

miR-204 is required for lens and retinal development via *Meis2* targeting

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MicroRNAs (miRNAs) are small noncoding RNAs that have important roles in the regulation of gene expression. The roles of individual miRNAs in controlling vertebrate eye development remain, however, largely unexplored. Here, we show that a single miRNA, miR-204, regulates multiple aspects of eye development in the medaka fish (*Oryzias latipes*). Morpholino-mediated ablation of miR-204 expression resulted in an eye phenotype characterized by microphthalmia, abnormal lens formation, and altered dorsoventral (D-V) patterning of the retina, which is associated with optic fissure coloboma. Using a variety of in vivo and in vitro approaches, we identified the transcription factor *Meis2* as one of the main targets of miR-204 function. We show that, together with altered regulation of the Pax6 pathway, the abnormally elevated levels of *Meis2* resulting from miR-204 inactivation are largely responsible for the observed phenotype. These data provide an example of how a specific miRNA can regulate multiple events in eye formation; at the same time, they uncover an as yet unreported function of *Meis2* in the specification of D-V patterning of the retina.

microRNA | eye development | medaka fish

Development of the vertebrate eye takes place through a series of morphogenetic events that are controlled by molecular networks in which transcription factors and signaling pathways have major roles (1). Specific components of these networks are reiteratively exploited in space and time to pattern eye tissues and to control the subsequent cellular programs, such as cell proliferation, differentiation, migration, and programmed cell death (2, 3). Most of these developmental processes are critically sensitive to gene dose, and variations in the normal levels of regulatory proteins appear to result in a variety of eye anomalies (4, 5). Posttranscriptional regulatory mechanisms maintain the appropriate levels of expression of these proteins and enable rapid changes in the cellular proteome; thus, they have fundamental roles in the development of the nervous system (6).

MicroRNAs (miRNAs) are a class of 20- to 25-nucleotide noncoding RNA molecules that mediate a newly recognized level of posttranscriptional control of gene expression. Indeed, miRNAs can impair either mRNA translation or stability by binding through imperfect base pairing to specific sites in the 3'-UTR of target mRNAs (7). Recently, many miRNAs have been shown to be required for vertebrate developmental processes, such as cell fate determination and patterning as well as cell death and proliferation (8).

A number of miRNAs show restricted expression patterns in the developing lens, retinal pigment epithelium (RPE), neural retina, and other ocular tissues, which suggests their potential relevance to eye development and function (9, 10). However, the precise roles of individual miRNAs and their specific influences on given mRNA targets that are important for vertebrate eye development remain unclear.

In the present study, we show that miR-204, which is highly expressed in the presumptive RPE, lens, ciliary body, and neural retina (11, 12), is required for correct lens and optic cup de-

velopment. Using a variety of gain- and loss-of-function approaches in the medaka fish [*Oryzias latipes* (ol)], we demonstrate that miR-204-mediated modulation of the *Meis2* gene dose has a significant impact on regulation of the genetic pathways controlling eye morphogenesis and differentiation.

Results

miR-204 Knockdown Causes Lens Abnormalities, Microphthalmia, and Eye Coloboma. We found that during early medaka development [stage (St) 23], ol-miR-204 was expressed in the lens placode and in the presumptive RPE, with an apparent dorsal^{high}-to-ventral^{low} gradient (Fig. S1A). At later stages, ol-miR-204 expression was also detected in the ciliary marginal zone, ciliary body, and presumed migratory neural crest cells (Fig. 1A–A'). This expression pattern suggested that miR-204 might modulate different aspects of eye development.

To investigate this further, we interfered with miR-204 processing and activity using a multiblocking morpholino (Mo)-based knockdown approach (13). To this end, we designed two Mos, Mo-miR-204- and Mo-miR-204-2, against the two ol-miR-204-1 and ol-miR-204-2 precursor sequences present in the medaka genome, which give rise to an identical mature miR-204 sequence. Embryos injected with either of these two Mos at the one-cell stage were morphologically indistinguishable from control embryos up to the optic-vesicle stage. In contrast, from St24 onward, an aberrant eye phenotype was clearly visible in most of the Mo-miR-204-injected embryos (65 ± 5% of 3,000 injected embryos). Growth of the eye cup was significantly impaired and culminated in evident microphthalmia at St40 (Fig. S2A and D). In 90% of the microphthalmic embryos, lens development was also impaired. Specifically, at St24, the monolayer of lens epithelial cells was positioned in the dorsal region of the lens vesicle instead of lining its distal surface in morphants (Fig. 2A–F, Fig. S3H, H', J, and J', and Fig. S4A, B, G, and H). Furthermore, the primary fiber cells that are located in the center of the lens vesicle were misplaced and disorganized, whereas those of the control embryos had begun to elongate to form the organized concentric layers (Fig. 2A–F and Fig. S4A, B, G, and H). This altered cellular organization was also evident at later stages, when abnormal ventral-distal herniations of the lens were also evident (Fig. S2E). Finally, a significant number of the microphthalmic morphant embryos (30%) were characterized by a ventral coloboma, through failure of optic fissure closure (Fig. S2C and F).

