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miR-17 family miRNAs are expressed during early mammalian development and regulate stem cell differentiation

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ABSTRACT

MicroRNAs are small non-coding RNAs that regulate protein expression by binding 3′UTRs of target mRNAs, thereby inhibiting translation. Similar to siRNAs, miRNAs are cleaved by Dicer. Mouse and ES cell Dicer mutants demonstrate that microRNAs are necessary for embryonic development and cellular differentiation. However, technical obstacles and the relative infancy of this field have resulted in few data on the functional significance of individual microRNAs. We present evidence that miR-17 family members, miR-17-5p, miR-20a, miR-93, and miR-106a, are differentially expressed in developing mouse embryos and function to control differentiation of stem cells. Specifically, miR-93 localizes to differentiating primitive endoderm and trophectoderm of the blastocyst. We also observe high miR-93 and miR-17-5p expression within the mesoderm of gastrulating embryos. Using an ES cell model system, we demonstrate that modulation of these miRNAs delays or enhances differentiation into the germ layers. Additionally, we demonstrate that these miRNAs regulate STAT3 mRNA in vitro. We suggest that STAT3, a known ES cell regulator, is one target mRNA responsible for the effects of these miRNAs on cellular differentiation.

Introduction

MicroRNAs (miRNAs) are short, non-coding RNAs that negatively regulate target mRNAs via binding to their 3′ untranslated regions (UTRs). Although miRNA silencing is mediated through the RNA-induced silencing complex (RISC), its binding does not generally result in mRNA cleavage, as is the case with siRNA. The miRNA-RISC association results in the formation of a bulge within the miRNA, preventing cleavage and explaining the relatively low level of target complimentarity necessary for silencing (Chu and Rana, 2006). Specifically, Watson–Crick base pairing between the target mRNA and nucleotides 2–8 at the 5′ end of the miRNA, frequently referred to as the "seed region," is generally believed to determine miRNA binding potential (Lewis et al., 2003). Following miRNA binding, mRNAs are silenced either by storage in P-bodies or degradation via mRNA decay pathways (Gregory et al., 2005; Rana, 2007; Zeng et al., 2003).

Newly published literature has implicated miRNA-mediated silencing as having important regulatory functions during embryonic development and embryonic stem (ES) cell proliferation and differentiation (Boyer et al., 2005; Hatfield et al., 2005; Houbaviy et al., 2003; Kanellopoulou et al., 2005; Krichevsky et al., 2006; Murchison et al., 2005; Shcherbata et al., 2006; Wang et al., 2007; Zhao et al., 2005). The miR-17 family of miRNAs, which is expressed as three polycistronic clusters, is highly conserved throughout species and is thought to have evolved along with vertebrates (Tanzer and Stadler, 2004). Through evolution, a series of duplications, deletions and mutations have given rise to the modern miR-17 family, which consists of 14 mature miRNAs located on chromosomes 13, X and 7 in humans. Hinting at the importance of these miRNAs, these 14 genes also exist in the same order and as three clusters in mouse, rat, and chimp (Tanzer and Stadler, 2004).

To date few miRNAs have been specifically investigated during embryonic development (Ventura et al., 2008; Zhao et al., 2007). When deleted via gene targeting, miR-1-2 null mice showed developmental defects including disrupted cardiac morphogenesis and failed electrical conduction in cardiomyocytes. Most recently, deletion of miR-17 family members miR-92, 106a, and 106b also revealed developmental defects (Ventura et al., 2008). In addition, new reports have shown these miRNAs to be involved in zebrafish development (Giraldez et al., 2005), cancer progression (Dews et al., 2006; He et al., 2005), and hematopoietic cell differentiation (Garzon et al., 2006). Within zebrafish, miR-430, a member of the miR-17 family, was shown to be important for development of both the heart and hindbrain (Giraldez et al., 2005). A second study examined the role of c-myc in the regulation of expression of the miR-17 cluster (O'Donnell et al., 2005). They demonstrated that c-myc directly activated transcription of miR-17 family microRNAs, which then bound to upstream regulators and downstream targets of myc.

