miR-322/-503 cluster is expressed in the earliest cardiac progenitor cells and drives cardiomyocyte specification

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Understanding the mechanisms of early cardiac fate determination may lead to better approaches in promoting heart regeneration. We used a mesoderm posterior 1 (Mesp1)-Cre/Rosa26-EYFP reporter system to identify microRNAs (miRNAs) enriched in early cardiac progenitor cells. Most of these miRNA genes bear MESP1-binding sites and active histone signatures. In a calcium transient-based screening assay, we identified miRNAs that may promote the cardiomyocyte program. An X-chromosome miRNA cluster, miR-322/-503, is the most enriched in the Mesp1 lineage and is the most potent in the screening assay. It is specifically expressed in the looping heart. Ectopic miR-322/-503 mimicking the endogenous temporal patterns specifically drives a cardiomyocyte program while inhibiting neural lineages, likely by targeting the RNA-binding protein CUG-binding protein Elav-like family member 1 (Celf1). Thus, early miRNAs in lineage-committed cells may play powerful roles in cell-fate determination by cross-suppressing other lineages. miRNAs identified in this study, especially miR-322/-503, are potent regulators of early cardiac fate.

microRNA | miR-322 | miR-424 | miR-503 | cardiomyocyte

MicroRNAs (miRNAs) are small noncoding RNAs 18–24 nt in length that regulate posttranscriptional gene expression (1). miRNAs are guided to the 3′ UTR of target mRNAs by partial sequence complement in the "seed" region, causing degradation of the mRNA transcript and/or translational inhibition. They are perceived to fine-tune gene expression but also function as molecular switches (2–5). miRNAs can also exert a fail-safe mechanism to silence mRNAs that are superfluous in specific cell lineages (6, 7). Hence, miRNAs are powerful regulators of cell fates (8).

A few miRNAs are expressed specifically in the heart and skeletal muscles, namely, miR-1, miR-133, miR-206, miR-208, and miR-499 (9). Genetic loss- and gain-of-function studies have revealed a critical role for the miR-1/-133 cluster in the mouse heart (5, 10–12). Deletion of miR-1-2 resulted in embryo lethality because of ventricular septal defects and in postnatal death because of conduction system defects (11). miR-208 and miR-499 are encoded in the introns of cardiac myosin heavy-chain genes (13, 14). A recent report described the use of striated muscle-specific miRNAs in reprogramming mouse fibroblasts into cardiomyocytes, although the efficiency was low (15). Because of the relatively late onset, these "myomiRs" may need partners that function early to turn on the cardiac program efficiently. miRNAs associated with early cardiac progenitor cells (CPCs), which are often depicted by mesoderm posterior 1 (Mesp1) and Flk1/Pdgfra expression (16, 17), are largely unknown.

Mesp1 sits high in the regulatory hierarchy of cardiac development. It is first expressed at the onset of gastrulation along the primitive streak and in the premesoderm that eventually gives rise to the heart. In homozygous *Mesp1*-deficient embryos, the Mesp1-lineage cells were delayed in migrating from the primitive streak to the heart field (18). Mesp1 drives ES cells (ESCs) toward the cardiac fate. Transient expression of Mesp1 accelerated and enhanced the appearance of cardiac progenitors (19–22). Despite its pivotal role in cardiogenesis, its expression is highly transitory. It disappears almost completely before the appearance of cardiac crescent at embryonic day (E) 7.0, suggesting that it is not required for cardiac morphogenesis (16, 18). Recent evidence indicates that Mesp1's role is broader than previously recognized. Our global survey of its transactivation targets shows that Mesp1 primarily regulates mesendoderm genes (23). Moreover, Mesp1-marked cells contribute to skeletal muscles and blood in addition to cardiovascular lineages (24). Despite this expanded role, its early onset and relative specificity make Mesp1 a suitable marker for the earliest CPCs.

Here we report the identification of early CPC-enriched miRNAs, many of which are direct transactivation targets of MESP1. The top candidate, the miR-322/-503 cluster, is expressed early and specifically in the looping heart during embryogenesis. Temporally controlled induction of miR-322/-503 triggered precocious and robust cardiomyocyte formation. The miR-322/-503 cluster targets an RNA-binding factor, CUG-binding protein Elav-like family member 1 (Celf1), which otherwise leads embryonic stem cells toward neural fates.

Significance

Compared with microRNAs (miRNAs) enriched in cardiac and skeletal muscles, little is known about miRNAs expressed in early cardiac progenitors. Here, we show that mesoderm posterior 1 (Mesp1) transactivates a large number of miRNAs that may promote cardiomyocyte formation. The miR-322/-503 cluster has the highest enrichment in the Mesp1 lineage of cardiac progenitor cells, is specifically expressed in the developing heart tube, and drives precocious cardiomyocyte formation by targeting an RNA-binding factor, CUG-binding protein Elav-like family member 1 (Celf1). This study fills a gap in our knowledge about miRNAs acting early in the cardiac program and identifies previously unreported candidates in promoting cardiac regeneration.

