**MicroRNA 218 Mediates the Effects of Tbx5a Over-Expression on Zebrafish Heart Development**

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Abstract

tbx5, a member of the T-box gene family, encodes one of the key transcription factors mediating vertebrate heart development. Tbx5 function in heart development appears to be exquisitely sensitive to gene dosage, since both haploinsufficiency and gene duplication generate the cardiac abnormalities associated with Holt–Oram syndrome (HOS), a highly penetrant autosomal dominant disease characterized by congenital heart defects of varying severity and upper limb malformation. It is suggested that tight integration of microRNAs and transcription factors into the cardiac genetic circuitry provides a rich and robust array of regulatory interactions to control cardiac gene expression. Based on these considerations, we performed an in silico screening to identify microRNAs embedded in genes highly sensitive to Tbx5 dosage. Among the identified microRNAs, we focused our attention on miR-218-1 that, together with its host gene, slit2, is involved in heart development. We found correlated expression of Tbx5 and miR-218 during cardiomyocyte differentiation of mouse P19CL6 cells. In zebrafish embryos, we show that both Tbx5 and miR-218 dysregulation have a severe impact on heart development, affecting early heart morphogenesis. Interestingly, down-regulation of miR-218 is able to rescue the heart defects generated by tbx5 over-expression supporting the notion that miR-218 is a crucial mediator of Tbx5 in heart development and suggesting its possible involvement in the onset of heart malformations.

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Introduction

The formation of the mature vertebrate heart with separated chambers and valves involves a complex orchestration of gene expression. Numerous genes are critical for cardiac morphogenesis, although their exact functions and their integration with other cardiac regulators is poorly understood [1].

The T-box gene tbx5 encodes a key transcription factor for vertebrate heart development [2,3]. Tbx5 function in the heart is gene dosage sensitive, as both haploinsufficiency and gene duplication give rise to Holt–Oram syndrome (HOS). HOS is a highly penetrant autosomal dominant disease characterized by congenital malformations of the heart and upper limbs, which are two sites of Tbx5 expression [4–7]. Nonetheless, the molecular mechanisms accounting for gene dosage sensitivity are not known. Mice heterozygous for mutations in Tbx5 display many of the phenotypic abnormalities of individuals with HOS [8,9]. Comparable defects are seen in the zebrafish Tbx5 mutant heartstrings, suggesting that Tbx5 expression and function have been conserved throughout vertebrate evolution [10,11]. In the murine model of HOS, gene expression profiling of a tbx5 allelic series demonstrated that Tbx5 could regulate hundreds of downstream genes [8]. Examination of the expression dynamics of mouse genes regulated by Tbx5 indicates that Tbx5 can act via an array of independent mechanisms, some of which include direct DNA binding as has been shown for Gja5 [9], and others via indirect mechanisms that may involve complex regulatory networks.

Several genes regulated by mouse Tbx5 encode transcription factors (TFs), or are involved in transcriptional regulation suggesting that HOX might in part be the consequence of “misregulation of regulators”. Besides TFs, microRNAs (miRNAs) play key roles in heart development and cardiac diseases [12–14]. TFs and miRNAs comprise two major layers of gene regulatory networks with strictly interconnected activities: TFs control miRNA expression and many miRNA targets are TFs. There is increasing evidence that TFs and miRNAs can work cooperatively through mutual cross-regulation [15]. Starting from these considerations, we performed an in silico screening to identify miRNAs embedded in genes highly sensitive to Tbx5 dosage. We focused our attention on miR-218-1 that, together with its host gene slit2, is involved in heart development [16]. We confirmed a correlation between tbx5 and miR-218 expression and showed that alterations of miR-218 expression have a significant impact on zebrafish heart development. Interestingly, down-regulation of miR-218 is able to rescue most of the defects generated by Tbx5 over-expression, demonstrating the pivotal role of miR-218 in mediating the effects of Tbx5 dosage on heart development. These data support the idea that a miRNAs/Tbx5 regulatory circuit is crucial in cardiac morphogenesis.
Results

Identification of Tbx5-modulated miRNAs

To identify miRNAs modulated by Tbx5, we developed a bioinformatic tool to search for miRNAs within introns of Tbx5-controlled genes (Fig. S1). As a source of Tbx5 targets, we used genes identified by microarray analysis of a conditional mouse allelic series of tbx5 [8] and of a mouse 1H cell line infected with adenovirus expressing tbx5 [17]. Four miRNAs were identified: miR-218-1, miR-678, mir-719 and miR-335 (Table 1). We focused our attention on miR-218 since: i) it is conserved from human to zebrafish (www.mirbase.org); ii) the miR-218-1 host gene, slit2, is highly sensitive to Tbx5 mis-expression [8]; iii) the secreted Slit ligands, together with their Robo receptors, contribute to the control of oriented cell tissue growth during chamber morphogenesis of the mammalian heart [18]; iv) Slit/Robo are part of a regulatory loop required during heart tube formation in zebrafish [16].

tbx5 and miR-218 are Co-expressed in Mouse Tissues and in Cardiomyocyte Differentiation of P19Cl6 Cells

We compared tbx5, slit2 and miR-218 expression in newborn mouse lung, liver, brain, aorta, skeletal muscle and heart. slit3 expression was also analyzed since its host miRNA, miR-218-2, cannot be separately quantified because it is identical to miR-218-1. In agreement with the literature [19], we observed tight co-expression of slit2 and miR-218, and a general correlation among tbx5, slit2, slit3 and miR-218 expression (Fig. S2).