Additionally, several members of the miR-17 family, including miR-17-5p, miR-20a, miR-93, and miR-106a have been identified as microRNAs that are specifically expressed in undifferentiated or

Abbreviations: EB, Embryoid body; ES(C), Embryonic stem (cell); ICM, Inner cell mass; miRNA, microRNA; RISC, RNA-induced silencing complex; STAT3, Signal transducer and activator of transcription 3; UTR, Untranslated region.

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differentiating embryonic stem cells (Houbaviy et al., 2003; Suh et al., 2004; Tang et al., 2006). Interestingly, these same 4 microRNAs, which exhibit high sequence similarity (Fig. 1A), were among those identified by at least 3 different algorithms as possible binding partners to the 3′-UTR of the Signal Transducer and Activator of Transcription 3 (STAT3) (Fig. 1B). Based on the current miRNA literature and the knowledge that STAT3 interacts with myc in ES cells (Cartwright et al., 2005), hematopoiesis (Hirano et al., 2000), and cancer (Barre et al., 2005), we hypothesize that miR-17 family members are expressed during embryonic development and regulate cellular differentiation by targeting of mRNAs such as STAT3.

STAT3, a downstream transcription factor in the JAK-STAT signal cascade, is an important player in both developmental and stem cell biology. Surprisingly, few investigations exist describing STAT3 prior to implantation (Antczak and Van Blerkom, 1997; Duncan et al., 1997); however, STAT3 knockout mice are embryonic lethal just prior to gastrulation at E6.0–6.5, and exhibit functional failure of the visceral endoderm and a lack of mesoderm formation (Takeda et al., 1997). While this developmental lesson alludes to the important role STAT3 plays in many tissue types, the constitutive activation of STAT3 in several cancers demonstrates that this protein must also be tightly regulated (Catlett-Falcone et al., 1999; Corvinus et al., 2005; Nefedova et al., 2004). STAT3 is negatively regulated by at least 4 known mechanisms, including various phosphatases, expression of its endogenous dominant negative isoform, STAT3β, and negative feedback loops created by expression of Suppressors of Cytokine Signaling (SOCS) and Protein Inhibitors of Activated STATs (PIAS) proteins (Horvath, 2000; Inagaki-Ohara et al., 2003). Previous work from our laboratory and others has demonstrated that several of these mechanisms operate simultaneously at the onset of ES cell differentiation to induce a rapid and drastic decrease in STAT3 activity (Chan et al., 2003; Feng, 2007; Foshay and Gallicano, 2008; Foshay et al., 2005; Li et al., 2005). Our previous data suggest that the degree and duration of this period of STAT3 inactivity may affect cell fate choices over the course of differentiation. Thus, as STAT3 regulation by several different methods appears to be critical for proper ES cell differentiation, we hypothesize that miRNAs may exert their effects on differentiation through this key developmental regulator.

Although as many as 50 miRNAs are predicted to bind the 3′-UTR of STAT3, there are only two reports of miRNA-mediated regulation of STAT3 in the current literature. In both cases, the investigators demonstrate that levels of STAT3 phosphorylation can be indirectly affected by miRNAs in neural cell differentiation and in cancer (Krichevsky et al., 2006; Meng et al., 2007). However, our data is the first to elucidate a STAT3/miR-17 family member(s) interaction, which results in a functional regulation of ES cell differentiation.

Materials and methods

ES cell culture and differentiation

CCE ES cells (Keller et al., 1993; Robertson et al., 1986), obtained from Stem Cell Technologies, Inc., were grown in feeder free conditions with media containing 15% ES cell qualified FBS (Invitrogen, Carlsbad, CA). Differentiation of ES cells was induced by removal of LIF and transfer to suspension culture.