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Results

Using Mesp1 Genetic Tracing to Identify miRNAs Enriched in Early CPCs. To identify miRNAs that regulate early cardiac cell fate, we used a genetic tracing strategy to label early CPCs. We crossed the *Mesp1*$^{Cre/+}$ and *Rosa26*$^{EYFP/EYFP}$ mouse strains to obtain *Mesp1*$^{Cre/+}$; *Rosa26*$^{EYFP/+}$ embryos in which *Mesp1* promoter-driven Cre mediates recombination in the *Rosa26* locus and permanently marks the Mesp1 lineage with YFP. In agreement with previous reports (16, 18), Mesp1-lineage cells contributed to the primitive streak, cardiac crescent, and head folds at E7.5. They localized to the developing heart, head mesenchyme and intersomatic vessels at E9.5 and to the heart and surrounding structures at E11.5 (Fig. 1*A*). We generated an Mesp1 lineage-tracking ESC line (genotype *Mesp1*$^{Cre/+}$; *Rosa26*$^{EYFP/+}$), UH3 cells (23). YFP$^+$ signal was absent in undifferentiated UH3 cells and started to appear from day 3 in a standard hanging-drop protocol. The signal reached 2.9% at day 5 and did not increase thereafter. In contrast, *Rosa26*$^{EYFP/+}$ ESCs did not express YFP at any time points, indicating that the reporter activity is contingent on Cre expression (Fig. 1*B*). We previously determined that MESP1 protein peaked at day 5 and that day 5 YFP$^+$ cells possessed an early CPC signature (23).

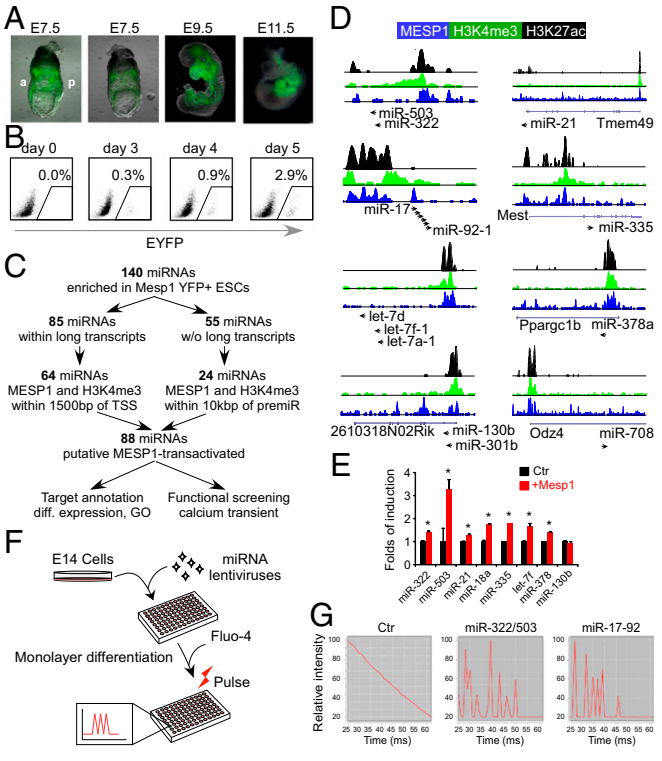

Fig. 1. Identification of Mesp1 lineage-enriched and MESP1-targeted miRNAs. (*A*) Mesp1 lineage tracking during early embryogenesis. (*B*) Mesp1 lineage tracking during ESC differentiation in UH3 (*Mesp1*$^{Cre/+}$; *Rosa26*$^{EYFP/+}$) cells. YFP$^+$ cells started to appear at day 3 and peaked at day 5, as measured by FACS. (*C*) Identifying Mesp1 lineage-enriched miRNAs, MESP-targeted miRNAs, and subsequent bioinformatical and functional assessment. (*D*) The majority of Mesp1 lineage-enriched miRNAs are MESP1-transactivation targets. Shown are alignments of MESP1-binding sites, H3K4me3 and H3K27ac signatures, and overlapping long transcripts. The body of the arrow indicates the position, and the arrowhead points to the direction of transcription. (*E*) MESP1 induced the expression of its putative target miRNAs. MESP1 was triggered by dox in Mesp1 Tet-On ESCs. miRNA levels were determined 48 h postinduction. (*F*) Functional assessment of miRNAs in a calcium transient-based screening assay. (*G*) The miR-322/-503 and miR-17–miR-92 clusters had the strongest cardiogenic activity in the calcium transient-based screening.

Next, we identified early CPC-enriched small RNAs by next-generation sequencing. We obtained 9,461,773 usable reads, of which 2,465,814 were aligned to miRNAs. We used an arbitrary cutoff of fold change >2 (YFP$^+$ vs. YFP$^-$) and reads >1,000 to obtain a list of early CPC-enriched miRNAs (Table S1). Most miRNAs on the list do not have established roles in cardiac development. A cluster of miRNAs—miR-322 and miR-503—had the highest enrichment (~25-fold). Members of the miR-17–miR-92 family and its paralogs, the miR-106b–miR-25 family and miR-106a–miR-363 family, were among the highly enriched. This superfamily is essential for cardiac development: The loss of both the miR-17–miR-92 and miR-106b–miR-25 clusters led to severe cardiac defects and embryonic death (25). The miR-302/-367 cluster also was highly enriched. This cluster reprograms somatic cells to induced pluripotent stem cells (iPSCs), whereas miR-302 promotes mesendoderm at the expense of neuroectoderm in human ESCs (26, 27). The high enrichment of these clusters attests to successful capturing of important regulatory miRNAs. The heart- and skeletal muscle-specific miRNAs, namely, miR-1, -133, -206, -208, and -499, were not expressed at this stage.