We did not observe any qualitative and quantitative phenotypic differences following the injection of Mo-miR-204-1 or Mo-miR-204-2; hence, subsequent studies were performed only with

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potential mRNA targets using the recently developed host-gene oppositely correlated target (HOCTAR) tool, which integrates expression profiling and sequence-based miRNA target recognition software (15). Among the predicted miR-204 targets, the homeobox transcription factors *Meis1* and *Meis2* appeared particularly attractive to explain the observed eye defects because they had been previously shown to regulate vertebrate eye development by modulating *Pax6* expression (16). Furthermore, the predicted target site of miR-204 within the 3'-UTR of *Meis2* (but not of *Meis1*) was highly conserved across all vertebrate species analyzed, including medaka (Fig. 1D). To validate this prediction, we cloned the 3'-UTR of the human *MEIS2* gene containing the miR-204 target site downstream of the coding region of the *Luciferase* (Luc) reporter gene, and tested the ability of miR-204 to affect reporter expression in vitro. The presence of the *MEIS2* 3'-UTR sequence specifically inhibited Luc activity in response to miR-204 (Fig. 1E). In addition, point mutations in the miR-204 binding site of the *MEIS2* 3'-UTR abolished this repression, indicating that miR-204 directly and specifically targets *MEIS2* (Fig. 1E). In agreement with these observations, the levels of MEIS2 protein in H36CE human lens epithelial cells were decreased in the presence of miR-204 duplexes and elevated on miR-204 inhibition (Fig. 1F and G). The specificity of this repression was confirmed by additional controls described in *SI Text* (i.e., a Luc construct containing the 3'-UTR of *PAX6* and miR-182, an unrelated miRNA expressed in the eye) (Fig. S3 K–N).

The miR-204 targeting of *Meis2* was also confirmed in vivo. miR-204 and *Meis2* showed overlapping expression patterns in the lens and in the peripheral optic cup (Fig. 1A–C'). Moreover, injections of miR-204 duplexes resulted in a decrease in endogenous *Meis2* mRNA and protein levels, whereas injections of Mo-miR-204 led to an increase in the optic cup of medaka embryos (Fig. 1H–M). The expansion of the *Meis2* domain in the miR-204 morphants was not caused by a generalized delay of eye development because (i) *Meis2* was correctly expressed at early stages of lens and retinal development in the morphants (Fig. S3 G–J') and (ii) *Ath5*, an early marker of retinal ganglion cell differentiation, was expressed normally at St26, when the retina begins to differentiate (Fig. S3 O and P). Altogether, these data strongly indicate that *Meis2* is a bona fide miR-204 target.

miR-204 Controls Lens Differentiation by Targeting *Meis2* and Modulating the *Pax6* Transcriptional Pathway. We next sought to determine whether the miR-204 morphant phenotype was indeed related to abnormal activation of *olMeis2* expression. *Meis2* has been reported to regulate *Pax6* activity in the lens in a direct and positive way (16). *Pax6*, in turn, controls the expression of genes involved in lens differentiation, including *Sox2*, *Prox1*, and α -*ACrystallin* (17, 18). With the exception of *Meis2*, none of these genes are predicted to contain miR-204 target sites. We thus reasoned that if miR-204 directly controls expression of *Meis2* in vivo, the levels of expression of all these genes should be increased in the miR-204 morphant as a consequence of alterations in *Meis2* expression.