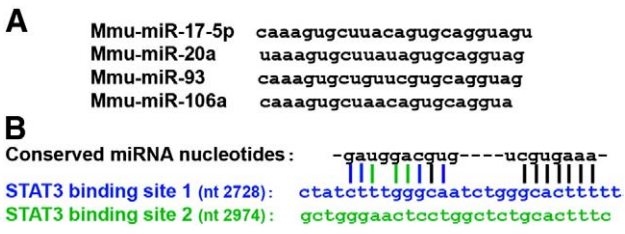


Fig. 1. (A) The mature miRNA sequences for miR-17-5p, miR-20a, miR-93, and miR-106a. These 4 miRNAs are predicted to bind STAT3. (B) The 2 predicted miR-17 family binding sites on the STAT3 3′UTR. Binding sites and miRNA nucleotides are aligned to shown conservation (black — both binding sites; blue — site 1; green — site 2). All 4 miRNAs share the same seed region, which is perfectly complementary to both STAT3 binding sites.

Embryo procurement

Blastocysts and implanted embryos were obtained as described in Gallicano and Capco (1995) and Gallicano et al. (1998). Briefly, one female is placed into a cage with one male to allow copulation. Females are checked the next day for vaginal plugs, which represents 0.5 days post coitum. Eighty four hours later, the mouse is sacrificed by CO2 asphyxiation, the female reproductive tract is removed, and blastocysts are flushed from the uterus using a 26 3/4 gauge needle attached to a 10 cm^3 syringe filled with medium. For in situ hybridization and confocal microscopy, blastocysts were transferred to 4.0% paraformaldehyde fixative for 1 h.

Implanted embryos were obtained from fertilized dames post-humously 5.5 days, 6.0 days, and 6.5 days post coitum by dissection from decidua and using Dumont #5 forceps. Isolated embryos were then placed in 4.0% para-formaldehyde and processed for either in situ hybridization or immunohistochemistry.

Removal of cells from blastocysts

Blastocysts were used to extract and isolate cells from three areas, trophectoderm, ICM, and presumptive primitive endoderm. To do so, blastocysts were placed in a watch glass containing 0.25% trypsin for 5–10 min until the cells comprising the blastocyst began dissociating. A pulled glass needle similar to those used for ES cell injection into blastocysts was used to extract three cells from each region of the blastocyst using an Eppendorf TransferMan NK2 micromanipulator set on negative pressure. This technique is simply the opposite of the technique used to inject blastocysts with ES cells. Once three cells were removed they were then placed into a microcentrifuge tube containing the qRT-PCR buffer supplied by the mirVana miRNA Isolation Kit (Ambion, Austin, TX).

Immunofluorescence and confocal microscopy

Embryos were fixed in 4% fresh paraformaldehyde for 30 min followed by permeabilization overnight at 4 °C. Following blocking for 1 h, embryos were incubated in primary antibody at a 1:50 dilution overnight at 4 °C. Incubation with secondary antibodies was conducted for 3 h at room temperature. Antibodies: STAT3 #9132 from Cell Signaling (Danvers, MA); CK8/18 #03-GP11 from ARP (Belmont, MA). Slides were viewed using an Olympus Fluoview 500 Laser Scanning Microscope (Olympus America Inc, Melville, NY) using 1.4 numerical aperture. Images were acquired and analyzed using the accompanying Fluoview software (version 4.3).

In situ hybridization

In situ hybridization probes were obtained from Exiqon (Woburn, MA) and experiments were performed according to the GEISHA protocol (from the University of Arizona) available on the Exiqon website. All reagents and apparatus used were DEPC treated. Hybridizations were conducted overnight at 58 °C.

