Published in final edited form as:
Appl Biochem Biotechnol. 2012 January ; 166(1): 64–71. doi:10.1007/s12010-011-9404-y.

Sox9 is upstream of microRNA-140 in cartilage

Yukio Nakamura[a,†], **Xinjun He**[b,†], **Hiroyuki Kato**[a], **Shigeyuki Wakitani**[c], **Tatsuya Kobayashi**[d], **Sumiko Watanabe**[e], **Atsumi Iida**[e], **Hideaki Tahara**[f], **Matthew L. Warman**[g], **Ramida Watanapokasin**[h,*], and **John H. Postlethwait**[b]

[a]Department of Orthopaedic Surgery, Shinshu University School of Medicine, Matsumoto, 390-8621, Japan [b]Institute of Neuroscience, University of Oregon, Eugene OR 97403, U.S.A [c]Department of Orthopaedic Surgery, Osaka City University Graduate School of Medicine, Osaka 545-8585, Japan [d]Endocrine Unit, Massachusetts General Hospital and Harvard Medical School, Boston, MA 02114, U.S.A [e]Department of Molecular and Developmental Biology, Institute of Medical Science, University of Tokyo, Tokyo 108-8639, Japan [f]Department of Surgery and Bioengineering, Advanced Clinical Research Center, Institute of Medical Science, The University of Tokyo, Tokyo 108-8639, Japan [g]Howard Hughes Medical Institute, Departments of Orthopaedic Surgery and Genetics, Children's Hospital Boston and Harvard Medical School, Boston, MA 02115, U.S.A [h]Department of Biochemistry, Faculty of Medicine, Srinakharinwirot University, Bangkok, Thailand

Abstract

MicroRNA-140 (miR-140) is Specifically expressed in developing cartilage tissues. We have previously reported that miR-140 plays an important role during palatal cartilage development by modulating *platalet-derived growth factor receptor alpha* (*pdgfra*) in zebrafish. However, the regulatory mechanism of miR-140 in cartilage is still unknown. Using developing zebrafish, *Sox9a* mutant (*Sox9a−/−*) and *Sox9b* mutant (*Sox9b−/−*) zebrafish and *Sox9* siRNA in human chondrocytes, T/C-28 Cells, we found that miR-140 is regulated by the cartilage master transcription regulator *Sox9* in zebrafish and mammalian cells.

Index Entry

microRNA-140 (miR-140); *Sox9*; cartilage; zebrafish; T/C-28; SiRNA; in situ hybridization; RT-PCR

Introduction

MicroRNAs (miRNAs) are a large family of non-coding Small RNAs. miRNAs regulate gene expression by promoting messenger RNA (mRNA) degradation and/or repressing translation through Sequence-specific interactions with the 3′-untranslated regions of Specific mRNA targets (1,2). One-third of human mRNAs Seem to be under miRNA regulation, Suggesting the essential role in regulating gene expression by miRNAs (3). Among known miRNAs, miRNA-140 (miR-140, mirn140) is Strongly expressed in cartilage of developing zebrafish, medaka and mice (4–6). However, the biological regulatory mechanism of miR-140 in cartilage is Still not known.

*Corresponding author: Department of Biochemistry, Faculty of Medicine, Srinakharinwirot University, Bangkok, Thailand., Telephone: +66 2 649 53 69, Fax+66 2 64 95 834, ramidawa@yahoo.com or yuwadee@swu.ac.th.
†Equal Contributions

The HMG domain-containing transcription factor, *Sox9,* functions as a transcription factor by recognizing a specific heptameric DNA Sequence (A/T)(A/T)CAA(A/T)G(7) as well as plays an essential role in cartilage development and chondrocyte differentiation at multiple steps (8). In humans, heterozygous null mutations of *Sox9* cause the human skeletal dysmorphologic Syndrome, campomelic dysplasia (9, 10). *Sox9* is also required for cartilage formation in craniofacial and fin development in zebrafish (11). These findings indicate that *Sox9* plays a central role in cartilage development across the species. Recent Studies have shown that *Sox9* regulates target gene transcription in concert with other proteins (12). The Nuclear RNA-binding protein, $p54^{zrb}$, modulates transcriptional activity of *Sox9* (12). Tat-interactive protein 60 (Tip60) and p300/CREB binding protein enhance *Sox9* transcription in chondrocytes (13, 14). Considering the Strong expression of miR-140 *in vivo* cartilage and the Specific roles played by miR-140 during palatogenesis, we hypothesized that miR-140 interacts with *Sox9* during chondrogenesis.