To assess whether there are functional regulatory interactions among Tbx5, Slit2 and miR-218, we first examined these genes in an in vitro model for cardiomyocyte differentiation. P19Cl6 cells proliferate in growth medium (GM) and differentiate into beating cardiomyocytes in differentiation medium (DM) [20,21]. P19Cl6 increased the expression of cardiac differentiation markers such as GATA4, α-MHC, CX40 and decreased the expression of the marker of pluripotency Oct4 after a few days of culture in DM, compared to cells maintained in GM (Fig. 1A). A progressive increase in tbx5 expression was also observed (Fig. 1B), which was paralleled by an increase in slit2, slit3 and miR-218 transcripts. To show that the slit/miR-218 increase was at least partially dependent on Tbx5, tbx5 was up- or down-regulated by transfecting P19Cl6 cells with a tbx5-carrying expression vector (CMV-Tbx5), or with a siRNA mix directed against tbx5, respectively. Tbx5 over-expression tripled slit2 expression, almost doubled miR-218 expression and had no effect on slit3 expression (Fig. 1C). On the other hand, tbx5 silencing, the effect of which was highest 2 days after silencing (6th day in culture, see Methods), caused significant reduction of slit2 and miR-218 expression 4 days after transfection (8th day in culture, Fig. 1D). Pre-miRNA 218-1 expression paralleled the increase in miR-218 level during cardiomyocyte differentiation (Fig. S3A) and after Tbx5 modulation (Fig. S3B). Moreover, the transfection of a siRNA mix against Slit2 cut the level of Slit2 by half without affecting miR-218 expression, supporting the idea that miR-218 expression depends on the regulation of Slit2 transcription rather than on its translation. Overall these results suggest that the expression of Slit2 and its embedded microRNA miR-218-1 are modulated by Tbx5 during cardiomyocyte differentiation.

miR-218a Over-expression Affects Zebrafish Heart Development

To analyze the role of miR-218 in heart development, we decided to use zebrafish since this model is particularly informative for studying cardiac early patterning networks due to its relatively simple two-chambered heart coupled with its ability to develop even in the absence of a functioning heart. Moreover, various data derived from Tbx5 knock-down experiments in zebrafish [10,11] have revealed developmental defects of the heart and limbs comparable to the defects observed in human with Tbx5 mutations [4,22] or in Tbx5 knock-down mice [9,23], suggesting that the functional role of this crucial transcription factor is evolutionarily conserved.

In zebrafish, as in mammals, two isoforms of miR-218, miR-218a-1 and miR-218a-2, are embedded in slit3 and slit2 genes, respectively. A third genomic copy of this miRNA, miR-218b, is present in fish. However miR-218b, an intergenic miRNA, has very low expression, suggesting that its contribution to the global miR-218 level might be irrelevant [16]. miR-218a1/2, slit2 and slit3 are highly expressed in zebrafish neural tissue (Fig. 2A, [24,25]) and Fig. 2B [26-28]. In cardiac tissue, slit2 and slit3 are clearly detectable at 48 hpf (Fig. 2B), while the expression of miR-218a1/2 is barely detectable by in situ hybridization (ISH) up to 48 hpf (not shown) but is visible around 72 hpf (Fig. 2A).

To over-express miR-218a1/2, we injected double stranded RNA oligonucleotide with a miR-218a sequence (miR-218a mimic) in Tg(mlc2:eGFP) embryos. Injection of 260, 135 and 35 pg of miR-218a mimic generated embryos with cardiac defects in a dose-dependent way (Fig. 2C). At the highest dose (2 ng in Fig. 2C), we observed a slight decrease in embryos with cardiac defects, which was balanced by the increased number of dead embryos and, to a lesser extent, of embryos with different morphological defects. As specificity controls, 2 ng of either miR-492, a control miRNA, that is not annotated in zebrafish, or miR-214, which is not heart-specific, were injected without generating embryos with cardiac defects in significant percentages (Fig. 2C). Conversely, in miR-218a over-expressing embryos we found different cardiac defects accounting for the ratios shown in Fig. 2C: hearts failing to complete looping, ventricles showing very irregular walls, and atria that were strongly reduced and sometimes stretched to a thin “string-like” morphology (Fig. 2F,G). To down regulate miR-218a, we injected either a morpholino targeting the mature form of miR-218a (MO1-218), or a longer morpholino also targeting the Drosha cleavage site of pre-miR-218a (MO2-218, see Methods and [16]). The knockdown efficiency of these morpholinos was

| Table 1. miRNAs identified by bioinformatic approach within introns of Tbx5-modulated genes. |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|
| Microarray | Gene symbol | miRNA | Tbx5 control | Proposed biological function |
| Mori/Plagman | Mest | miR-335 | positive | Trabeculation, mouse embryonic cardiac expression [54] |
| Mori | Nupl 1 | miR-719 | negative | Nuclear pore complex |
| Mori | Slit 2 | miR-218-1 | positive | Secreted negative regulator of axonal extension |
| Mori | Hrml 1 | miR-278 | positive | S-adenosylmethionine-dependent methyltransferase activity |

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confirmed by their ability to down-regulate mature miR-218 (Fig. S4A), and to rescue the phenotype caused by miR-218a overexpression (Fig. S4B). Nonetheless, injection of either MOD-218 or MOM-218 (not shown) generated no cardiac edema or cardiac defects even at very high dose (12 ng) in any of the genetic backgrounds we tested (Fig. 2D,H). This is consistent with the extremely low level of miR-218a during early stages of development (data not shown and [16,29]).

Injection of miR-218a mimic or MOs-218 in Tg(flk1:eGFP), which express GFP driven by the endothelial-specific enhancer of flk1, allowed a direct visualization of vascular integrity. As previously reported [16], MO-218 microinjection did not cause gross alteration in vascular structures (Fig. S5C) nor the hemorrhagic events described in mice after miR-218a knock down [19]. miR-218a over-expression did not affect vessel morphology either (Fig. S5B), supporting the idea that in zebrafish miR-218a does not overtly influence the organization of blood vessels during embryogenesis.

