-140*, caused craniofacial truncation due to the impaired chondrocyte differentiation[3,4]. *miR-140* regulates several target genes including *Adamts-5* and *Dnpep* and in turn governs the proliferation and differentiation of chondrocyte[3,4]. Interestingly, knockout (KO) mice of an ubiquitin E3 ligase *Wwp2*, the host gene of *miR-140*, was subsequently reported in *Nature Cell Biology* (*Wwp2*$^{GT/GT}$ mouse) with the similar craniofacial truncation phenotype[5]. In this report, Zou et al. demonstrated that WWP2 mono-ubiquitylates a transcription factor Goosecoid and this modification is indispensable for the transcription of *Sox6*, a key transcription factor for chondrocyte differentiation, and thus *Wwp2*$^{GT/GT}$ mice displayed craniofacial phenotype due to

Author contributions

M.I., S.M. and T.S. analyzed the KO mice, M.T. performed the microinjection, M.I., S.T. and H.A. planned the experiments and wrote the manuscript.

Competing financial interests

The authors declare no competing financial interests.

malformation of the cartilage[5]. These reports suggested an intriguing possibility that *miR-140* and its host gene *Wwp2* have overlapping or coherent function in craniofacial morphogenesis. At the same time, the methodology to generate $Wwp2^{GT}$ allele, the insertion of a gene-trap cassette into 3rd intron of *Wwp2* gene[5], attracted our attention (Fig. 1A). The gene-trap cassette prevents the expression of downstream exons and introns of its insertion site, and indeed Zou et al. showed that the *Wwp2* transcript that correspond to downstream exons were absent from their KO mice[5]. As such, *miR-140* which is encoded further downstream might also be down-regulated in this mice (Fig. 2A) and in this scenario, the craniofacial phenotype observed in $Wwp2^{GT/GT}$ mice might be in part caused by the loss of *miR-140*.

To explore these two possibilities, we took advantage of CRISPR/Cas9 system to generate the KO mice of *miR-140* and *Wwp2*, and double knockout mice of *miR-140/Wwp2*. sgRNAs which target 2nd exon of *Wwp2* gene and/or *miR-140–5p* sequence were microinjected into mouse zygotes together with hCas9 mRNA (see Method for the details). As a result, we isolated the alleles with small insertions or deletions (indels) in *Wwp2* locus ($Wwp2^{1ins}$; $miR\text{-}140^{+}$), *miR-140* locus ($Wwp2^{+}$; $miR\text{-}140^{14del}$), or in both loci ($Wwp2^{1del}$; $miR\text{-}140^{132del}$) (Fig. 1B, C). Western blot and qPCR analyses confirmed that no WWP2 protein was detected in the chondrocyte of mice with mutated *Wwp2* alleles (Fig. 1E) and mature *miR-140* was absent in the chondrocyte of mice with mutated *miR-140* alleles (Fig. 1D). From this point in this manuscript we designate miR-140 KO to refer $Wwp2^{+/+}$;$miR\text{-}140^{14del/14del}$, Wwp2 KO and miR-140/Wwp2 double KO to refer $Wwp2^{1ins/1ins}$; $miR\text{-}140^{+/+}$ and $Wwp2^{1del/1del}$; $miR\text{-}140^{132del/132del}$, respectively.