Indeed, RNA ISH demonstrated that in the morphant optic cup, *olPax6*, *olSox2*, *olProx1*, and α -*ACrystallin* showed up-regulated expression and/or were misexpressed in both lens ectoderm and primary lens fiber cells when compared with control embryos (Fig. 2A–F and Fig. S4 A, B, G, and H). To demonstrate a direct interaction between miR-204 and *Meis2* further, and to dissect out the role of this interaction in lens development, we increased the levels of miR-204 by injecting miR-204 duplexes. From St20 onward, duplex-injected embryos were severely microphthalmic, with small lenses (Fig. S2 J–L), which strongly resembled the phenotype reported for *Meis2.2* zebrafish morphant embryos (19). Consistent with the hypothesis that *olMeis2* is an important miR-204 target, expression of the *olMeis2*, *olPax6*, *olSox2*, *olProx1*, and α -

ACrystallin genes was significantly reduced in the lens placode of all the duplex-injected embryos (Fig. 2G–I and Fig. S4 C and J), as confirmed by quantitative real-time qRT (PCR) (Fig. S4M). Of note, inhibition of miR-204 activity by Mo-miR-204 injections was accompanied by an opposite trend in the relative transcript levels (Fig. S4N). Interestingly, both the lens epithelial monolayer and the primary fiber cells were normally localized in duplex-injected embryos (Fig. 2G–I and Fig. S4 C and J).

If most of the changes in lens differentiation marker expression caused by miR-204 knockdown are mediated by *Meis2*, coinjection of a Mo against *olMeis2* (Mo-*Meis2*) should reestablish normal expression levels in miR-204 morphants. Consistent with this, Mo-*Meis2* injection was sufficient to rescue the normal expression of lens differentiation markers in a substantial proportion of miR-204 morphants (Fig. 2J–L, Fig. S4 D and J, and Table S2), although defects in epithelial and lens fiber cell organization were not rescued (Fig. 2J–L and Fig. S4 D and J). This suggests that miR-204 regulates additional, and as yet unidentified, genes that are important for correct lens development.

To obtain additional support for the importance of miR-204-mediated regulation of *olMeis2*, we disrupted the interaction of miR-204 with its target site in *olMeis2* 3'-UTR by injecting a *Meis2* "target protector" (20) Mo (*Meis2*-TPmiR-204) in WT embryos (*SI Text* and Fig. S3 A–F). A significant percentage of *Meis2*-TPmiR-204-injected embryos were morphologically indistinguishable from miR-204 morphants and characterized by similar defects in the lens, optic cup size, and optic fissure coloboma (Fig. S2 G–I and Table S2). Moreover, protection of the miR-204 target site within *Meis2* mRNA resulted in expansion of the lens placode expression domains of *olMeis2*, *olPax6*, and *olSox2* and mislocalization of *olProx1* and α -*ACrystallin*, the mRNAs of which were ectopically detected in the epithelial cell monolayer (Fig. 2M–O and Fig. S4 E and K). Unlike our observations in miR-204 morphant embryos, these changes were not associated with defects in epithelial and lens fiber cell organization (Fig. 2M–O and Fig. S4 E and K), further supporting the possibility that these alterations are mediated by other mRNA targets. Finally, protection of *Meis2* targeting was sufficient to rescue the correct expression of lens differentiation markers in miR-204-overexpressing embryos (Fig. 2P–R and Fig. S4 F and L).

Altogether, these data indicate that miR-204 controls lens cell differentiation by modulating the expression of lens placode differentiation genes via the *Meis2*/*Pax6* pathway.

miR-204 Has an Active Role in Establishment of Dorsoventral Polarity of the Optic Cup. Defects in optic fissure closure were observed in both the Mo-miR-204 and *Meis2*-TPmiR-204 morphants (Fig. S2 D–I). Colobomas have been frequently described as a consequence of impaired dorsoventral (D–V) polarity of the optic cup (21). As observed in the lens, this defect was associated with concomitant up-regulation of *olMeis2* (Fig. 1H, J, K, and M and Fig. S5B) and *olPax6* expression. In particular, *olPax6* expression extended to the ventral retina, where it is normally expressed at relatively low levels (Fig. 3A, D, and M). Previous studies have shown that expansion of *Pax6* expression in the ventral retina results in alterations in D–V polarity of the optic cup (22, 23). Therefore, we asked whether expression of D–V optic cup markers was modified in Mo-miR-204 and *Meis2*-TPmiR-204 morphants. In all the injected embryos, the expression domain of the ventral marker *olVax2* and the ventral expression domain of *olPax2* (24) were reduced or absent (Fig. 3B, E, and N and Fig. S5 E and H), whereas the expression domains of the dorsal markers *olBmp4* and *olTbx5* were ventrally expanded (Fig. 3C, F, and O and Fig. S5 K and N). A reciprocal molecular phenotype was observed after miR-204 overexpression: The *olBmp4* and *olTbx5* domains were largely reduced, whereas those of *olPax2* and *olVax2* were dorsally expanded (Fig. 3H and I and Fig. S5 F and L). Furthermore, coinjection of Mo-*Meis2* with Mo-miR-204 (Fig. S2 M–O,