miRNA binding predictions

Binding predictions were made by comparing results of three different miRNA prediction programs, each of which utilizes a different prediction algorithm. The miRBase website (http://microrna.sanger.ac.uk/targets/v4/), run by the Sanger Institute, employs the miRanda algorithm to predict miRNA:mRNA pairs. MicroInspector, another web-based miRNA search program (http://mirna.imbb.forth.gr/microinspector/), run by the Institute for Molecular Biology and Biotechnology, Heraklion, Greece, uses

a different algorithm that allows for G:U wobbles in seed matches. Finally, we used a custom made miRNA prediction program, written by Bill Foshay, which utilizes a variation of the TargetScanS algorithm (Lewis et al., 2005) and components of the Vienna RNA secondary structure programming library (RNAlib) (http://www.tbi.univie.ac.at/~ivo/RNA/RNAlib.html), which obtains RNA energy parameters from the Turner laboratory (http://rna.chem.rochester.edu/). The PicTar miRNA website (http://pictar.bio.nyu.edu/) was also used for generating Table 2.

qRT-PCR

All RNA samples were prepared by extraction with the mirVana miRNA Isolation Kit (Ambion, Austin, TX). Samples were treated with DNaseI to digest any contaminating genomic DNA and diluted to 10 ng/μL. All primers were designed by and purchased from Applied Biosystems (Foster City, CA). All RT and qPCR reagents were also purchased from Applied Biosystems. No-RT controls were performed for both miRNA and mRNA qRT-PCRs. Individual samples were run in triplicate, and each experiment was repeated at least 3 times. All samples were run on the Applied Biosystems ABI Prism 7700 system using SDS 2.1 software. Relative gene expression was calculated using the $2^{-\Delta\Delta C}{}_T$ method (Livak and Schmittgen, 2001).

Western blots

Western blots were performed using precast 7.5% Tris–HCL polyacrylimide gels and run on the BioRad mini gel system (Hercules, CA). Proteins were transferred to PVDF membranes and blocked in a solution of 2% BSA and 5% milk in PBS-T. The STAT3 antibody, available from Cell Signaling (Danvers, MA), was used at a 1:1000 dilution. Bands were visualized using colorimetric detection and exposure to autoradiography film. Similar exposure times were used for STAT3 and tubulin blots to ensure appropriate normalization. Quantification of bands was performed using Adobe Photoshop CS to calculate the pixel intensity of each band. Pixel intensities for STAT3 and tubulin were expressed as a ratio to generate a normalized value for STAT3 expression.

DNA constructs and transient transfections

All transient transfections were performed using the TransIT-LT1 or TransIT-TKO reagents from Mirus Bio Corporation (Madison, WI). Transfection reactions were prepared according to protocols provided with the reagent. For differentiation experiments, undifferentiated ES cells were transfected and 24 h post-transfection (d0) cells were harvested or placed into suspension culture for differentiation. The pMIR-REPORT and pRL-CMV constructs are commercially available from Ambion (Austin,TX) and Promega (Madison, WI), respectively. pMIR-REPORT 3′UTR inserts are as follows: pMIR-miR20 contains two synthesized binding sites that are complimentary to miR-20, pMIR-Hoxa11 contains the Hoxa11 3′UTR, pMIR-S3 contains part of the STAT3 3′UTR including the two putative miRNA binding sites, pMIR-S3st1mt contains the STAT3 3′UTR with 4 point mutations in the first miRNA binding site, pMIR-S3st2mt contains 4 point mutations in the second miRNA binding site, pMIR-S32xmt contains 4 point mutations in each of the two miRNA binding sites. All miRNA mimics and inhibitors were designed by and purchased from Dharmacon (Lafayette, CO). The mimics are duplexes and therefore the concentration of active miRNA mimic (strand loaded into RISC) within the cell is about 50 nM or half of the total transfected concentration (100 nM). Inhibitors are single stranded.

Luciferase assays

Luciferase assays were conducted following transient transfections of pMIR and pRL-CMV constructs at a 15:1 ratio. Cells were harvested according to specifications of the Dual Luciferase Reporter Assay Kit (Promega) and 20 μL of the lysate was transferred to each well of a 96 well plate for analysis. For experiments in undifferentiated ES cells, lysates were prepared 48 h post-transfection. For differentiation experiments, undifferentiated cells were transfected and placed into suspension culture without LIF 24 h post-transfection (d0). The luciferase reactions were conducted using the Wallac VICTOR^2 96-well plate reader at the Lombardi Cancer Center Shared Resource. All luciferase data are presented as a normalized ratio of luciferase/*Renilla*.