Next we examined the craniofacial phenotype of these mice. The skulls of 30-days-old mice are observed with micro-CT (Fig. 1F). First, we confirmed that the skulls of miR-140 KO mice were truncated as in the previous report (Fig. 1F). However, for our surprise, the skull of Wwp2 KO mice was undistinguishable from that of WT mice (Fig. 1F). The miR-140/Wwp2 double KO mice had truncated skull, but no apparent difference was seen when compared with the miR-140 KO skull (Fig. 1F). These results suggest, at least in our system, that the loss of WWP2 protein alone does not cause craniofacial truncation phenotype. Furthermore, simultaneous depletion of WWP2 protein and *miR-140* does not promote the craniofacial phenotype caused by loss of *miR-140* (Fig. 1F). In sum, our results suggest that the truncation of skull correlates with loss of *miR-140* expression, but not with WWP2 protein level, and that the functional coherence between these two factors does not take place during the craniofacial skeletal development. Our results also suggested that the craniofacial phenotype observed in $Wwp2^{GT/GT}$ mice was not due to the loss of WWP2 protein, but to the unintentional loss of *miR-140* expression by the Gene-trap cassette. Consistent to this idea, not only WWP2 protein but also *miR-140* is significantly reduced with $Wwp2^{GT}$ allele (Fig. 2B, C). To further elucidate if the loss of WWP2 protein or *miR-140* is responsible for the craniofacial phenotype of $Wwp2^{GT/GT}$ mouse, we took advantage of compound heterozygous strategy: we have crossed $Wwp2^{GT/GT}$ mice with our miR-140 KO or Wwp2 KO mice and examined the correlation between craniofacial phenotype and the expression level of *miR-140* and WWP2 protein (Fig. 2A). As shown in Figure 2D, while compound heterozygous between $Wwp2^{GT}$ and Wwp2 KO alleles ($Wwp2^{GT/1ins}$; $miR\text{-}140^{GT/+}$) showed NO craniofacial phenotype with NO WWP2 protein

and reduced but significant *miR-140* expression (Fig. 2B, C), compound heterozygous between $Wwp2^{GT}$ and miR-140 KO ($Wwp2^{GT/+}$; $miR\text{-}140^{GT/14del}$) showed clear craniofacial truncation with reduced but significant WWP2 protein and NO *miR-140* expression (Fig.2B-D). These results demonstrated that the craniofacial truncation appeared when the expression of *miR-140*, but not of WWP2 protein, was depleted from the allele combined with $Wwp2^{GT}$. From these results, we concluded that *miR-140*, not WWP2 protein, is required for the proper craniofacial development.

The host-miRNA relationship between *miR-140* and *Wwp2* is evolutionary conserved among the vertebrates, and *miR-140* and WWP2 are highly expressed in embryonic and adult chondrocyte in Zebrafish and mouse[6–8]. Moreover, both loss of miR-140 and WWP2 are linked with palatogenesis in Zebrafish[8,9], therefore, although we did not find cooperative function between *miR-140* and WWP2 in craniofacial development in mice, it remains to be an attractive possibility that these two factors cooperate in other contexts also in mammals, such as cartilage homeostasis or pathogenic process of osteoarthritis, rheumatoid arthritis and other diseases.

Gene-trap is a powerful genetic screen tool to identify the knockout mice with the phenotypes of interests, assuming that the gene-trap cassette blocks the expression of a protein coding gene[10]. However, our result implied that we should be cautious when evaluating the phenotypes of KO mice generated by gene-trap or similar strategy, since they may also affect the expression of downstream elements of targeted protein coding genes, such as miRNAs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank Dr. Masuda (UCSD) for the kind cooperation in microCT analysis. This research is supported by AMED-CREST from AMED (JP15gm0410001, JP17gm0810008), Daiichi Sankyo Foundation of Life Science, JSPS KAKENHI [Grant Number: 26113008, 15H02560, 15K15544], and grants from the National Institutes of Health [Grant Number: AR050631, AR065379] to H.A., the Grant from the National Center for Child Health and Development (Grant Number 25–1), the Uehara memorial foundation, the Nakajima Foundation and JSPS KAKENHI (16K18558) for M.I.