Previous Studies have shown that miR-140 targets osteoarthritis (OA)-related mRNAs, *ADAMTS-5, MMP13, COL2AI,* and *ACAN* in human articular chondrocytes (15). We have also previously reported that miR-140 modulates palatogenesis in zebrafish (16), and a single nucleotide polymorphism in miR-140 precursor has recently been linked with human non-Syndromic cleft palate (17). Also, miR-140 knockout mouse study shows that miR-140 plays a role in cartilage development and homeostasis, partly regulating *ADAMTS-5,* a major cartilage matrix-degrading protease in OA (18). These findings Strongly suggest that miR-140 plays a role in skeletal biology.

We herein report that *Sox9* acts upstream of miR-140 in developing zebrafish and mammalian cells. Our findings provide a new insight of the biological regulatory mechanism of miR-140 in cartilage and of the transcriptional machinery in *Sox9*-regulated chondrogenesis.

Materials and Methods

Zebrafish care and use

Developing and adult zebrafish were maintained using the Standard method. Embryos from natural mating were staged as described previously (19). Wild type mating was obtained from the Oregon AB line. To Suppress pigmentation, embryos were raised in the zebrafish aquatic system water containing 1-phenyl-2-thiourea (0.003%) (*Sigma*). Zebrafish care was in accordance with IACUC approval of Children's Hospital Boston, University of Oregon, and Murayama Medical Center.

In situ hybridization of zebrafish embryos

Whole-mount and section *in situ* hybridization was carried out with zebrafish embryos at different stage (20). The probes for zebrafish miR-140, *col2ala,* and *Sox9a* were used as described previously (11). Digoxigenin-labeled RNA probes were generated using the T7 and T3 labeling kit (*Roche*), following the manufacturer's instructions. The signals were detected with an alkaline phosphatase conjugated anti-digoxigenin antibody (*Roche*) and 4-nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate. The reaction Was Stopped by replacing Substrate with Several rinses of PBT (phosphate-buffered Saline: PBS, 0.1% Tween 20) and then Stored in PBS containing 0.02% sodium azide in the dark at 4°C until photographed.

siRNA for Sox9 and semi-quantitative RT-PCR analysis of human miR-140

SiRNA experiments were performed as described previously (21). The siRNA construct for *Sox9* and the control were kind gifts from Dr. Ikegawa, Riken, Tokyo. T/C-28 cells were

plated at a density of 5×10^4 cells/well in 24-well tissue culture plates. 0.5–1 μg of the siRNA construct or the Control vector was transfected using the LipofectAmine (*Invitrogen*). Two days after transfection, cell lySates were collected in the extraction buffer (10 mM Tris, pH 7.2, 1% Triton-X100, 0.1% SDS, 1% Na-deoxycholate, 5 mM EDTA, 50 mM NaCl) and Subjected to semi-quantitative RT-PCR. PCR primers for human miR-140 are as follows: Forward 5′-GTACTTTAACAACCAGGCCAGCAGCATCT-3′ and reverse 5′-ACCATCCCCGTTCCCACACTTCC-3′.

Semi-quantitative RT-PCR analysis in developing Zebrafish

Gene Tools (Philomath, OR, USA, http://www.gene-tools.com) Supplied Morpholino Oligonucleotides (MOs) with the sequences: zebrafish miR-140 Drosha blocker-MO: 5′-AAACCACTGACGGGACaCaGGaGaC-3′, and with the Sequences: zebrafish miR-140 Dicer blocker: 5′ CCCTGTGGTAGAACAGCATGACGTA-3′. We injected one or two-cell stage zebrafish embryos with approximately 1–2 nL of morpholinos (3–5ng Drosha or Dicer blocker was used) PCR primers for zebrafish *Sox9a, Sox9b, col2ala, coll0al,* and *β-actin* are as follows: *Sox9a* (forward) 5′-CCATGCCGGTGAGGGTGAAC-3′ and *Sox9a* (reverse) 5′-CTTATAGTCGGGGTGATCTTTCTTGTG-3′; *Sox9b* (forward) 5′-AGGTGCTGAAGGGCTACGACTGGT-3′ and *Sox9b* (reverse) 5′-GATTTCCTCCGTCTGGGCTGGTATT-3′; *col2ala* (forward) 5′-CTTCCAGGACCTTCTGGTGA-3′ and *col2ala* (reverse) 5′-CAGGTCCAGTCAATCCAGGT-3′, *coll0al* (forward) 5′-ACAAGGGATTTGTGGACCAG-3′ and *coll0al* (reverse) 5′-GCGTGCATTTCTCAGAACAA-3′, and *β-actin* (forward) 5′-GTTTTCCCCTCCATTGTTGGAC-3′ and *β-actin* (reverse) 5′-CAGGATCTTCATCAGGTAGTCTGTCA-3′, respectively.