MESP1 Transactivates Early CPC-Enriched miRNAs. To understand the transcriptional and epigenetic regulation of the identified miRNAs, we examined our recently reported datasets of MESP1 ChIP sequencing (ChIP-seq) and histone 3 trimethylated at lysine 4 (H3K4me3) ChIP-seq in day 5 CPCs (23) and histone 3 acetylated at lysine 27 (H3K27ac) ChIP-Seq in enriched mesoderm cells (28). Because miRNA transcription start sites (TSS) are not well defined, we focused on two classes of upstream regulatory regions. Of the 140 miRNAs enriched in Mesp1-lineage cells, 85 fall within a long transcript. Overlapping MESP1 and H3K4me3 peaks were present within 1.5 kb of the TSS in 64 (45.7%) of these miRNAs. Of the remaining 55 that do not fall within a long transcript, overlapping MESP1 and H3K4me3 peaks were present in 28 (20.0%) within a distance of 10 kb of the miRNAs. Together, 65.7% of CPC-enriched miRNAs have both MESP1 and H3K4me3 signatures on their TSSs (Fig. 1 *C* and *D*). Frequently, these signatures also overlap with H3K27ac peaks (alignment of representative findings are shown in Fig. 1*D*). Using a Tet-On tetracycline conditional gene-expression system, we induced MESP1 in ESCs and detected increased expression of the putative downstream miRNAs (Fig. 1*E*). These results strongly suggest that MESP1 directly transactivates miRNAs enriched in early CPCs.

To assess the function, the targets of the top 25 MESP1-activated miRNAs were predicted using miRanda (www.microrna.org/microrna/). Next, we compared their fragments per kilobase of exon per million fragments mapped (FPKM) expression profiles in day 5 YFP$^+$ and YFP$^-$ cells (23). Gene Ontology (GO) terms enriched in the down-regulated genes (796 counts) (Fig. S1*A*) include neuroectoderm and some endoderm derivatives. In contrast, terms related to the recently expanded Mesp1 lineages (heart, skeletal muscle, and blood) were not enriched (Fig. S1*B*). These data suggest that Mesp1 drives miRNAs to suppress non-Mesp1 lineages.

Using Calcium Transient-Based Screening to Identify Cardiomyocyte-Promoting miRNAs. Next, we set up a screening assay for miRNAs that drive cardiomyocyte differentiation in ESCs. Because calcium transients can be recorded in immature cardiomyocytes (29) and in CPCs when pulsed (30), we designed the following screening procedure: (*i*) monolayer wild-type E14 ESCs were transduced with miRNA lentiviral vectors; (*ii*) at a series of time points, Fluo-4 fluorescent calcium indicator was loaded; (*iii*) an electric pulse was applied to the culture; and (*iv*) calcium transients were recorded (Fig. 1 *F* and *G*).

Wild-type and miRNA-transduced E14 ESCs were cultured as a monolayer in serum-free medium, yielding little spontaneous cardiac differentiation. No calcium transients were recorded in wild-type ESCs at day 5 and 6 and were rare at day 7 and 8. Starting from day 5, ESCs transduced with miR-322/-503 or miR-17–miR-92 showed multiple (more than four) calcium transients after pulse (Fig. 1*G*). Other miRNAs which consistently displayed

calcium transients at any time point include the miR-130b/-301b cluster, the miR-23a–miR-24-2 cluster, miR-340, -378, -335, -31, -708, -542, -152, and -382, but fewer than four transients were recorded in any of these miRNAs (Fig. S2). We concluded that the miR-322/-503 and miR-17–miR-92 clusters have the strongest cardiomyocyte-promoting activity. Table S2 summarizes selected CPC-enriched miRNAs, their folds in enrichment, the presence of MESP1-transactivating sites, and activities in the screening assay.

The calcium-transient screening did not include myomiRs, because they are not enriched in the early CPCs. Next, we used the hanging-drop method to validate our screening, with miR-1 and miR-208 as controls. miR-322/-503, miR-1, and miR-208 all induced higher expression of *Tbx5*, *Nkx2-5*, and *α-MHC* and increased formation of cardiomyocytes (Fig. S3). However, the relative potential of these miRNAs can be addressed only when they are expressed in their natural timing and cell types; that investigation is beyond the scope of this study.

miR-322/-503 Specifically Drives a Cardiomyocyte Differentiation Program. The miR-322/-503 cluster is encoded in an intergenic region on the X-chromosome [miR-322 stem–loop, chromosome X: 53054255–53054349 (−); miR-503 stem–loop, chromosome X: 53053984–53054054 (−)]. The coding sequences for both miRNAs are highly conserved among placental mammals. There are additional miRNAs in this locus, but they either lack placental mammal conservation (miR-351) or were enriched to a much lower degree (miR-542/-450b) in CPCs. We first used a LacZ-knockin reporter allele (31) to determine the expression pattern of miR-322/-503. β-Galactosidase activity was detected in the truncus arteriosus, bulbus cordis, the wall of common ventricular chamber, and sinus venosus of the primitive heart at E8.5 and E9.5 and in the heart and somites at E10.5 (Fig. 2*A*). To decide if miR-322/-503 persists in later stages, we microscopically dissected E14.5 organs and assayed miR-322/-503 expression by real-time RT-PCR. Both miRNAs had the highest expression in the heart and tongue and the lowest expression in the brain (Fig. 2*B*). Together, these data indicate that the miR-322/-503 cluster initially expresses in the Mesp1 lineage of progenitor cells and persists in the heart and some skeletal muscles.