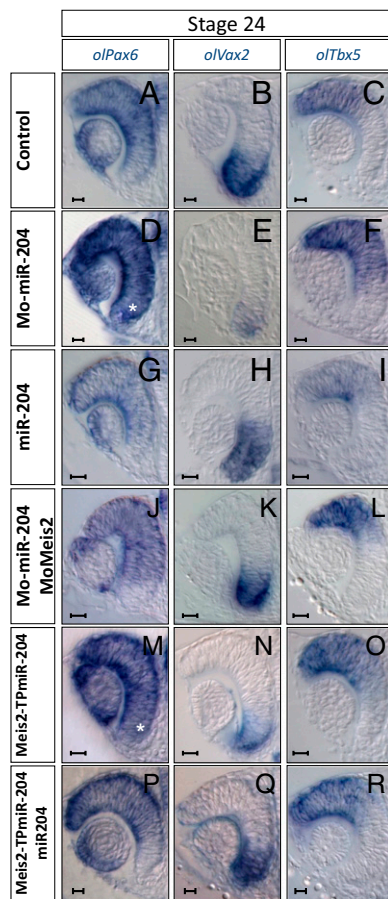


Fig. 3. miR-204 is required for establishment of D-V polarity of the optic cup. Frontal vibratome sections of control (A–C), Mo-miR-204 (D–F), miR-204 (G–I), Mo-Meis2/Mo-miR-204 (J–L), Meis2-TPmiR-204 (M–O), and Meis2-TPmiR-204/miR-204 (P–R)-injected embryos processed for whole-mount RNA ISH with probes specific for *olPax6* (A, D, G, J, M, and P), *olVax2* (B, E, H, K, N, and Q), and *olTbx5* (C, F, I, L, O, and R). *olPax6* is up-regulated and ectopically expressed in ventral retina of morphant embryos (D and M, asterisk). Expression of ventral gene *olVax2* is reduced, whereas that of dorsal marker *olTbx5* is expanded ventrally in morphant embryos (E, F, N, and O) when compared with control embryos (B and C). miR-204-overexpressing embryos show reciprocal alterations in *Pax6* and D-V marker expression (G–I). Defects in D-V optic cup polarity are completely rescued by coinjection of Mo-miR-204 with Mo-Meis2 (J–L) and Meis2-TPmiR-204 with miR-204 (P–R). (Scale bars: 20 μ m.)

Fig. S54, and Table S2) or that of miR-204 with the Meis2-TPmiR-204 target protector (Fig. S2 S–U, Fig. S5C, and Table S2) fully rescued the coloboma phenotype and the normal levels of D-V markers and *Pax6* expression (Fig. 3 J–L, P–R and Fig. S5 G, I, M, and O).

Altogether, these data demonstrate that miR-204-mediated control of the Meis2/*Pax6* pathway contributes to D-V patterning of the optic cup. Moreover, our findings reveal a previously unidentified role for *Meis2* in this morphogenetic event.

Discussion

MiRNAs appear to function as “master regulators” of key molecular pathways through their ability to fine-tune gene dose (25); consequently, they have basic roles in vertebrate organogenesis and pathogenesis. The general importance of this class of molecule in eye development is supported by effects observed in mice after genetic inactivation of *Dicer*, a key enzyme of miRNA biogenesis (26). However, there is little information available on individual miRNAs that contribute to the correct development

and function of the eye. Examples exist with miR-24a, miR-124, and miR-26a for the regulation of apoptotic pathways, retinogenesis, and circadian rhythms of mRNAs in the retina, respectively (27–29). Here, we showed that a single miRNA can regulate multiple aspects of eye development. Indeed, miR-204 is required for lens differentiation, optic cup development, and optic fissure closure.