Results

miR-17 family miRNAs are differentially expressed in developing mouse embryos

Based on our hypothesis that miR-17 family miRNAs are important for embryonic development we sought to determine their expression patterns in early mouse embryos. Because this family of miRNAs is transcribed from the genome as three polycistronic clusters, which are regulated by c-myc, we expected to find the mature miR-17 family miRNAs expressed at similar levels and within the same regions of the embryos.

In our first set of experiments, we identified the expression patterns of miR-17-5p, miR-20a, miR-93 and miR-106 in E4.0 blastocysts by in situ hybridization (ISH) using locked nucleic acid (LNA) miRNA specific probes. Despite their small size and sequence similarity, the highly stable LNA probes allowed us to use increased hybridization temperatures relative to conventional in situ protocols (Kloosterman et al., 2006; Nelson et al., 2006). As a result, we determined distinct embryonic localization patterns for each miRNA. Specifically, miR-17-5p and miR-20 were seen throughout the blastocyst, with a slight increase in hybridization seen within cells of the trophectoderm (Fig. 2A). Low levels of miR-106a signal were also observed throughout blastocysts; however, elevated levels of miR-106a hybridization were clearly evident in the inner cell mass (ICM) (Fig. 2A). The most striking expression pattern was that of miR-93. This miRNA was restricted to the trophectoderm and the future primitive endoderm. Additionally, miR-93 expression was barely detectable within the ICM (Fig. 2A). We also noticed that the signals for miR17-5 and miR-20 were scattered throughout the cytoplasm. However the signal for miR-93 was enriched to one side of the cytoplasm in select cells. Although we were surprised to see both subtle as well as distinctly different miRNA expression patterns between family members, our observations fit well with our original hypothesis; that the highest miRNA expression would be found in differentiating tissues. These data suggest that miR-17 family miRNAs may play a role in embryonic development and differentiation.

Because we hypothesized that these miRNAs may exert their effects via STAT3, we next looked at STAT3 expression in the blastocysts. As expected, STAT3 expression was specific to the ICM with little or no staining seen in the trophectoderm and the presumptive primitive endodermal ICM cells that line the blastocoelic cavity (Fig. 2B). This expression pattern was opposite to that of miR-93, suggesting that endogenous miR-93 may downregulate STAT3 in specific cells of the blastocysts.

We continued our investigation of miR-17 family expression patterns, conducting in situ hybridization in E5.5 and E6.5 embryos (Figs. 3 and 4). At E5.5 we saw strong hybridization of all probes in the extraembryonic cells (Figs. 3A–D). These cell types include the extraembryonic ectoderm and the visceral endoderm, which, at E5.5, are differentiating into specialized tissues that will form the yolk sac and placenta, and facilitate nutrient and gas exchange for the embryo. In contrast, the primitive embryonic ectoderm, or epiblast, is still undifferentiated at this stage. These cells, which are rapidly proliferating in preparation for gastrulation, showed little or no