Cartilage staining

Zebrafish embryos at 4–7 day post-fertilization (dpf) were stained with Alcian Blue and Alizarin Red and then were flat-mounted as described previously (22). After photography of the entire larvae, the neurocraniums were dissected free from Surrounding tissues, mounted on glass slides in 75% glycerol, and sealed with coverslips. The images of the Stained, flat-mounted cartilage were captured using the Leica DMLB upright microscope and Spot camera (*Diagnostic Instruments, Inc*).

Results and Discussion

We investigated the biological roles of miR-140 in developing zebrafish and human chondrocytic cell lines, T/C-28 cells. Since miR-140 is expressed strongly *in vivo* in Cartilage and plays a role in palatal cartilage development, we hypothesized and tested if the transcription factor *Sox9* regulates miR-140. Our Study using *Sox9* mutant zebrafish and *Sox9* siRNA in human chondrocytes revealed that *Sox9* is upstream of miR-140.

During chondrogenesis, *Sox9* is expressed in all chondroprogenitors and all differentiated chondrocytes except hypertrophic chondrocytes (11, 23). Interestingly, the whole-mount in situ hybridization showed that the expression of miR-140 was in mesoderm and endoderm in 1 day post-fertilization (dpf) old zebrafish embryos (16) regulating mir 140 is strongly expressed in facial Cartilage at 2 dpf in zebrafish when zebrafish chondrogenesis starts (Fig. 1A). The expression correlation of *Sox9* and mir140 Suggesting that mir-140 may be downstream of *Sox9.* In addition, the Section *in situ* hybridization revealed that the expression of zebrafish miR-140 (*pri-mirn-140*) and *col2al* was substantially decreased in *Sox9a* mutant (*Sox9a−/−)* and *Sox9b* mutant (*Sox9b−/−*) zebrafish at 3dpf (Fig. 1B and 24), Suggesting that *Sox9* plays an essential role for miR-140 as an up-regulator. To further

Study if the regulation of miR-140 by *Sox9* was also present in other species, we examined the expression of human miR-140 in the human chondrocytic cells T/C-28 with *Sox9*-knocked down. Our results showed that *Sox9* SiRNA transfection into T/C-28 cells inhibited the expression of miR-140 in a dose dependent fashion (Fig. 2A), implying that the regulation of miR-140 by *Sox9* is generally conserved between zebrafish and humans.

The network of transcriptional regulation is complex. It is common that a gene regulated by another one can have a feed-back regulation (25). Thus, we next tested if miR-140 affects the expression of *Sox9* in zebrafish. First, we injected into zebrafish embryos with MO that blocks the Dicer or Drosha processing of miR-140 and examined the expression of *Sox9a, Sox9b,* and other cartilage markers. Semi-quantitative RT-PCR showed that Dicer or Drosha blocker significantly reduced the expression of miR-140 in developing zebrafish (data not shown). *In situ* hybridization and semi-quantitative RT-PCR results revealed that the knockdown of miRNA by inhibiting the expression of Dicer or Dorsha did not affect the expression of *Sox9a* or *Sox9b, col2ala,* and *coll0al* (Fig. 2B and Fig. 3A). These observations suggest that miR-140 does not have a feedback effect on *Sox9* expression.