Starting from *in silico* predictions and using a variety of *in vitro* and *in vivo* experimental approaches, we have shown that *Meis2* is a bona fide target of miR-204 activity. Our data also show that the specific miR-204-mediated regulation of *Meis2* modulates the function of the *Pax6* transcriptional network. These data indicate that miR-204 is an essential component of the Meis2/*Pax6* molecular pathway, and hence is an important element of the molecular network that regulates eye development in vertebrates (Fig. 4). This regulatory cascade is strongly supported by a number of observations. *Meis2* directly activates *Pax6* expression during lens and retina development in the zebrafish, chick, and mouse (16, 19, 30). In medaka fish embryos, expression levels of *olMeis2* and, concomitantly, those of *olPax6* and its downstream targets are up-regulated on miR-204 knock-down or inhibition of the miR-204 interaction with its specific target site in the *Meis2* 3'-UTR. Furthermore, the phenotypic alterations observed in miR-204 morphants strongly resembled those reported for *Meis2* or *Pax6* gain-of-function models (4, 16, 19, 30), which include aberrant lens differentiation and microphthalmia. Conversely, miR-204 over-expression led to a significant down-regulation of *Meis2* activity, with phenotypic consequences similar to those observed after *Meis2* loss-of-function, in which a reduction of *Pax6* gene dose was also observed (16, 19, 30). Finally, concomitant knock-down of *olMeis2* and miR-204 and protection of the *olMeis2* target site in miR-204-overexpressing embryos, significantly rescued expression of *olPax6*, *olSox2*, *olProx1*, and *ol α -Crystallin* in the lens, which is consistent with a pathway in which miR-204 controls lens differentiation through regulation of *olMeis2* levels (Fig. 4).

During lens development, the progeny of epithelial cells migrate into the transitional zone and elongate and differentiate into lens fiber cells (18, 31). Timely differentiation and correct migration of lens fibers are crucial for continuous addition of fiber mass and formation of a correctly organized lens. The miR-204-mediated control of *olMeis2* appears to modulate gene expression programs that control these events because its over-expression or inactivation produces significant changes in the expression of lens differentiation markers. miR-204 also appears to control lens morphogenesis, because lens epithelial cells were abnormally positioned in Mo-204 morphants. However, this miR-204 activity is very likely to be *Meis2*-independent, because protection of *Meis2* mRNA did not alter epithelial and lens fiber cell organi-

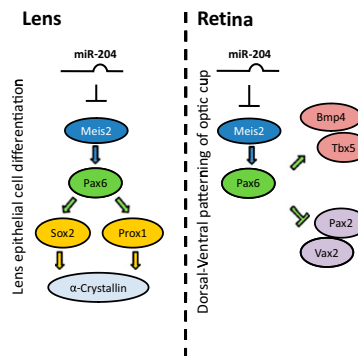


Fig. 4. Model for function of miR-204 in lens and retina development. miR-204 controls expression of the *Meis2* gene, contributing to regulation of the Meis2/*Pax6* pathway in both lens and retina development.

zation. Thus, miR-204 might ensure correct control of lens morphogenesis by targeting other genes involved (e.g., in the control of cell polarity, cell-cell signaling, tissue polarity, or cell migration). This last possibility is particularly attractive, because cells with elevated miR-204 levels are highly mobile and have invasive properties (32, 33).

The miR-204 contributed to other aspects of eye morphogenesis, as expected by its specific distribution in ocular tissues other than the lens. The requirement for miR-204 in establishment of D-V polarity in the optic cup and in optic fissure closure can also be explained by its control of *Meis2* expression. Indeed, the defects in D-V polarity and optic fissure closure in the miR-204 and Meis2-TPmiR-204 morphants were rescued by coinjection with Mo-Meis2 or miR-204, respectively. This is an as yet unreported aspect of Meis2 function that is probably mediated by the observed ectopic ventral expression of *Pax6*, the overexpression of which correlates with significant down-regulation of ventral determinant genes, which leads to the formation of optic fissure coloboma (22, 23). However, the relatively low frequency of optic fissure defects in miR-204 morphants suggests that miR-204 might not serve as an “on-off” switch for the genetic program required for correct optic fissure closure.