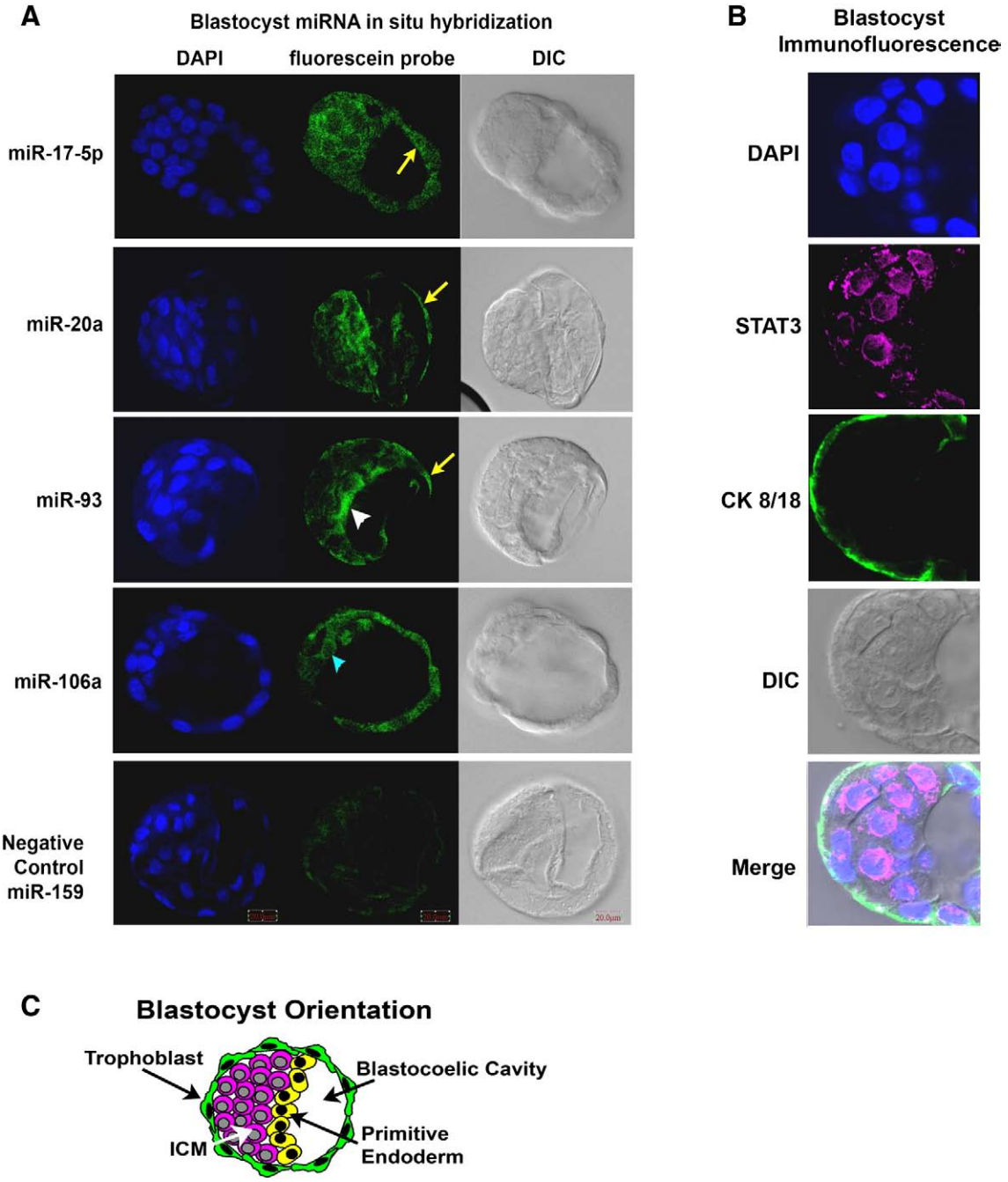


Fig. 2. (A) In situ hybridization of mature miRNAs in blastocysts stage embryos. miR-17-5p and miR-20a showed generally even expression through the cells of the blastocyst. A mild increase in hybridization of both miRNAs was detected in the trophectoderm (yellow arrow). miR-93 showed the most distinctive expression pattern with very little signal in the cells of the ICM and a drastic upregulation in both the putative primitive endoderm (white arrowhead) and trophectoderm (yellow arrow). miR-106a showed very specific staining in the ICM (blue arrowhead), although levels appeared relatively equal in all cell types. Cells with the highest miRNA expression were those undergoing differentiation (i.e. putative primitive endoderm and trophectoderm). A probe to cel-miR-159 (a *C. elegans* miRNA not found in mouse) is used as a negative control. The embryos shown are representative of 6–10 embryos for each miRNA tested. (B) Immunofluorescence of blastocyst stage embryos showed that STAT3 is localized to the ICM, an expression pattern opposite of miR-93. Arrowheads point to putative primitive endoderm that has little or no STAT3 staining. Anti CK8/18 staining clearly labeled the trophectoderm, which shows low levels of STAT3 staining. The embryo shown is a representative of 10 embryos from three experiments. (C) A schematic drawing of a blastocyst stage embryo to demonstrate the orientation of the blastocysts shown in panels A and B.