Reference

1. van Rooij E et al. A family of microRNAs encoded by myosin genes governs myosin expression and muscle performance. Dev Cell 17, 662–673 (2009). [PubMed: 19922871]
2. Najafi-Shoushtari SH et al. MicroRNA-33 and the SREBP host genes cooperate to control cholesterol homeostasis. Science 328, 1566–1569 (2010). [PubMed: 20466882]
3. Miyaki S et al. MicroRNA-140 plays dual roles in both cartilage development and homeostasis. Genes Dev 24, 1173–1185 (2010). [PubMed: 20466812]
4. Nakamura Y, Inloes JB, Katagiri T & Kobayashi T Chondrocyte-specific microRNA-140 regulates endochondral bone development and targets Dnpep to modulate bone morphogenetic protein signaling. Mol Cell Biol 31, 3019–3028 (2011). [PubMed: 21576357]
5. Zou W et al. The E3 ubiquitin ligase Wwp2 regulates craniofacial development through mono-ubiquitylation of Goosecoid. Nat Cell Biol 13, 59–65 (2011). [PubMed: 21170031]

6. Wienholds E et al. MicroRNA expression in zebrafish embryonic development. Science 309, 310–311 (2005). [PubMed: 15919954]
7. Tuddenham L et al. The cartilage specific microRNA-140 targets histone deacetylase 4 in mouse cells. FEBS Lett 580, 4214–4217 (2006). [PubMed: 16828749]
8. Nakamura Y et al. Wwp2 is essential for palatogenesis mediated by the interaction between Sox9 and mediator subunit 25. Nature Communications 2, 251 (2011).
9. Eberhart JK et al. MicroRNA Mirn140 modulates Pdgf signaling during palatogenesis. Nat Genet 40, 290–298 (2008). [PubMed: 18264099]
10. Stanford WL, Cohn JB & Cordes SP Gene-trap mutagenesis: past, present and beyond. Nat Rev Genet 2, 756–768 (2001). [PubMed: 11584292]