Next, we used an ESC differentiation model to study the cardiogenic function of miR-322/-503. First, we determined that endogenous miR-322 and miR-503 levels started to rise at day 3 and peaked at day 5 (Fig. 2*C*), following a pattern similar to that of *Mesp1* (23, 32). To mimic this temporal pattern, we generated stable miR-322/-503 Tet-On ESC lines in which a single dose of doxycycline (dox) increased miR-322 and miR-503 expression by more than 10-fold within 24 h (Fig. 2 *D* and *E*). Day 3 dox supplementation resulted in precocious cardiomyocyte formation. At day 6.5, spontaneously contracting clusters with well-formed sarcomeric structures were widely present in cultures with dox but were absent in cultures without dox (Fig. 2*F*). At day 10, when the cultures were further differentiated, cultures with dox showed significantly increased cardiac troponin T (cTnT)-positive cardiomyocytes compared with the dox-free cultures (23.4 vs. 7.6% by FACS) (Fig. 2*G*). We assayed developmental markers at a series of time points: Dox induced significantly increased levels of cardiomyocyte markers (*Tbx5*, *Mef2c*, *Nkx2-5*, and *α-MHC*) but had little effect on pluripotency (*Oct4* and *Sox2*) or mesendoderm markers (*Eomes*, *T*, *Gsc*, and *Mesp1*) (Fig. 2*H*). Thus, miR-322/-503 acts at a specific developmental stage and is a potent inducer of the cardiomyocyte program.

We asked if ectopic miR-322/-503 also affects other lineages. Skeletal muscle progenitor markers *Myf5* and *Pax3* were significantly up-regulated, indicating that miR-322/-503 induce skeletal muscle differentiation (Fig. 3*A*). Endothelial cell markers *Flk1* and *Pecam1* showed modest increases, but the prevalence of positive VE-Cadherin staining was indistinguishable in cultures with and without dox (Fig. 3 *B* and *C*), suggesting that the role of miR-322/-503 in endothelial cell differentiation is complex and perhaps is not as powerful as in cardiomyocyte differentiation. There was no differential expression of the smooth muscle marker SM-actin in cultures with and without dox (Fig. 3 *B* and *C*).

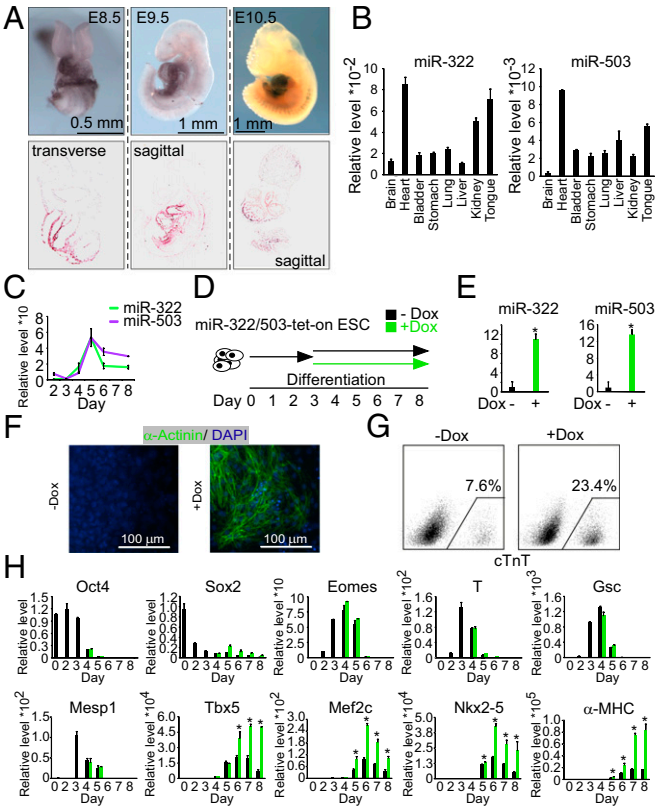

Fig. 2. miR-322/-503 specifically drives a cardiomyocyte differentiation program. (*A*) miR-322/-503 is specifically expressed in embryonic hearts at E8.5, E9.5, and E10.5, as determined by a knockin LacZ reporter. (*B*) miR-322 and miR-503 expression is highest in the heart and skeletal muscles. The expression levels were determined by real-time RT-PCR and normalized to *GAPDH*. (*C*) Temporal patterns of miR-322 and miR-503 expression during ESC differentiation. (*D*) Schematic diagram of monolayer differentiation of miR-322/-503 Tet-On ESCs. Dox (1 μg/mL) was supplemented at day 3. (*E*) Verification of induced miR-322 and miR-503 expression 24 h after dox supplementation. (*F*) Ectopic miR-322/-503 induced the precocious appearance of cardiomyocytes. α-Actinin was stained at day 6.5. (*G*) Ectopic miR-322/-503 augmented total yields of cardiomyocytes. cTnT was stained and analyzed by FACS at day 10. (*H*) Ectopic miR-322/-503 induced significant increases in cardiac transcription factors (*Tbx5*, *Mef2c*, and *Nkx2-5*) and structure the gene *α-MHC*, with no significant effect on pluripotent markers (*Oct4* and *Sox2*) or mesoderm markers (*Eomes*, *T*, *Gsc*, and *Mesp1*). $n \geq 3$; $^*P < 0.05$ vs. control cells.