expression of miR-17-5p, miR-20a, miR-93, or miR-106a. These data correlate with the blastocyst ISH data (Fig. 2) and demonstrate the idea that miR-17 family miRNAs are upregulated in differentiating cell types of the developing embryo.

In addition to the in situ studies, we performed immunohistochemistry for STAT3 expression on paraffin embedded sections of E5.5 embryos (Fig. 3E). These sections showed high levels of specific nuclear STAT3 staining throughout the epiblast, suggesting that STAT3 is active in this area. We detected little or no STAT3 in the visceral endoderm and low levels in the extraembryonic ectoderm. Taken together, these data present an expression pattern for STAT3 that is opposite of miR-17 family expression in E5.5 embryos (Figs. 3A–E).

At E6.5, embryos showed highly specific miRNA expression patterns. All four of the miRNA investigated were localized to the posterior end of the primitive streak (Figs. 4A–D). Interestingly, the hybridization was restricted to ectoderm cells at least one cell layer removed from the primitive streak. Thus, the posterior primitive

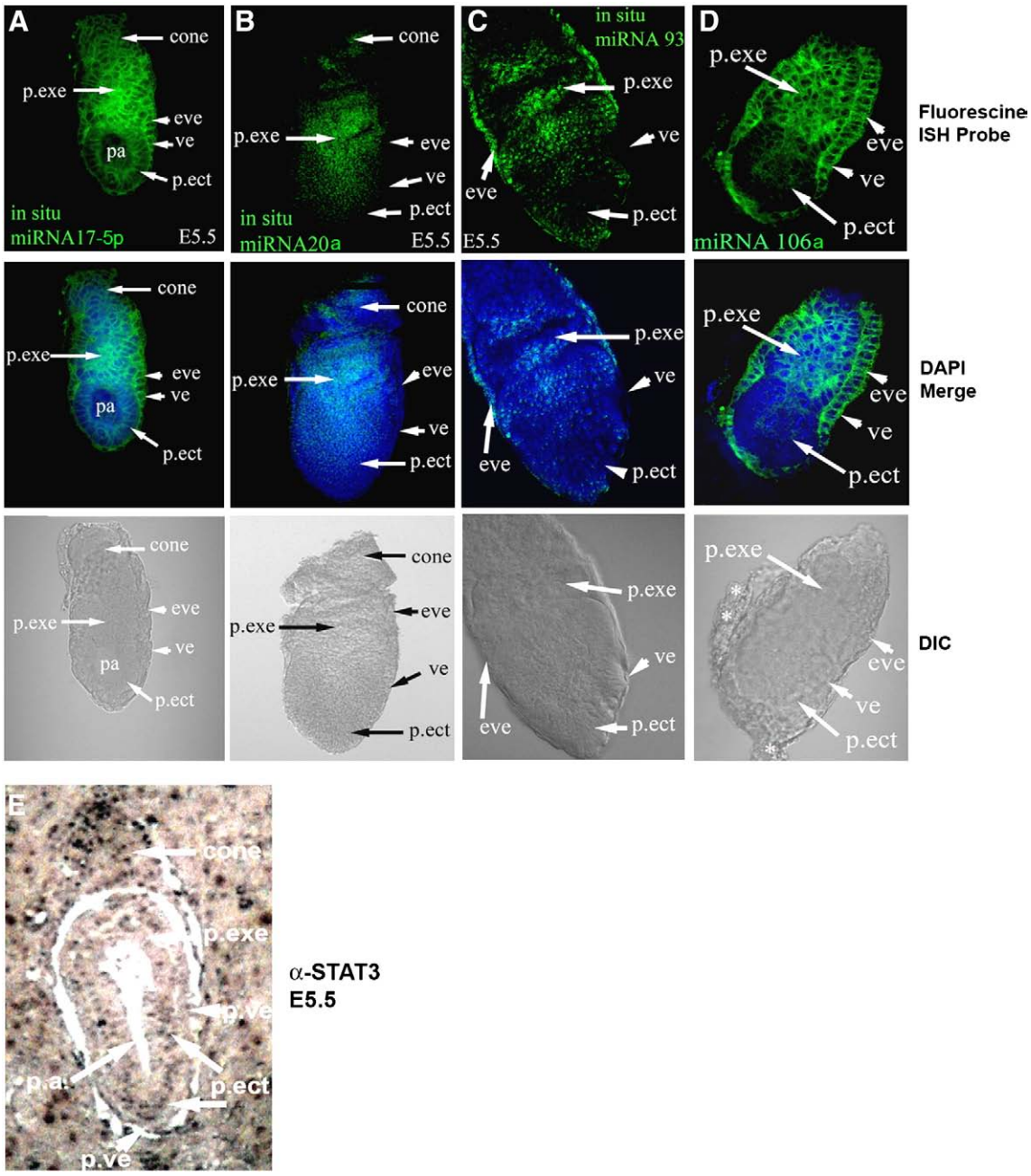


Fig. 3. (A–D) Whole mount in situ hybridization of E5.5 mouse embryos. At this stage in development, expression of the miR-17-5p, miR-20a, miR-93 and miR-106a is high in the differentiating extraembryonic cells that will form the tissues that support growth of the epiblast. The embryonic ectoderm, or epiblast, is relatively undifferentiated at this stage of development. These cells exhibit little or no miR-17 family miRNA expression. (E) Immunohistochemistry for STAT3 expression in an E5.5 embryo section. STAT3 (brown) is localized to the nuclei within the epiblast or primitive ectoderm. Little if any staining is observed in the visceral endoderm or extraembryonic ectoderm. (A–E) cone — ectoplacental cone; eve — extraembryonic visceral endoderm; ve — visceral endoderm; p-ect — primitive ectoderm; p-exe — primitive extraembryonic ectoderm; pa — proamniotic cavity.