Then we wondered if there is functional relationship between *Sox9* and miR-140. In zebrafish, mutation of *Sox9a* inhibits, most of the cartilage tissue development while mutation of *Sox9b* reduces cartilage development, especially having malformed small palates due to that the neural crest cells with *Sox9b* mutation fail to differentiate (11). Our previous study has shown that miR-140 knockdown and overexpression in zebrafish have effects on palatal formation by regulating the early stage of neural crest cell migration, which is relatively independent of later cartilage differentiation during *Sox9*-regulated palatogenesis (11). Thus, we expected that miR-140 and *Sox9* would have additive effects if both *Sox9* and miR-140 were misregulated. To test this, we overexpressed *in vitro* synthesized miR-140 duplex into zebrafish embryos with mutation of *Sox9* genes. Our results showed that miR-140 overexpression induced the cleft palate phenotype both in the uninjected control (UIC) and *Sox9b* mutant fish, confirming that although miR-140 is regulated by *Sox9*, it acts functionally independent of *Sox9b* (Fig. 3B).

Most of the intergenic miRNAs are spliced out from introns on their host genes using their own promoters (26, 27). The intergenic miRNAs use the same transcription strategies with mRNAs using sets of transcription factors and RNA polymerase II to control their transcription as described previously (28). Thus different miRNAs have their own unique transcriptional machineries depending on their genomic context. The regulation of miR-140 by *Sox9* in our study put miR-140 in the context of *Sox9*-regulated transcriptional network. *Sox9* regulates its downstream genes by physically binding to the specific sequence on the promoter region (29). We have identified several *Sox9* binding sites further upstream of miR-140 site within the *Wwp2* gene, which contains miR-140, in human, mouse and zebrafish (Fig. 4). Currently, it is not clear whether or not miR-140 is immediately downstream of *Sox9*. Therefore, it is possible that miR-140 expression is several steps regulated by *Sox9* expression. Interestingly, the depletion of miR-140 or *Wwp2* did not alter the expression of *Wwp2* or miR-140 in zebrafish, respectively (Nakamura et al, unpublished data). Recent report also shows that miR-140 knockout mouse did not change the expression of *Wwp2* (18). In addition, miR-140 or *Wwp2* knockdown has caused different cartilage phenotypes in zebrafish (Nakamura et al, unpublished data and 16). Taken together, these results indicate that miR-140 has its own promoter and regulatory mechanism. Further studies are needed to determine whether *Sox9* is a direct modulator for miR-140 and whether miR-140 functions independently of the *Wwp2* gene.

In conclusion, we provide the evidence that miR-140 is downstream of the transcription factor *Sox9* in developing zebrafish and in mammalian cells.

Acknowledgments

We thank Dr. Keiko Tamai at Case Western Reserve University, Cleveland, OH for her valuable discussions.