Finally, we analyzed the expression of some cardiac markers in embryos over-expressing miR-218a. Despite the strong cardiac morphological alterations, miR-218a over-expressing embryos showed normal expression of the ventricular myosin heavy chain and the atrial myosin heavy chain (Fig. S6), as previously described in Tbx5 (hot) mutants [10]. It has been shown that Tbx5 activity is crucial for the specification of the AV boundary and valve formation [30]. To verify whether miR-218 over-expression can affect cardiac valve development, we analyzed the expression of the tie-2 gene, a member of the Tie family of tyrosine kinase receptors, which is expressed mainly in endothelial cells [31] and is up regulated during atrio-ventricular canal differentiation [32,33]. In hemizygous Tg(tie-2:GFP) embryos, the transgene has low expression [34] and it is easy detectable only in the atrio-ventricular canal thus allowing to visualize the valve tissues (Fig. 3A, a,a'). Hemizygous Tg(tie-2:GFP) embryos injected with miR-218a showed an increase in Tie-2 expressing cells in the ventricle and atrium (Fig. 3A, b,b' and Fig. 3B,C). A similar Tie-2 dysregulation was observed in hemizygous Tg(tie-2:GFP) embryos after Tbx5 misexpression (Fig. 3A, c,c',d,d' and Fig. 3B).

All together these data indicate that correct expression of miR-218 is crucial for proper cardiac development.

miR-218 Over-expression Decreases the Migration of Myocardial Precursors

Recent data have shown that, in zebrafish, delayed heart field migration was caused by either miR-218a reduction or by silencing of Robo1, an established target of miR-218a [16,19]. In line with data showing rob1 as target of miR-218a [16,19], we analyzed the expression of the tie-2 gene, a member of the Tie family of tyrosine kinase receptors, which is expressed mainly in endothelial cells [31] and is up regulated during atrio-ventricular canal differentiation [32,33]. In hemizygous Tg(tie-2:GFP) embryos, the transgene has low expression [34] and it is easy detectable only in the atrio-ventricular canal thus allowing to visualize the valve tissues (Fig. 3A, a,a'). Hemizygous Tg(tie-2:GFP) embryos injected with miR-218a showed an increase in Tie-2 expressing cells in the ventricle and atrium (Fig. 3A, b,b' and Fig. 3B,C). A similar Tie-2 dysregulation was observed in hemizygous Tg(tie-2:GFP) embryos after Tbx5 misexpression (Fig. 3A, c,c',d,d' and Fig. 3B).

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All together these data indicate that correct expression of miR-218 is crucial for proper cardiac development.
Embryos and the migration of the GFP-expressing cells was followed by confocal analysis. Myocardial cells migrated more slowly in miR-218a than in miR-Ct injected embryos (Fig. 4). Even after injection of high miR-218 doses, cardia bifida was never observed, suggesting that miR-218 over-expression slows down but does not arrest the migration of cardiomyocytes to the midline. On the contrary, MOD-218 injection did not affect cmlc2-positive cells migration (Fig. 4C), further confirming that reducing the level of this miRNA during the first hours of development has no overt effects on heart development.

These data show that the cardiac defects observed in miR-218a over-expressing embryos might be at least in part related to defects in heart field migration.

Tbx5 Over-expression can be Rescued by Down-regulation of miR-218

To further investigate the functional interaction between Tbx5 and miR-218a, we assessed whether the morphological alterations generated by Tbx5 misexpression might be compensated for the
We reasoned that if the phenotype induced by Tbx5 over-expression was due, at least in part, to increased expression of miR-218a, the co-injection of MO-218 should rescue the Tbx5 gain of function phenotype. To test this hypothesis, Tbx5a was over-expressed by injecting its mRNA into the cytoplasm of one–cell-stage embryos. Tbx5 over-expression affects heart development in humans [35,36], mice [37] and chicks [5], while no reports describe the effect of Tbx5 over-expression in zebrafish. Over-expression of tbx5a mRNA induced a range of cardiac defects, from cardiac edema in 25% of embryos injected with low doses of mRNA (35 pg), to mild cases of looping defects or absence of looping and alteration of chamber morphology in 60% of embryos injected with higher doses (100 pg, Fig. 5A and C, panels b’–d’). Pectoral fins were occasionally asymmetric in embryos injected with 200 pg of tbx5a transcript (Fig. 5C, panel d).

miR-218a over-expression was observed as a consequence of Tbx5 up regulation (Fig. 5B). Co-injection of 8 ng of either MOD-218a or MOM-218a along with 100 pg of mRNA Tbx5a restored normal heart morphology and looping in a high percentage of injected embryos and reduced the number of embryos with eye defects as compared to control-injected embryos (Fig. 6A and B, panels b,b’). Co-injection of MO-218 was also able to counteract the tie-2 expansion observed in Tbx5 dysregulated embryos (Fig. 6 C,D). This result strengthened our hypothesis that

Figure 3. miR-218a over-expression leads to the expansion of tie-2 expression. A, confocal images of 72 hpf Tg(tie-2:GFP) embryos injected with 260 ng of control miRNA (a,a’), 260 ng of miR-218a mimic (b,b’), 2 ng of MO-Tbx5a (c,c’) or 100 pg of mRNA Tbx5a (d,d’). A, magnification of the control valve is shown in the inset in panel a’. Labels: A, atrium, V, ventricle. B, FACS analysis of cells dissociated from 72 hpf Tg(tie-2:GFP-cmlc2:eRFP) embryos injected as described in A. C, confocal images of 72 hpf Tg(tie-2:GFP-cmlc2:eRFP) embryos injected with 260 ng of miR-Ct (top) or with miR-218a mimic (bottom). The control valve is magnified in the inset in panel a’. White scale bars: 100 μm, red scale bars 25 μm.

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analyzed. from time-lapse images. Five embryos for each experiment were
migration velocities of myocardial Tg(cmlc2:eGFP) were screened for the presence of edema. White scale bars: 150
A,B, images of Tg(cmlc2:eGFP) embryos injected with 260 pg of miR-Ct (A) or with 260 pg of miR-218a mimic (B) at different
times of development. Dorsal view, anterior at the bottom. After
confocal analysis, embryos were left to develop until 72 hpf when they
were screened for the presence of edema. White scale bars: 150 µm. C,
migration velocities of myocardial Tg(cmlc2:eGFP) cells as quantified from time-lapse images. Five embryos for each experiment were
analyzed.
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the effect of Tbx5 over-expression on heart development might, at
least in part, be be mediated by miR-218.