To determine if inhibition of miR-322 or miR-503 impairs cardiac differentiation, we generated "miR-Zip"-expressing E14 ESCs. The control culture showed evident cardiomyocyte formation at day 8, with expression of cardiac factors (*Tbx5*, *Mef2c*, *Nkx2-5*, and *α-MHC*) and sarcomeric α-actinin. Either miR-322 or miR-503 miR-Zip down-regulated these markers significantly, suggesting an obligatory role of miR-322/-503 in cardiomyocyte formation in ESCs (Fig. S4).

Global Assessment of Transcriptome upon miR-322/-503 Induction Indicates Specific Inhibition of Ectoderm Lineages. To gain a global picture of reactive transcriptome changes upon miR-322/-503 expression, we surveyed the mRNA in day 4 (24 h after dox induction) cultures with and without dox by microarray. A total of 618 genes were up-regulated, and 892 genes were down-regulated. The most significant GO terms enriched in up-regulated genes were "heart development" and "skeletal system development" (Fig. 3*D*). The most significantly up-regulated cardiac genes include *Hand2*, *Wnt2*, *Isl1*, *Tbx20*, *Foxc1*, and *Tbx5*, among others (Fig. 3*E*). Interestingly, "blood vessel development" and "vasculature development" were enriched in both up- and down-regulated

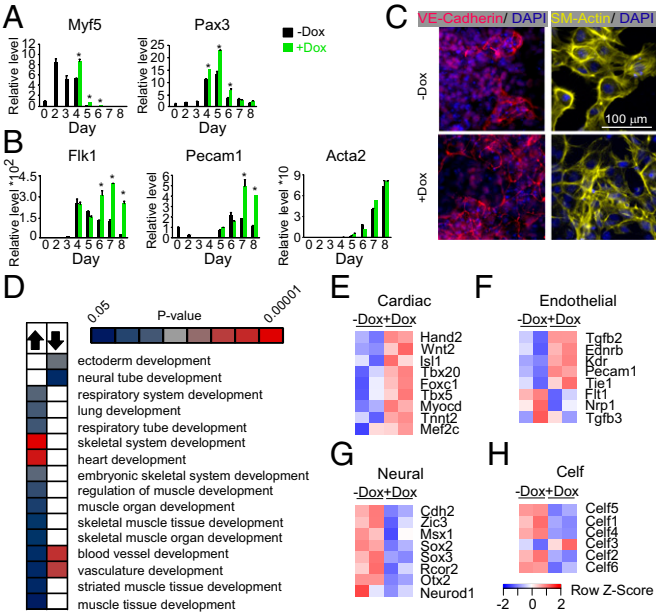

Fig. 3. miR-322/-503 selectively inhibits neuroectoderm differentiation. (*A*) Ectopic miR-322/-503 augmented the expression of skeletal muscle progenitor genes *Myf5* and *Pax3*. (*B*) Ectopic miR-322/-503 augmented endothelial cell markers (Flk1 and Pecam1) but not the smooth muscle cell marker *Acta2*. $n \geq 3$; $^*P < 0.05$ vs. control cells. (*C*) Ectopic miR-322/-503 did not change the prevalence of either endothelial or smooth muscle cells. (*D*) GO terms enriched in up-regulated and down-regulated genes by miR-322/-503. (*E–H*) Heatmaps of representative cardiac genes (*E*), endothelial cell genes (*F*), neural genes (*G*), and Celf family members (*H*) regulated by miR-322/-503.

genes, supporting a complex role of miR-322/-503 in vascular endothelial cell differentiation (Fig. 3 *D* and *F*). The most significantly enriched GO terms in down-regulated genes were "ectoderm development" and "neural tube development," strongly suggesting that miR-322/-503 drives the cardiomyocyte program at the cost of neuroectoderm lineages. The most suppressed genes pertinent to the neuroectoderm were *Cdh2*, *Zic3*, *Msx1*, *Sox2*, *Sox3*, *Rcor2*, *Otx2*, and *Neurod1* (Fig. 3*G*).

Celf1 Is a Direct Target of miR-322/-503. In our search for the miR-322/-503–targeted cell-fate regulators, our attention was drawn to a family of Celf proteins. Every member of this family except Celf3 was down-regulated upon miR-322/-503 induction (Fig. 3*H*). Celf binds RNA and regulates RNA decay, alternative splicing, and translation. Celf proteins are stage- and lineage-specific, implying that they have roles in lineage determination. We studied the expression patterns of the relatively abundant members. In E7.5 embryos, Celf1, Celf2, and Celf4 were localized in the ectoderm. In E9.5 and E10.5 embryos, they were located predominantly in neural tissues (Fig. 4*A* and Fig. S5*A*). Among a range of E14.5 embryonic organs, all Celf proteins had the highest expression in the brain (Fig. 4 *B* and *C* and Fig. S5*B*). We reasoned that although all Celf members may be targets of miR-322/-503, Celf1 is a chief candidate, because (*i*) it is widespread but differentially expressed among organs; (*ii*) it is regulated mainly through posttranscriptional mechanisms; (*iii*) it inhibits skeletal muscle differentiation and induces muscle wasting in myotonic dystrophy (33, 34); and (*iv*) it was biochemically determined to be a degradation target of miR-503, although the biological significance of this finding is unclear (35).