References

1. He L, Hannon GJ. Nature Rev Genet. 2004; 5:522–531. [PubMed: 15211354]
2. He X, Eberhart JK, Postlethwait JH. J Cell Mol Med. 2009; 13:606–618. [PubMed: 19220576]
3. Berezikov E, Guryev V, Van de Belt J, Wienholds E, Plasterk RH, Cuppen E. Cell. 2005; 12:21–24. [PubMed: 15652478]
4. Ason B, Darnell DK, Wittbrodt B, Berezikov E, Kloosterman WP, Wittbrodt J, et al. PNAS. 2006; 103:14385–14389. [PubMed: 16983084]
5. Tuddenham L, Wheeler G, Ntounia-Fousara S, Waters J, Hajihosseini MK, Clark I, et al. FEBS Lett. 2006; 580:4214–4217. [PubMed: 16828749]
6. Wienholds E, Kloosterman WP, Miska E, Alvarez-Saavedra E, Berezikov E, de Bruijn E, et al. Science. 2005; 309:310–311. [PubMed: 15919954]
7. Oh, C-d; Maity, SN.; Lu, JF.; Zhang, J.; Liang, S.; Coustry, F., et al. PLoS ONE. 2010; 5:1–12.
8. Akiyama H, Chaboissier MC, Martin JF, Schedl A, de Crombrugghe B. Genes Dev. 2002; 16:2813–2828. [PubMed: 12414734]
9. Foster JW, Dominguez-Steglich MA, Guioli S, Kwok C, Weller PA, Stevanovic M, et al. Nature. 1994; 372:525–530. [PubMed: 7990924]
10. Wagner T, Wirth J, Meyer J, Zabel B, Held M, Zimmer J, et al. Cell. 1994; 79:1111–1120. [PubMed: 8001137]
11. Yan YL, Willoughby J, Liu D, Crump JG, Wilson C, Miller CT, et al. Development. 2005; 132:1069–1083. [PubMed: 15689370]
12. Hata K, Nishimura R, Muramatsu S, Matsuda A, Matsubara T, Amano K, et al. JCI. 2008; 118:3098–3108. [PubMed: 18677406]
13. Furumatsu T, Tsuda M, Taniguchi N, Tajima Y, Asahara H. J Biol Chem. 2005; 280:8343–8350. [PubMed: 15623506]
14. Hattori T, Coustry F, Stephens S, Eberspaecher H, Takigawa M, Yasuda H, et al. Nuc Acids Res. 2008; 36:3011–3024.
15. Tardif G, Hum D, Pelletier JP, Duval N, Martel-Pelletier J. BMC Musculoskelet Disord. 2009; 10:148. [PubMed: 19948051]
16. Eberhart JK, He X, Swartz ME, Yan YL, Song H, Boling TC, et al. Nat Genet. 2008; 40:290–298. [PubMed: 18264099]
17. Li L, Meng T, Jia Z, Zhu G, Shi B. Am J Med Genet. 2010; 152A:856–862. [PubMed: 20358594]
18. Miyaki S, Sato T, Inoue A, Otsuki S, Ito Y, Yokoyama S, et al. Genes Dev. 2010; 24:1173–1185. [PubMed: 20466812]
19. Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF. Dev Dyn. 1995; 203:253–310. [PubMed: 8589427]
20. Thisse C, Thisse B, Schilling TF, Postlethwait JH. Development. 1993; 119:1203–1215. [PubMed: 8306883]
21. Kou I, Ikegawa S. J Biol Chem. 2004; 279:50942–50948. [PubMed: 15456769]
22. Walker MB, Kimmel CB. Biotech Histochem. 2007; 82:23–28. [PubMed: 17510811]
23. Wright E, Hargrave MR, Christiansen J, Cooper L, Kun J, Evans T, et al. Nat Genet. 1995; 9:15–20. [PubMed: 7704017]
24. He X, Yan YL, Delaurie A, Postlethwait JH. Zebrafis. 2011; 8(1):1–8.
25. Kageyama R, Niwa Y, Shimojo H.) Mol Cells. 2009; 27:497–502. [PubMed: 19466597]
26. Monteys AM, Spengler RM, Wan J, Tecedor L, Lennox KA, Xing Y, et al. RNA. 2010; 16:495–505. [PubMed: 20075166]
27. Ying SY, Lin SL. J Biol Sci. 2006; 13:5–15.

28. Lee Y, Kim M, Han J, Yeom KH, Lee S, Baek SH, et al. EMBO J. 2004; 23:4051–4060. [PubMed: 15372072]

29. van de Wetering M, Clevers H. EMBO J. 1992; 11:3039–3044. [PubMed: 1639073]