The apparent lack of phenotype that we observed after MO-
218a injection (Fig. 2D,I) and the very low expression of miR-218
at early developmental stages, suggest that decreased miR-218a
should not contribute to the phenotype generated by Tbx5
knockdown. To verify this assumption we downregulated Tbx5
using a morpholino directed against the tbx5a translational start
site (MO-Tbx5a). The effectiveness of this morpholino was
confirmed by the injection of GFP-carrying a chimeric target
(Fig. S6A,B) and its specificity was confirmed by rescuing the MO-
Tbx5a phenotype co-injecting in vivo transcribed Tbx5a-mRNA
(not shown). Tg(cmlc2:eGFP) embryos injected with MO-Tbx5a
showed the heart and limb defects characteristic of the well
described heartstring phenotype (Fig. S8E,G and [10,11]). In
addition to tbx5a, a second tbx5 isoform has been recently
identified in zebrafish, tbx5b, which has lost the characteristic
for limb/pectoral fin expression of tbx5 genes [38]. Consequently,
we also designed a morpholino against this tbx5 isoform, MO-
Tbx5b (Fig. S8C,D). Similarly to MO-Tbx5a injection, MO-
Tbx5b injection caused looping defects and stretched cardiac
chambers, but it did not induce Tie-2 mis-expression and lack of
fins (Fig. S8F). For these reasons and since the penetrance of the
MO-Tbx5b phenotype was much lower compared to MO-Tbx5a
(compare Fig. S8G,H) we chose MO-Tbx5a for further analysis.
Co-injection of mimic miR-218a did not rescue the cardiac edema
and looping defects generated by MO-Tbx5a (Fig. S9A). Instead,
the co-injection increased both the frequency and the severity of
the mutant phenotype, doubling the number of heartstring-like
embryos (Fig. S9A) and the number of embryos that showed
a particularly extended edema at 48 hpf (Fig. S9B). This synergism
was further demonstrated by MO-Tbx5a and miR-218a mimic co-
injection in sub-phenotypic doses. In fact, zebrafish embryos
tolerated the injection of either 35 pg of miR-218a or 0.5 ng of
MO-Tbx5a well and only rarely revealed heart looping defects as
compared to un-injected siblings (Fig. 2A and Fig. S9C,D).
However, the injection of both MO-Tbx5a and miR-218a mimic at
these doses dramatically increased the number of embryos with
looping defects (Fig. S9C,E).

As a whole, these data support a functional interaction between
Tbx5 and miR-218a in heart morphogenesis.

Discussion

Our data show that miR-218 is part of a regulatory circuit
through which Tbx5 controls heart morphogenesis. Previous
studies in mice identified slit2, which encodes miR-218-1 within
one of its introns, as one of the genes highly sensitive to Tbx5
dosage [8]. Moreover, the coordinate expression of miR-218-1 and
its host genes has been largely documented both in physiological
(mouse development [19]) and pathological (cancer progression
[39,40]) conditions. We showed a functional relation between
Tbx5 and slit2 and fish

slit2 regulate miR-218. Tbx5 deregulation affects
expression directly or indirectly. However the
presence of T-box consensus sequences upstream of both mouse
and fish slit2 transcription start site, as identified by the Transfac
program (http://www.biobase-international.com; not shown),
supports the hypothesis that Tbx5 might directly bind to and
activate the slit2 promoter. To demonstrate that the Tbx5/miR-
218 regulatory circuit is also functional during development, we
used the zebrafish as a model system. Through functional assays in
zebrafish, we showed that either over-expression or down-
regulation of Tbx5 affects heart morphogenesis. In line with the
hypothesis that miR-218 might be a Tbx5 effector, we demon-
strated that miR-218a deregulation generates cardiac defects
(Fig. 2A). As in zebrafish the expression level of miR-218a is

Figure 4. miR-218 over-expression causes a delay in early heart
field migration. A,B, images of Tg(cmlc2:eGFP) embryos injected with
260 pg of miR-Ct (A) or with 260 pg of miR-218a mimic (B) at different
times of development. Dorsal view, anterior at the bottom. After
confocal analysis, embryos were left to develop until 72 hpf when they
were screened for the presence of edema. White scale bars: 150 µm. C,
migration velocities of myocardial Tg(cmlc2:eGFP) cells as quantified from time-lapse images. Five embryos for each experiment were
analyzed.
Figure 5. tbx5 over-expression causes eye, cardiac and fin defects. A, phenotypes generated by increasing doses of tbx5a mRNA. The percentage of embryos with the indicated defects was averaged across multiple independent experiments. The total number of embryos analyzed was as follows: mRNA-Tbx5a (35 pg) n = 48; mRNA-Tbx5a (100 pg) n = 199; mRNA-Tbx5a (200 pg) n = 131. B, qRT-PCR analysis of miR-218a relative expression in 24 and 34 hpf embryos injected with 100 pg of tbx5a mRNA compared with embryos injected with 100 pg of GFP mRNA. C, phase-contrast and confocal images of representative transgenic Tg(cmlc2:eGFP) embryos at 72 hpf showing eye, heart and fin morphological defects induced by the injection of 100 pg (b,b'–c–c') or of 200 pg (d,d') of tbx5a mRNA. Arrowheads indicate eye alteration, arrows show fin absence. Labels: A, atrium, V, ventricle. Dotted lines encircle ventricle (white) and atrium (red). Scale bars: black 100 μm, red 25 μm.

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extremely low during the first stages of development (our observation is supported also by the data of Fish et al. [16] and by data regarding miRNA microarray of Thatcher et al. [29], the introduction of very low amounts of miR-218a mimic into embryos at the one- or two-cell stage generated a severe cardiac phenotype. miR-218a over-expressing hearts were not able to complete the looping process, showed marked alteration of the cardiac chamber morphology and mis-expression of a marker of valve cardiac tissues. A large pericardial edema was visible after 48 hpf. The frequency and severity of all these cardiac phenotypes were dose-dependent (Figs. 2 and 3). Our data suggest that at least part of these morphological anomalies may be due to a reduced migration rate of myocardial cells (Fig. 4). Conversely, miR-218a down-regulation, even through injection of high doses of two different morpholinos, did not affect heart development.