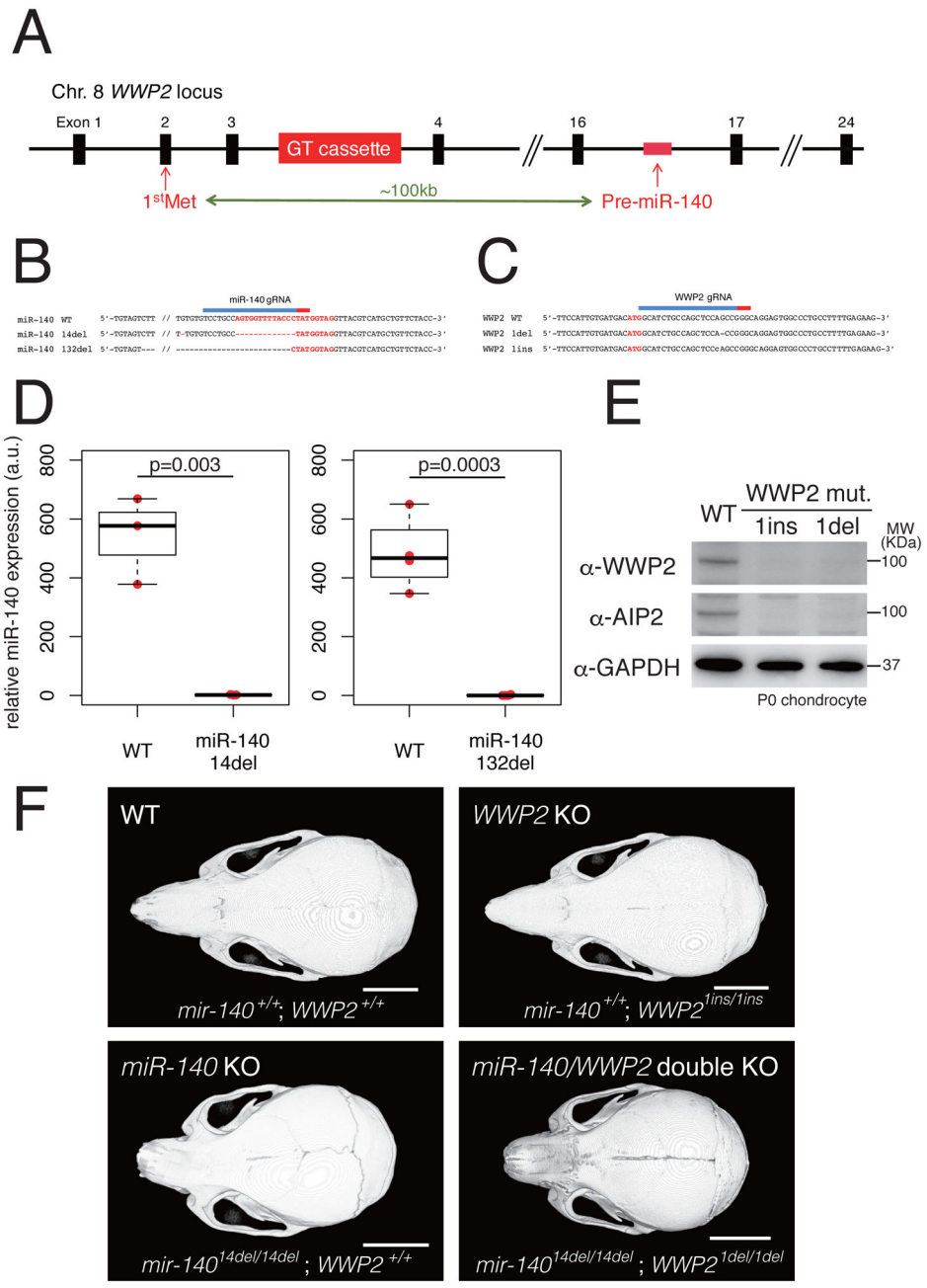

Figure 1.

(A) A schematic illustration demonstrating *Wwp2* and *pre-miR-140* loci on mouse chromosome 8. Black boxes indicate the exons of *Wwp2* gene and red box indicates the *pre-miR-140* gene. (B) An alignment of DNA sequences of wild type (WT) and mutant *miR-140* alleles. Red letters and hyphens indicate the mature miR-140–5p sequence and deleted bases, respectively. Blue bar and red bar indicate the position of the target of miR-140 gRNA and the PAM sequence. 14del: 14 base deletion, 132del: 132 base deletion. (C) An alignment of DNA sequences of wild type (WT) and mutant *Wwp2* alleles. Red letters and a hyphen indicate the initiation codon of WWP2 and the deleted base, respectively. Blue bar

indicates the position of the target of WWP2 gRNA and red bar indicates the PAM sequence. 1del: 1 base deletion, 1ins: 1 base insertion. (D) qPCR analysis of mature miR-140–5p expression in the chondrocytes of newborn mice. 14del: *miR-140*$^{14del/14del}$, 132del: *miR-140*$^{132del/132del}$. The expression levels of *miR-140* are normalized with those of RNU6B (left panel) or snoRNA135 (right panel). mut.; mutant, a.u.: arbitrary unit. Statistical analysis: two-tailed two-sample student t-test, n=3 (independent animals) for WT (left panel) and miR-140 14 del, n=4 (independent animals) for WT (right panel) and miR-140 132del. Box plots show median and 25–75th percentiles (middle line and box, respectively). Whiskers extend to the maximum and minimum data points. (E) Western blot analysis of WWP2 protein in the chondrocytes of newborn mice. α-WWP2 and α-AIP2 antibodies detect the same protein (WWP2) with distinct epitopes. GAPDH serves as a loading control. 1del: *Wwp2*$^{1del/1del}$, 1ins: *Wwp2*$^{1ins/1ins}$. (F) microCT analysis of the skulls of 30-days-old mice. The skulls are shown from the top and anterior side (nose) are at the left. Scale bars: 0.5 cm. Data shown in E and F are representative results from 2 independent experiments.