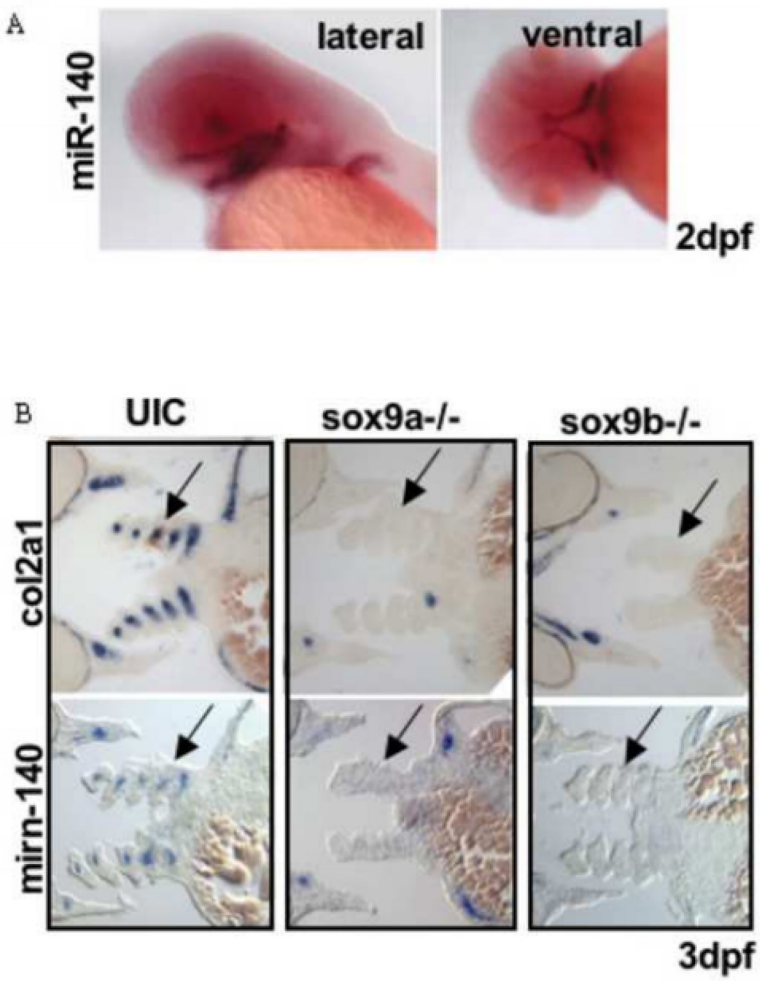

Fig. 1.

In-situ hybridization of zebrafish embryo at different stages. The probes for zebrafish miR-140 and *col2ala* were used and digoxigenin-labeled RNA probes were generated using the T7 and T3 labeling kit. The signals were detected with an alkaline phosphatase conjugated anti-digoxigenin antibody and 4-nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate. (A) The whole-mount in situ hybridization shows that miR-140 is strongly expressed in facial cartilage at 2dpf in zebrafish. (B) The section in situ hybridization reveals that the expression of zebrafish miR-140 (mirn-140) and col2al is substantially decreased in *Sox9a* mutant (*Sox9a−/−*) and *Sox9b* mutant (Sox9b−/−) zebrafish at 3dpf. Arrows indicate the expression of each gene, *Sox9a, Sox9b, col2al,* or mirn-140. UIC stands for uninjected controls.

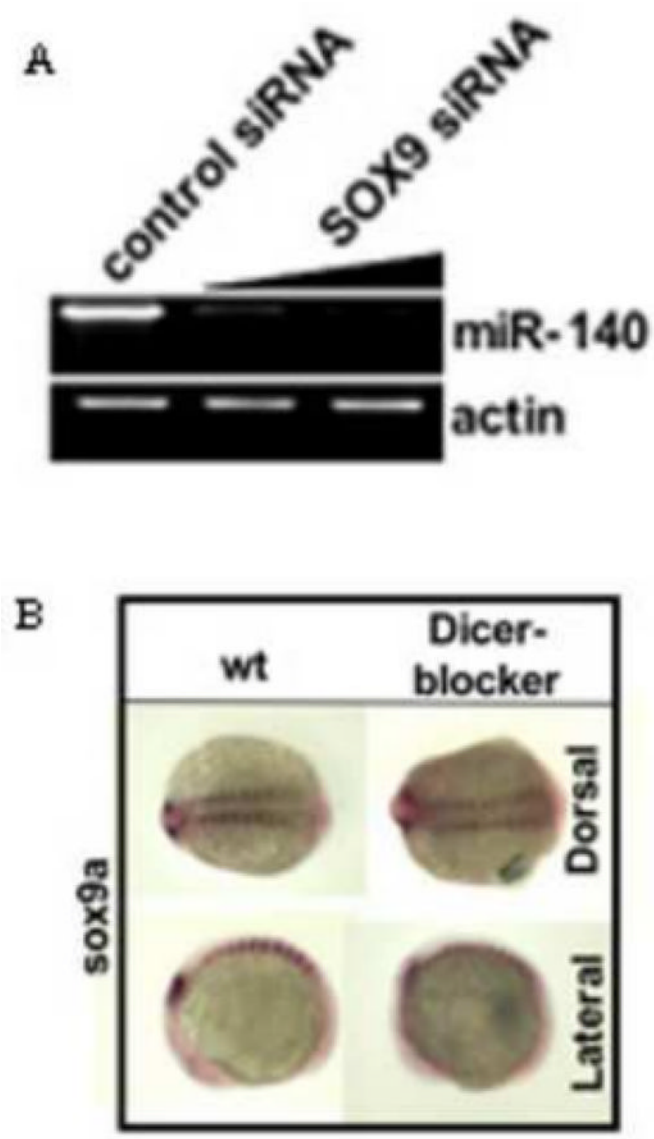

Fig. 2.