Since Tbx5 and Slit2 are both expressed in myocardial cells [16] we hypothesized that early Tbx5 over-expression might cause heart malformation through early activation of miR-218a and silencing of the target genes of this miRNA. This hypothesis is supported by the observation that miR-218 down-regulation by MO-218a injection rescues the effects of Tbx5 over-expression (Fig. 6). robo1 has been identified as a target of miR-218 in many different organs and tissues [19,59,41]. Fish et al. [16] showed that robo1 is a miR-218a direct target and that early robo1 down-regulation by morpholino injection in zebrafish embryos induces severe pericardial edema and heart defects caused by reduced migration rate of endocardial cells. Thus, robo1 is a candidate gene through which Tbx5 and miR-218a early over-expression affects heart development. Fish et al. [16] reported that miR-218a down-regulation, performed by the microinjection of one of the two morpholinos that we used in our study, causes severe cardiac defects and cardiac edema through reduced migration of endocardial and myocardial cells. This is inconsistent with both our data and with the results of Fish et al. [16] who observed that Robo1 knock-down generates the same phenotype, because the same authors also showed that Robo1 is targeted by miR-218. In fact, an effect of miR-218a on the migration of endocardial and myocardial cells is not expected, as miR-218a seems not to be substantially expressed before 24 hpf [16,29], when the fusion of migrating cardiac cells is about to be completed. We speculate that the timing of miR-218a upregulation during heart development is crucial for heart morphogenesis. miR-218 and Robo1 are supposed to be upregulated and downregulated, respectively, when myocardial cell migration is about to end. In this view, Tbx5 mis-expression by mRNA microinjection at the one-cell stage might speed up the up-regulation of miR-218 and reduce the migration of myocardial cells precociously, which in turn might affect heart morphogenesis by impairing the correct interaction between myocardial and endocardial cells.

A negative role of miR-218 on cell migration has been highlighted in different biological contexts. In mice, it has been shown that miR-218 regulates vascular patterning by modulating Slit-Robo signaling [19]. The authors showed that miR-218-driven repression of Robo1/2 and of heparan sulfate proteoglycans (HSPGs), which are proteins essential for Slit/Robo signaling, negatively affects endothelial cell (EC) migration. miR-218 has also been shown to affect cancer progression by inhibiting tumor cell migration and metastasis via the repression of the Slit2/Robo1 pathway in gastric [40] and in nasopharyngeal [39] tumors, respectively. Therefore our data showing a migration delay of cnmt2-positive cardiac precursors in embryos over-expressing miR-218 (Fig. 4), but not in embryos in which miR-218 was down-regulated by MO-218 injection (Fig. 4C), are in line with these findings.

In the human heart, Tbx5 is expressed not only in the myocardium, but also throughout the embryonic epicardium and coronary vasculature. Using chick development as a model system, Hatcher et al. [42] showed that, in vivo, over-expression of biologically active Tbx5 inhibits proepicardial cell migration. Although we do not know whether the siRNA2 is expressed in proepicardial cells, it is tempting to speculate that the up-regulation of the Tbx5-miR-218 circuit might also impact the proepicardial cell migration by targeting robo1 or other cell migration regulators such as Semaphorins, some members of this large class of molecules being predicted targets of miR-218 (not shown).

Tbx5 over-expression affects heart development in different organisms. In humans, tbx5 gene duplication produces cardiac abnormalities [35,36]; in mice, persistent cardiac Tbx5 over-expression results in heart looping defects and abnormalities of early chamber development [37]. In chicks, Tbx5 over-expression determines a significant decrease in heart size and a marked decrease in ventricular trabeculation [5]. We also observed looping and cardiac chamber alterations in zebrafish after injection of tbx5a mRNA in embryos at one-cell stage. Unexpectedly, the severity of cardiac morphology defects was paralleled by the severity of eye defects (Fig. 5). Tbx5 is expressed in optic primordia from zebrafish to humans [43,44] and its mis-expression has been shown to affect eye morphogenesis and the visual projection in chicks [45]. Moreover, ophthalmological examination of HOS patients revealed alteration of dorso-ventral polarity in developing eye vesicles [46]. However, our Tbx5 over-expressing embryos showed particularly severe eye defects such as asymmetrically positioned eyes, fusion of eyes, and even unilateral anophthalmia (Fig. 5). Comparable eye defects are observed in Brg1 over-expressing zebrafish embryos [47]. Recently the importance of the balance between Brg1, a member of the SWI/SNF chromatin-remodeling complex, and several cardiac transcription factors including Tbx5, has been demonstrated [48]. Since Brg1 is maternally expressed [47], the over-expression of Tbx5 might generate a strong imbalance between these two factors during the first hours of development, and this imbalance might be at the root of eye defects. At the moment we do not know how miR-218 might also partially rescue eye defects. It is interesting to know that Pax2, that is negatively controlled by Tbx5 [45], is a predicted target of miR-218. Overall, these observations suggest that Tbx5 over-expression affects heart and eye development and that this might be at least partially mediated by miR-218. Our observation that MO-218 co-injection is able to ameliorate both heart and eye defects caused by Tbx5 injection is consistent with this model.