In miR-204 morphants, eye formation was initiated and progressed normally up to the optic cup stage; thereafter, it did not advance correctly, leading to micropthalmia. This phenotype might be a consequence of alterations in apoptosis and/or cell proliferation, because previous studies have shown that members of the *Meis* gene family can directly regulate these events. However, we found that the concomitant knock-down of *Meis2* and miR-204 was insufficient to restore normal eye size (Fig. S2), possibly because Mo-Meis2 reduces the levels of *Meis2* expression well below normal levels. Indeed, a micropthalmic phenotype has been reported for loss-of-function *Meis* mutants in the chick and zebrafish (19, 29). Alternatively, the miR-204-mediated regulation of eye size involves other, as yet unidentified, transcriptional pathways.

Although miRNAs have the potential to regulate the expression of hundreds of genes, we have shown that the specific miR-204-*Meis2* interaction has multiple consequences in eye development. Of note, this action is mediated by a single miR-204 target site within the 3'-UTR of the *Meis2* gene, as demonstrated by the specific target protector assay. Previous reports have proposed that the presence of multiple target sites for the same miRNA within the 3'-UTR of a given mRNA is a strong indication of the “strength” and biological relevance of these interactions (34). However, in agreement with our data, it has also been reported that point mutations in a single miRNA recognition site have a pathogenetic role in human genetic diseases (35). Thus, the mode of action of miRNAs and their relevance in the control of basic biological processes may be more complex than initially envisaged.

In conclusion, we have begun to unravel the function of miR-204 during eye development, and we have demonstrated that this is largely mediated by *Meis2* targeting. As shown by our data, miR-204 may have additional target genes in the eye. It will be of the utmost importance to identify these and to determine whether

alterations in miR-204 expression contribute to the pathogenesis of eye malformations in humans.

Materials and Methods

Medaka Stocks. Samples of the Cab strain of WT medaka fish were kept and staged as described (36).

Computational Analysis. Prediction of miRNA targets was performed using the Host gene Opposite Correlated TARgets (HOCTAR) tool (<http://hocr.tigem.it>) (15).

Mo and miR-204 Duplex Injections. Mos (Gene Tools, LLC) were designed and injected into fertilized one-cell embryos, as detailed in Table S1. The specificity and inhibitory efficiency of each Mo were determined as described (14). Optimal Mo concentrations (Tables S1 and S2) were determined on the basis of morphological criteria. miRIDIAN (Dharmacon) miRNA Mimics for miR-204 were injected at a final concentration of 4 μ M. Embryos injected with mm-Mo-miR-204 were used as controls.

Whole-Mount ISH. Whole-mount RNA ISH was performed, photographed, and sectioned as described (37). Digoxigenin-labeled antisense and sense riboprobes for *olMeis2*, *olPax6*, *ol α -Crystallin*, *olSox2*, *olProx1*, *olBmp4*, *olTbx5.2*, *olVax2*, and *olPax2* were used. The miRCURY detection miR-204 probe (Exiqon) was used according to Karali et al. (11).

Western Blotting. Immunoblotting was performed as described (38), with a rabbit polyclonal antibody against Meis2 (1:1,000) or an anti- β -tubulin monoclonal antibody (1:1,000; Sigma).

Immunolabeling. Medaka embryos were cryostat-sectioned, and immunocytochemistry was performed as described (39) using an anti-phospho-histone H3 monoclonal antibody (1:100; Cell Signaling Technology) and an anti-Meis2 rabbit polyclonal antiserum. Alexa-488-conjugated goat anti-rabbit or anti-mouse (1:1,000; Invitrogen) IgGs were used as secondary antibodies. Alternatively, a peroxidase-conjugated anti-rabbit antibody (1:200; Vector Laboratories) was used, followed by diaminobenzidine staining, as described previously (40).

Cell Transfection, qRT-PCR, and Luc Assays. The H36CE human lens epithelial cell line was grown as described (41). Cell transfections and qRT-PCR experiments were performed as described (15). Cells were transfected with either 50 nM miRIDIAN miRNA Mimics or 80 nM miRIDIAN miRNA Inhibitor (Dharmacon). Plasmids containing the 3'-UTR of the human *MEIS2* gene and psiUx plasmid constructs containing the hsa-premiR-204 sequence were used in Luc assays, as described previously (15). Each assay was performed in duplicate, and all the results are shown as means \pm SD of at least three independent assays. The primer sequences used to amplify each transcript are shown in Table S1.

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