Using several computational programs (www.microrna.org/microrna/), including Miranda and TargetScan, we predicted the putative miR-322/-503 target sites on the *Celf1* 3′ UTR (Fig. 4*D*). Because the seed sequences of miR-322 and miR-503 differ by only 1 nt, they likely share the same target site. We cloned a fragment containing *Celf1* 3′ UTR into pmirGLO for luciferase reporter assays. When miR-322/-503 was present, the pmirGLO-Celf1 3′ UTR showed more than 50% decrease in luciferase activity, but mutations in the target site showed little decrease (Fig. 4*E*). During ESC differentiation, a reverse correlation between the expressions of *Celf1* and miR-322/-503 was evident (Fig. 4*F*). Celf1 protein was reduced by dox-induced miR-322/-503 in miR-322/-503 Tet-On ESCs (Fig. 4*G*). In addition, endogenous Celf1 was down-regulated in a dose-dependent manner by increasing amounts of ectopic miR-322/-503 in 293T cells (Fig. 4*H*). These findings strongly suggest that Celf1 is a direct target of miR-322/-503.

We reasoned that, if Celf1 is one of the chief targets of miR-322/-503, inhibition of Celf1 should mimic the function of ectopic miR-322/-503. We asked if Celf1 knockdown would promote the cardiomyocyte program. In the presence of shRNA against Celf1, which showed high efficacy in inhibiting Celf1 protein expression (Fig. 4*I*), the cardiac program was significantly enhanced, as evidenced by increased expression of cardiac transcription factors (*Tbx5*, *Mef2c*, and *Nkx2-5*) and cardiac structure genes (*α-actinin* and

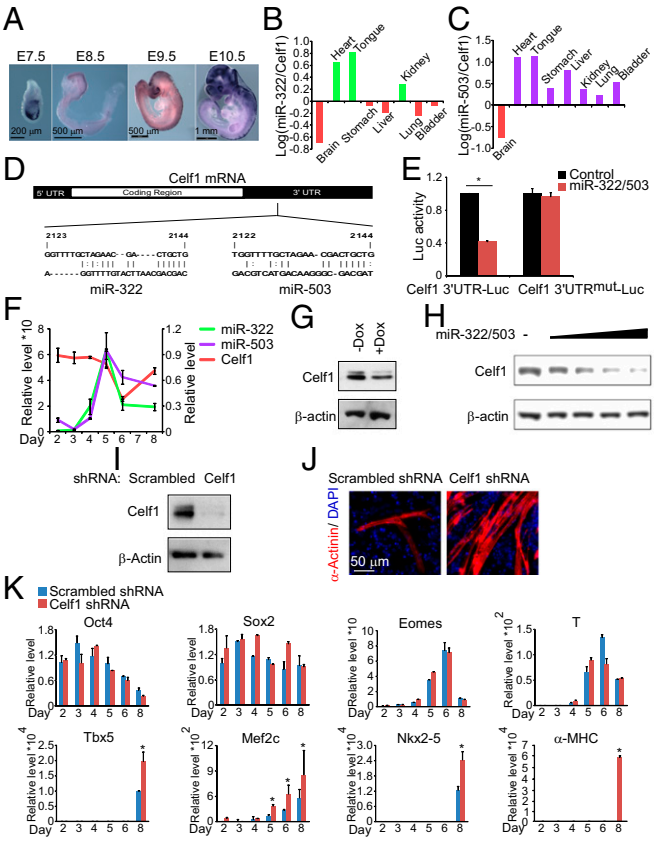

Fig. 4. Celf1 is a direct target of miR-322/-503. (*A*) Celf1 was detected mainly in neuroectoderm lineages during embryogenesis. (*B* and *C*) Relative expression of miR-322/-503 vs. Celf1 in a range of embryonic organs at E14.5. miR-322, miR-503, and *Celf1* expression levels (relative to that of *GAPDH*) were individually determined by real-time RT-PCR; then data were plotted as the ratio of miR-322/-503 to *Celf1*. (*D*) A putative miR-322/-503 recognition site on the 3′ UTR of *Celf1*. (*E*) miR-322/-503 inhibited luciferase activity of a firefly luciferase reporter fused with the *Celf1* 3′ UTR. Mutation of the putative recognition site abolished the inhibition. (*F*) The *Celf1* level drops when miR-322/-503 peaks during ESC differentiation. (*G*) CELF1 protein expression dropped upon dox-induced miR-322/-503 expression. (*H*) Ectopic miR-322/-503 down-regulated endogenous CELF1 expression in a dose-dependent manner. (*I*) Verification of *Celf1* knockdown in ESCs. (*J*) Celf1 shRNA increased cardiomyocyte differentiation in ESCs. α-Actinin was stained at day 8. (*K*) *Tbx5*, *Mef2c*, *Nkx2-5*, and *α-MHC* were all up-regulated in Celf-knockdown cells, whereas *Oct4*, *Sox2*, *Eomes*, and *T* were not significantly changed. $n \geq 3$; $^*P < 0.05$ vs. control cells.

α-MHC), whereas pluripotent genes (*Oct4* and *Sox2*) and mesoderm genes (*Eomes* and *T*) were largely unaffected (Fig. 4 *J* and *K*).