Our data suggest that the haplo-insufficiency of the Tbx5 gene, at the moment the most significant cause of HOS, does not impact heart and upper limb formation through miR-218 misregulation. The simplest explanation for this might be that other key RNAs controlled by Tbx5 than miR-218 might be necessary for heart morphogenesis by regulating mechanisms other than myocardial cell migration. This would still be consistent with the requirement of a tight tbx5 gene dosage regulation in space and time for proper heart morphogenesis. More rarely, HOS has been associated with increased Tbx3 expression by partial chromosome duplication [6,28] or mutation resulting in Tbx3 gain of function [49]. However advances in DNA sequencing technology also highlighted the potential role of non-coding variants in congenital malformations. Recent studies in mice uncovered three enhancers that collectively recapitulate the endogenous expression pattern of tbx3 but that singularly have specific and compartmentalized expression in the heart and forelimbs [50]. Interestingly,
MiR-218 is a Tbx5 Effector in Heart Development
Figure 6. Down-regulation of miR-218 can rescue the defects generated by tbx5 over-expression. A, quantification of the phenotypes induced by the injection of 100 pg of tbx5a mRNA (n = 199), 8 ng of MO-218 (n = 182) or by the co-injection of 100 pg of tbx5a mRNA and 8 ng of MO-218 (n = 241). As control, non injected embryos were quantified. Each experimental point in the graph represents the mean ± SE of at least three independent experiments. Comparisons between groups were performed by one-way analysis of variance, followed by Bonferroni’s post-hoc for multiple comparisons. B, phase-contrast and confocal images of representative transgenic Tgf(cmlc2:eGFP) embryos at 72 hpf comparing the phenotype of a control embryo (upper panels) to the rescued phenotype generated by the co-injection of 100 pg of tbx5a mRNA and 8 ng of MO-218 (lower panels). C, quantification of tie-2 mis-expression in 72 hpf Tg(tie-2:GFP) embryos after the co-injection of tbx5a mRNA (100 pg) and MO-Ct (8 ng, n = 60) or of tbx5a mRNA (100 pg) and MO-218 (8 ng, n = 62). D, Confocal images of representative 72 hpf Tg(tie-2:GFP) embryos co-injected with tbx5a mRNA and MO-Ct (a-a') or with tbx5a mRNA and MO-218 (b-b'). Labels: A, atrium, V, ventricle. Scale bars: black 100 μm, red 25 μm.

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a mutation in one of these enhancer sequences was identified in a cohort of non-syndromic patients and it has been shown to affect the enhancer activity in mice and zebrafish transgenic models. The population-wide frequency of this variant suggests that a significant number of congenital heart defects (CHD) associated with Tbx5 dysregulation might arise from non-coding mutations in Tbx5 heart enhancers effectively decoupling the heart and hand phenotypes of HOS syndrome. Therefore it is likely that modulation of Tbx5 in general, and over-expression of miR-218 as a consequence of Tbx5 up-regulation in particular, might have a higher impact on CHD population than previously hypothesized.

Finally, our results highlight the potential advantage of using miRNAs as target molecules for heart disease therapies. Their potential to restore the expression of hundreds of dysregulated mRNAs to their pre-pathological level in one shot and, in so doing possibly reverse the disease, places miRNAs among the most exciting molecules for the development of new therapeutic strategies.

Methods

Bioinformatic Analysis

Ensembl [http://www.ensembl.org/index.html], was used to obtain information about chromosome location, position and segment of the selected genes. Ensembl is a joint scientific project of the European Bioinformatics Institute (EBI; http://www.ebi.ac.uk/clusterw/) and the Wellcome Trust Sanger Institute (http://www.sanger.ac.uk/). EBI provides a centralized resource with annotations on genomes of sequenced species and the Ensembl Perl API (Application Programming Interface) models for access to biological objects, such as genes and proteins. Moreover EBI allows the execution of Perl programs for retrieving data from a public database MySQL. [http://www.mysql.it/]. We generated two local databases, one for genes, and one for microRNAs. By applying the Perl program that uses Ensembl API, we compared the gene databases with the microRNA database using a standard database interface module for Perl.

Reagents/Mature miRNA mimics (mmu-miR-218a, mmu-miR-492, mmu-miR-214 and miR-CT) were synthesized by GenePharma (Shanghai, China), morpholinos (Gene Tools, LLC USA), Lipofectamine 2000 TRIZol reagent, DNaseI amplification grade, SuperScript II reverse transcriptase, RNase out, t-minimal Essential Medium (Invitrogen), HyPreFect Transfection Reagent, miRNAeasy mini kit, miScript Reverse Transcription kit Quantitect Reverse Transcription kit and Quantilum SYBR Green PCR kit (QIAGEN, Milan, Italy), LightCycler 480 SYBR Green I Master, anti-DIG antibody-alkaline phosphatase Fab fragment, Blocking reagent, BM Purple and DIG-RNA labeling kit (Roche Diagnostics, Mannheim, Germany), DIG-labeled miRCURY LNA microRNA detection probe (Exiqon), SP6 RNA polymerase, RNase free DNase I (Fermentas International Inc), Herculase DNA polymerase, (Stratagene), pGEM Teasy vector, (PRO-MEGA), Fetal Bovine Serum (Lonza), zebrafish diet (SDS, Dietex, France), Tetramisole (Sigma), mMESSAGE mMACHINE® Kit (Applied Biosystems).

Zebrafish Lines

Current Italian national rules: no approval needs to be given for research on zebrafish embryos. Wild-type AB and transgenic Tgf(lk1:eGFP), Tg(cmlc2:eGFP), Tg(cmlc2:eRFP) and Tg(tie-2:GFP) transgenic lines were used in these studies. Zebrafish were raised and maintained under standard laboratory conditions (Westerring M zebrafish book) in a ZEBTEC Zebrafish Housing System (Tecniplast, Varese, Italy).

Cell Culture and Transfection

P19CL6 cells were obtained from Dr.Antonio Baldini (Telethon Institute of Genetics and Medicine, Napoli, Italy). Cells were cultured in growth conditions as previously described [20,21]. For differentiation conditions, growth medium was supplemented with 1% DMSO and the medium was replaced every two days. Cells were grown at 37°C in a humidified atmosphere containing 6% CO2. The pCMV-Tbx5 plasmid expressing mouse Tbx5 (Kindly provided by Prof. Mona Nemer, Université d’Ottawa) or the pCMV empty vector as control were transfected using Lipofectamine 2000. To downregulate mouse Tbx5 the following siRNAs were designed according to previously identified criteria [51]: siRNA-Tbx5-1 (5'-CUGG ACCCGUUUGGACACAUU-3'/5'-UGUGUCACAAACGGGCGAGUU-3') and siRNA-Tbx5-2 (5'-GCCC CGAUUCACAUCGUGUU-3'/5'-CACGAGUUGUAAUCGGGGCUU-3'). To downregulate mouse slit2 the following siRNAs were designed: siRNA-Slit2-1 (5'-GUCACU- CUGAAACAUAACU-3'/5'-UAUUGUUCAGAGAGGCUU-3') and siRNA-Slit2-2 (5'-GGCGACAGAAGAAAGAA GAUAUU-3'/5'-AUUGUUCUUCCUCUGAGGCUU-3'). For the silencing experiments in P19CL6, cells were transfected with control siRNA or a siRNA-Tbx5-1 and siRNA-Tbx5-2 mix in GM. 6 hrs after transfection the GM was substituted with the DM. Four days after the first round of transfection, the cells were transfected with the respective siRNA again. Cells were pelleted 6 h after the first transfection, 48 h and 96 h after the second round of transfection. SiRNAs were transfected using HiPerFect Transfection reagent.