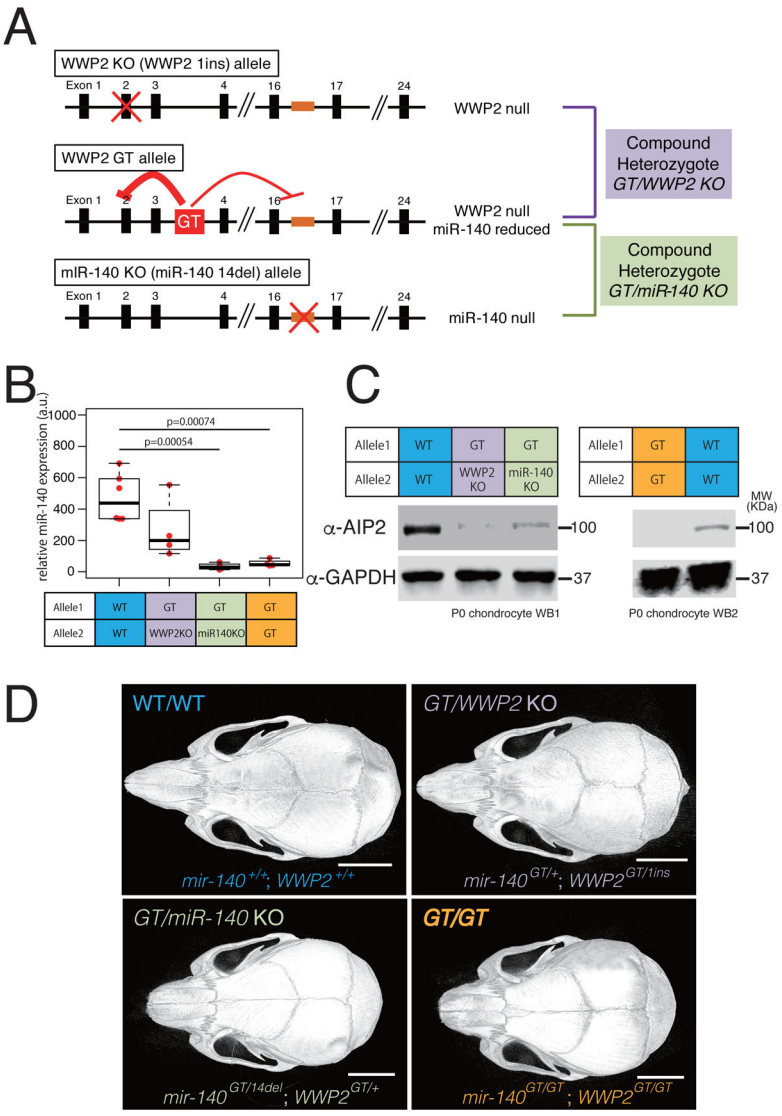

Figure 2.

(A) A schematic illustration demonstrating the compound heterozygous strategy among *Wwp2*^{GT}, *miR-140*^{14del} and *WWP2*^{1ins} alleles. (B) qPCR analysis of mature *miR-140–5p* expression in the chondrocytes of newborn mice. Two alleles of each mouse are separately shown at the bottom. The expression levels of *miR-140* are normalized with those of snoRNA135. a.u.: arbitrary unit. Statistical analysis: two-tailed two-sample student t-test, n=6 (independent animals) for WT/WT, n=4 (independent animals) for GT/WWP2KO, GT/miR-140KO, and GT/GT. Box plots show median and 25–75th percentiles (middle line and box, respectively). Whiskers extend to the maximum and minimum data points. (C) Western blot analysis of WWP2 protein in the chondrocytes of newborn mice. α-AIP2 antibody detect WWP2 protein. GAPDH serves as a loading control. Two alleles of each mouse are separately shown at the top. (D) microCT analysis of the skulls of 30-days-old mice. The genotypes of the mice are shown on the top left. The skulls are shown from the top and

anterior side (nose) are at the left. Scale bars: 0.5 cm. Data shown in C and D are representative results from 2 independent experiments.