Celf1 Drives Neuroectoderm and Inhibits Cardiac Differentiation. Celf1 expression drops sharply at day 5 when both miR-322 and miR-503 peak during ESC differentiation (Fig. 4). We asked how cell fates are affected if the Celf1 expression pattern is perturbed. We generated Celf1-Flag Tet-On ESCs and induced Celf1-Flag with dox (1 μg/mL) at day 4 (Fig. 5 *A* and *B*). At day 8, spontaneous contractility was evident in control cultures but was rare in cultures containing dox. By straining α-actinin, we observed well-formed sarcomeric structures in control cultures but not in cultures containing dox (Fig. 5*D*). In contrast, the early neural marker Tuj1 was frequently present in cultures containing dox, which also contained well-formed Tuj1^{+} neurites (Fig. 5*F*). Survey of lineage markers showed that Celf1 inhibited cardiac transcription factors (*Tbx5*, *Mef2c*, and *Nkx2-5*) and the structure gene *α-MHC*, whereas it promoted early neural markers (*Notch3*, *Sox1*, *Nestin*, and *Pax6*) (Fig. 5 *E* and *G*).

To test further the notion that miR-322/-503 targets Celf1 and impacts cell-fate decisions, we induced simultaneous miR-322/-503 and Celf1 expression in miR-322/-503/Celf1 Tet-On ESCs (Fig. 5 *H–J*). The ectopic Celf1 does not contain the original 3′ UTR and thus is not subjected to miR-322/-503 targeting. miR-322/-503 and Celf1 coexpression largely reproduced the results of Celf1 expression alone (Fig. 5 *J* and *K*), but the effect of miR-322/-503 was abolished. There was a slight delay in differentiation even in the uninduced culture, suggesting low-level (undetectable) leakage of Celf1, but induction of both miR-322/-503 and Celf1 led to reduced cardiac myocyte formation (Fig. 5*K*) and expression of cardiac genes (Fig. 5*L*).

Discussion

In this study we used a lineage-tracking system to isolate the Mesp1 lineage of CPCs and captured their enriched miRNAs. Reprogramming human somatic cells to cardiomyocytes often involves Mesp1 (30, 36, 37), justifying the identification of effectors downstream of Mesp1. The miRNAs identified here represent early regulators of cardiac fate, likely at the stages of cardiac mesoderm formation and cardiac program initiation. In agreement with this notion, miRNAs that specify mesoderm (let-7, miR-18, and miR-302) and/or are essential for cardiac development (the miR-17–miR-92 superfamily) were among the most highly enriched (25, 38, 39). Previously identified heart- and skeletal muscle-specific miRNAs—miR-1, -133, -208, -499, and others—were not expressed in Mesp1 CPCs. Mesp1 lineage-enriched miRNAs represent a valuable resource for dissecting early cell fate and cardiomyocyte differentiation. However, it will be critical to distinguish which miRNAs act at prerequisite steps, and which are directly involved in cardiac fate establishment.

Our epigenetic data suggest that Mesp1 directly transactivates a number of miRNAs. A majority of predicted targets of the top 25 miRNAs were down-regulated in the Mesp1 lineage, with GO terms outside Mesp1-regulated lineages, supporting a hypothesis that Mesp1 uses miRNAs to inhibit other lineages. The best candidate, the miR-322/-503 cluster, exemplifies this notion by a strong inhibitory effect on neural differentiation. Such a role has not been previously appreciated. miR-322(-424; miR-424 is the human ortholog of miR-322)/-503 is implicated in angiogenesis (40, 41), mammary epithelial involution after pregnancy (42, 43), cancers (acting as a tumor suppressor) (44, 45), and myotube formation (46). Our data suggest that its primary role during embryogenesis is regulating cardiac muscle differentiation. It is worth noting that null mutants of miR-322/-503 were not embryonically defective but had adult-onset anomalies (white fat accumulation) (43). However, such phenotypes may be the outcome of masking by multiple miRNAs (e.g., the miR-15 family) sharing the same seed sequence (47).

The identification of a cardiac- and skeletal muscle-specific X-chromosome locus is of fundamental importance. One of the future directions is to determine if miR-322/-503 and other miRNAs in this locus contribute to gender differences in cardiac development and diseases. Notably, the immediate upstream gene, *Plac1*, is paternally imprinted (48). miR-322/-503 and other miRNAs in this locus also may be imprinted and subjected to tight dose control.