Quantitative Real Time RT-PCR

Total RNA was extracted from ~30 embryos or from P19CL6 cells cultured in 6-well plates to 80-90% confluence at using the RNeasy mini kit. After DNase treatment, 1 μg of total RNA was retro-transcribed using miScript Reverse Transcription kit (for miRNA analysis) and Quantitect Reverse Transcription kit (for gene analysis) following the manufacturer’s instruction. Real-time PCR (qRT-PCR) was carried out using either Quantifast SYBR Green kit with Rotor gene (Quiagen) or LightCycler 480 SYBR Green I Master with LightCycler 480 (Roche) following the manufacturer’s instructions. Primers used for miRNA analysis were as follows: for mmu-Tbx5 5’-5’-CACTGGGATGCAGACACTT-
The morpholino antisense oligonucleotide MO-Tbx5a (5'-GGACACCTGTTAGATCGCAACTCA-3') and MO-D-218 (5'-GGATCCCATTGGATCCCATATTC-3') were designed against the mature form of miR-218a and the Drosha cleavage site of pre-miR-218a respectively. The sequence of the control MO was 5'-CCTCTTACTGTTACATTAC-3'.

Microinjection

The one- and two-cell stage embryos were injected with a constant injection volume (~1 nl, confirmed by volume analysis) using a microinjector (Tritech Research, Los Angeles CA USA).

Optical Microscopy and Confocal Analysis

Optical microscopy was performed with Olympus SZH microscope, images were acquired with Nikon DS-Fi1 camera and NIS-Elements F 3.0 software. Images were processed with Gimp-2.6 software. For confocal analysis embryos were fixed in 4% PFA for 1 h at room temperature under slow agitation and embedded in 2% low-melt agarose. Images were acquired with a Leica DMI 2000 confocal microscope. Image stacks were processed with FIJI-WIN32 by projection. For migration analysis, embryos were injected with the indicated miRNA mimics or morpholinos and allowed to develop at 26°C until the 14-somite stage. Then they were embedded in 1% low-melting soft agarose and imaged at 26°C with Leica DMI 2000 confocal microscope to obtain stacks. To calculate cell velocity stacks were processed with FACS caliber BD.

FACS Analysis

Zebrafish Tg(he-2:GFP-mic22:RFP) embryos were injected and raised in standard conditions until 72 hpf. Next embryos (n = 15–25 embryos for each analysis) were collected and treated with trypsin 0.125 mg/l at 37°C. After a 30 min incubation, trypsin was inactivated, samples were filtered with 50 μm cell-strainer and processed with FACS caliber BD.

Whole Mount in situ Hybridization

shb2 and shh3 clones for in situ hybridization were kindly provided by Dr. Hitoshi Okamoto (Laboratory for Development of Gene Regulation, RIKEN, Brain Science Institute Japan).

Whole mount in situ Hybridization

shb2 and shh3 clones for in situ hybridization were kindly provided by Dr. Hitoshi Okamoto (Laboratory for Development of Gene Regulation, RIKEN, Brain Science Institute Japan). Whole mount in situ hybridization was performed as previously described [33] with some modification: pre-hybridization temperature was 62°C; hybridization temperature was 62°C for gene probes and 52°C for miR-218 probe. 1.0 ng of antisense DIG labelled RNA probe was added to the hybridization mix. The anti-DIG antibody-alkaline phosphatase Fab fragment was diluted 1:4,000 in MABlock buffer (2% Blocking reagent in 100 mM Maleic acid and 150 mM NaCl) and incubation was performed at 4°C for gene probes and at room temperature for miR-218 probe. After incubation with the anti-DIG antiserum, embryos were washed in PBT and then incubated in the alkaline Tris buffer solution containing 2 mM Tetramisole (Sigma). The final staining step was performed in BM Purple AP-Substrate, precipitating buffer according to the manufacturer’s recommendations. Labeled
embryos older than 24 h were bleached as follows: embryos were
washed at room temperature under slow agitation in PBS changed
2x at 5 min. intervals. PBS was removed and embryos were
incubated for 10 minutes in pre-bleaching buffer (SSC 0,5X and
6% Formamide). After discarding the pre-bleaching buffer,
bleaching buffer (pre-bleaching buffer containing 33% H2O2)
was added to the embryos which were subsequently exposed to
a bright source of light for 15 minutes. Afterwards embryos were
transferred back to 87% glycerol in PBS incubated at room
temperature under slow agitation for 4 h and then stored at –
20°C.

Statistical Analysis
Data were analyzed using GraphPad Prism (GraphPad Soft-
ware, San Diego, CA USA). Statistical differences were de-
termined by unpaired t-test, with values of P<0,05 considered
statistically significant. Each experimental point in the graph
represents the mean ± SE of at least three independent
experiments. In the graph of Fig. 6A comparisons between groups
were performed by one-way analysis of variance, followed by
Bonferroni’s post-hoc for multiple comparisons.