Effect of *Sox9* siRNA in human chondrocyte cell line and in situ-hybridization. T/C-28 cells were plated at a density of 5×10^4 cells/well in 24-well tissue culture plates. 0.5–1 μg of the siRNA construct or the control vector was transfected using the LipofectAmine. Two days after transfection, cell lysates were collected in the extraction buffer and subjected to semi-quantitative RT-PCR. (A) *Sox9* siRNA in human chondrocyte cell line shows that miR-140 is markedly decreased, suggesting that miR-140 is regulated by *Sox9* across the species. Actin serves as a loading control. (B) miR-140 Dicer blocker does not alter the *sox9a* mRNA expression in 10-somite zebrafish embyors.

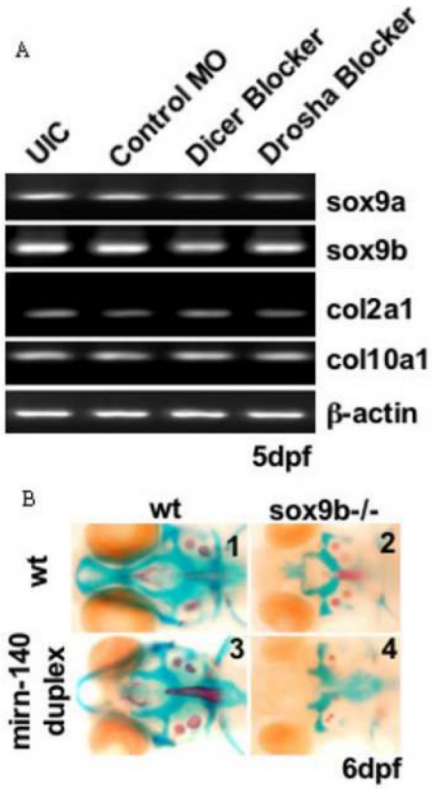

Fig. 3.

Semi-quantitative RT-PCR analysis in developing zebrafish and cartilage staining of zebrafish embryo. One or two-cell stage zebrafish embryos were injected with approximately 1–2 nL of morpholinos (3–5ng Drusha or Dicer blocker). PCR primers for zebrafish *Sox9a, Sox9b, col2ala, coll0al,* and *β-actin* were used. (A) Semi-quantitative RT-PCR reveals that miR-140 Dicer or Drusha blocker does not change the expression of cartilage markers; *Sox9a, Sox9b, col2al, coll0al* in zebrafish embryos. (B) Zebrafish embryos at 4 -7 day post-fertilization (dpf) were stained with Alcian Blue and Alizarin Red and then were flat-mounted. The alcian-blue staining at 6dpf zebrafish shows that the palatogenesis in wild-type (wt) and *Sox9b* mutant zebrafish is both affected by mirn-140 duplex.

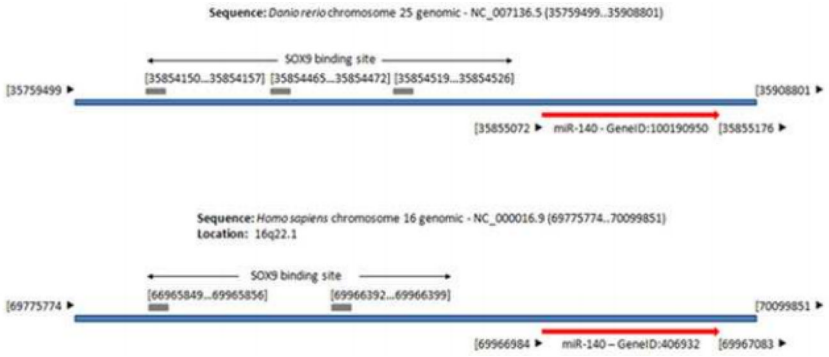

Fig. 4.

Schematic mapping of the chromosomal location of *Sox9* binding sites, further upstream of miR-140 site within the *Wwp2* gene, which contains miR-140 in human and zebrafish.