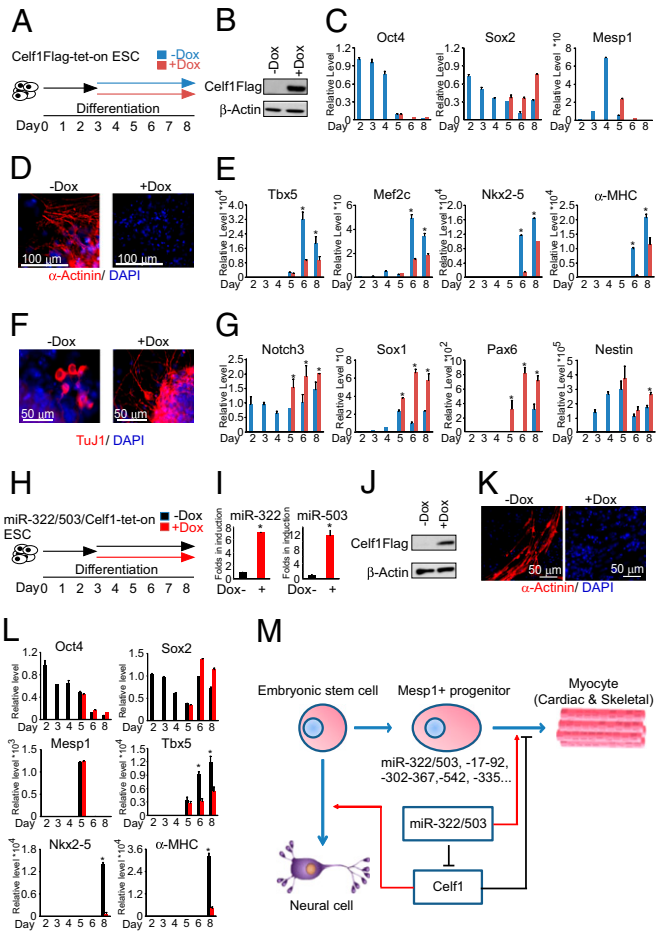

Fig. 5. Celf1 inhibits cardiomyocyte and induces neuroectoderm differentiation in ESCs. (*A*) Schematic diagram of ectopic Celf1 induction during ESC differentiation. (*B*) Verification of Celf1-Flag expression by Western blot. (*C*) Ectopic Celf1 did not affect the expression of pluripotent genes (*Oct4* and *Sox2*) or *Mesp1*. (*D*) Ectopic Celf1 expression impaired α-actinin^{+} cardiomyocyte formation. (*E*) Ectopic Celf1 suppressed the cardiomyocyte markers *Tbx5*, *Mef2c*, *Nkx2-5*, and *α-MHC*. (*F*) Ectopic Celf1 induced the expression of the pan-neural marker Tuj1 and promoted neurite formation. (*G*) Ectopic Celf1 significantly increased neuroectoderm gene expression. $n \geq 3$; $^{*}P < 0.05$ vs. control cells. (*H*) Schematic diagram of the simultaneous induction of miR-322/-503 and Celf1 during ESC differentiation. (*I* and *J*) Verification of simultaneous induction of miR-322/-503 and *Celf1* by real-time RT-PCR (*I*) and Western blot (*J*). $n \geq 3$; $^{*}P < 0.05$ vs. control cells. (*K*) Ectopic Celf1 impaired α-actinin^{+} cardiomyocyte formation despite coexpressed miR-322/-503. (*L*) Ectopic Celf1 suppressed cardiomyocyte markers (*Tbx5*, *Mef2c*, *Nkx2-5*, and *α-MHC*) despite coexpressed miR-322/-503. $n \geq 3$; $^{*}P < 0.05$ vs. control cells. (*M*) A working model.

Celf1 may be one of the important targets that mediate the function of miR-322/-503. Its best-known function is in the pathogenesis of myotonic dystrophy 1 (DM1), a common multisystem disorder that chiefly affects the skeletal and cardiac muscles. In DM1, the disease gene *DMPK1* harbors an expansion of CUG triplet repeats on its 3′ UTR which misregulate the RNA-binding factors MBNL1 and Celf1 (49). Both MBNL1 and Celf1 contribute to DM1 pathogenesis, but only Celf1 is responsible for muscle wasting and weakness. Ectopic Celf1 impairs myoblast differentiation (50, 51). Neurite overgrowth was recently observed in DM1 ESCs, suggesting uncontrolled neural differentiation (52). Here we show that Celf1 impairs cardiomyocyte differentiation but promotes neural lineages. Such a function agrees with Celf1's high expression in the neural lineages.

Celf1 binds to mRNAs of muscle genes and regulates their stability, as assayed in C2C12 myoblasts (53). However, understanding the lineage-specification mechanisms of Celf1 will require a full-scope future study in an unbiased system.

In summary, we have identified miRNAs downstream of Mesp1 that may suppress non-Mesp1 lineages during differentiation. These miRNAs are a valuable resource and a significant addition to the network of early cardiac-fate regulators. The miR-322/-503 cluster, which specifically regulates the cardiomyocyte program, is the most promising candidate of these miRNAs. Further study of these miRNAs may lead to new drug candidates for treating cardiac and skeletal muscle injuries, such as myocardial ischemia and muscular dystrophies.

Methods

Cell Culture. ESC lines bearing the *Mesp1*$^{Cre/+}$; *Rosa26*$^{EYFP/+}$ genotype, produced by crossing *Mesp1*$^{Cre/+}$ and *Rosa26*$^{EYFP/EYFP}$ mouse strains, were established using the E3.5 blastocyst outgrowth methods. ESC differentiation was performed under serum-free conditions (17, 19). For cardiac differentiation, activin (10 ng/mL) (R&D Systems) was added for the first 4 d. For neural differentiation, SB431542 (10 μM) (TOCRIS) and FGF2 (12 ng/mL) (R&D Systems) were added.

Calcium Transient-Based Screening. miRNA inserts were cloned into lentiviral vector pLL3.8 (19). ESCs were infected at a multiplicity of infection of 100. In screening, miRNA-transduced cells were cultured in cardiac differentiation medium in glass-bottomed 96-well plates. On differentiation days 5–8, Fluo-4 NW calcium dye (1:1,000) (Life Technologies) was added, and the plates were scanned by a high-throughput microscope (Vala Sciences) to monitor calcium transients following an electrical pulse (6 v, 2 s).

Additional technical details are provided in *SI Materials and Methods*. Sequences of primer sets and probes used in real-time RT-PCR are provided in Table S3.

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