Supporting Information
Figure S1 Schematic representation of the bioinforma-
tical tool developed to identify in silico Tbx5-controlled
miRNAs. Ensembl was used to obtain information about
chromosome location, position and segment of the selected genes.
Ensembl is a joint scientific project between the European
Bioinformatics Institute (EBI; http://www.ebi.ac.uk/chstahle/)
and the Wellcome Trust Sanger Institute (http://www.sanger.ac.
.uk/). EBI provides a centralized resource with annotations on
genomes of sequenced species and the Ensembl Perl API
(Application Programming Interface) models for access to bi-
ological objects, such as genes and proteins. Moreover EBI allows
the execution of Perl programs for retrieving data from a public
database MySQL (http://www.mysql.com/). We generated two local
databases, one for genes, and one for microRNAs. By applying the
Perl program that uses Ensembl API, we compared the gene
databases with the microRNA database using a standard database
interface module for Perl.
(TIF)

Figure S2 tbx5 and miR-218 are co-expressed in mouse
tissues. A-D, relative expression of tbx5, slit2, slit3 and miR-218 as
evaluated by q-RT-PCR in different newborn mouse tissues.
Results are standardized against GAPDH for genes, and against
U6 for miRNAs. Values represent the averages and standard
deviations of at least two independent experiments.
(TIF)

Figure S3 Expression of pre-miR-218-1 parallels that of
mature miR-218 during mouse differentiation and tbx5
modulation. Q-RT-PCR detection of pre-miR-218-1 and pre-
miR-218-2 relative expression in P19CL6 cells during differentia-
tion (A), or 48 h after plasmids or siRNA transfection (B). In B
fold changes of CMV-Tbx5 and siRNA-Tbx5 are relative to
CMV-empty and siRNA-Ct values, respectively. Results are
standardized against GAPDH. *, P<0.05 (Student’s t-test).
C, Q-RT-PCR detection of slit2 and mature miR-218 in P19CL6 cells
transfected with a mix of two siRNAs against slit2 or with a siRNA-
Ct.
(TIF)

Figure S4 Rescue of cardiac defects induced by miR-
218a over-expression was accomplished by co-injecting
MO-218a. A, qRT-PCR analysis of miR-218a relative expression
in 24 hpf embryos microinjected with 12 ng of control morpholino
(MO-Ct) or MO218a and with 260 pg of miR-Ct or miR-218a
mimic. miR-218a relative expression was calculated as the ratio
between the expression of injected and the expression of non
injected embryos. B, representative transgenic Tg(mlc2:eGFP)
embryos at 72 hpf showing heart morphological defects induced
by the injection of 260 pg of miR-218a mimic in the absence (a,b)
Ct and MOD-218a, 3 ng MO-Tbx5a) after mRNA
hybridization. Scale bar 100 μm. (TIF)

Figure S5 miR-218 dysregulation does not affect vascular
integrity. Confocal images of representative 72 hpf
Tg(fkh1:eGFP) embryos injected with 260 pg of miR-Ct (A),
260 pg of miR-218 mimic (B) or 3 ng MO5-218 (C). Black and
white scale bars: 100 μm. (TIF)

Figure S6 tbx5 and miR-218a misexpression does not alter
amhc and vmhc cardiac marker expression in zebrafish embryos.
Ventral views of 48 hpf embryos injected with the indicated miRNA mimics (260 pg) or MOs (12 ng MO-
Ct and MO5-218a, 3 ng MO-Tbx5a) after miRNA in situ
hybridization. Scale bar 100 μm. (TIF)

Figure S7 miR-218 targets the 3’ UTR of robo1 in zebrafish embryos. Top: schematic representation of sensors and miRNAs used for in vivo sensor assay. Bottom: examples of 24 hpf embryos microinjected with 40 pg of RFP mRNA, 400 pg of 3’UTR_robo1 sensor and 160 pg of miR-218 (a,c,e) or miR-218a (b,d). In figures C and D the percentage of the relative
phenotypes were indicated. ~30 embryos for each thesis were
injected. Scale bars 50 μm.
(TIF)

Figure S8 MO-Tbx5a and MO-Tbx3b effectively knock-
down the two zebrafish tbx5 isoforms. A-D, 35 pg of pCS2 plasmid expressing GFP fused with MO-Tbx5a or MO-Tbx3b
target sequences were injected in one-cell stage embryos in the
absence (A and C) or in the presence (B and D) of 1,5 ng of the
relative morpholino. Representative fluorescent images of 24 hpf
embryos. ~20 embryos for each thesis were analysed. E-F, Tbx5
morphants analysis. Phenotypic analysis of Tbx5a (E) and Tbx3b
(F) morphants: 2 ng of MO-Tbx5a, or 4 ng of MO-Tbx3b, were
injected in Tg(mlc2:eGFP) embryos. Phase-contrast images showing
pericardial edema (arrowheads) and fin absence (brackets) or
presence (arrows); in the bottom right corner of figures E and F,
fluorescent images showing heart morphology. Quantification of
Tbx5a (G) and Tbx5b (H) morphant phenotypes. The percentage
of embryos with the indicate defects was averaged across multiple
independent experiments. ~100 embryos for each thesis were
analysed. Black scale bars: 100 μm, red scale bars 25 μm.
(TIF)

Figure S9 Injection of miR-218a in Tbx5a morphants
increases the severity of heartstring phenotype. A, phenotypic analysis of Tbx5a morphants co-injected with 1 ng of MO-Tbx5a and either 130 pg of miR-218a mimic or 130 pg of miR-Ct. B, representative images of 48 hpf embryos showing the
dema expansion caused by the co-injection of miR-218a mimic. C, phenotypic analysis of embryos co-injected with sub-phenotypic
doses of both MO-Tbx5a(0,5 ng) and miR-218a mimic (35 pg). For
comparison the same dose of MO-Tbx5a was co-injected with
35 pg of miR-Ct. (D-E) Representative confocal images showing
heart morphology of transgenic Tg(mlc2:eGFP) embryos injected
with a sub-phenotypic dose of MO-Tbx5a and 35 pg of either miR-218 (D) or miR-218a (E) mimic (E). Embryo in D has normal looping while co-injected embryos in E show absence of looping, although with different degrees of heart defects. a, atrium; v, ventricle, η, cardiac edema. Black and white scale bars 100 μm, red scale bars 25 μm.

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Author Contributions

Conceived and designed the experiments: LP. Performed the experiments: EC LD LV FM AM ME. Analyzed the data: GG FC LP. Contributed reagents/materials/analysis tools: SWW FC LP. Wrote the paper: FC LP.
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