streak ectodermal cells bordering the mesoderm did not express any of the miRNAs. However, gastrulating cells, which were in the process of differentiating into mesoderm or mesendoderm expressed high levels of miR-93 and miR-17-5p (Figs. 4A, B). Although, in some cases brightly positive cells were observed within the endoderm, generally the visceral endoderm had low miRNA expression. These cells may be intercalating mesendoderm cells that will form the definitive endoderm. From these data we suggest that miR-17 family miRNAs are expressed in cells that are approaching the primitive streak, followed by virtually complete downregulation as they prepare to pass through the primitive streak. As these cells engress and differentiate, miR-17-5p and miR-93 are upregulated, most likely downregulating translation of mRNAs that are specific to the ectoderm or are involved in proliferation. The patterns of ISH and STAT3 expression from blastocysts, E5.5 and E6.5 embryos are summarized in Table 1.

Of note is the drastic difference in miRNA expression within extraembryonic and embryonic visceral endoderm of E6.5 embryos. While the embryonic visceral endoderm has low expression of all tested miRNAs, miR-20a and miR-106a are expressed at relatively high levels in the extraembryonic visceral endoderm (Figs. 4C, D, eve). The hybridization patterns are extremely specific with miRNAs being detected specifically in the cytoplasm and at the periphery of cells. In fact, the lack of signal in the nucleus for any of the miRNA probes would suggest that they are relatively specific for the processed, mature miRNA and not the unprocessed Pri-mRNA.

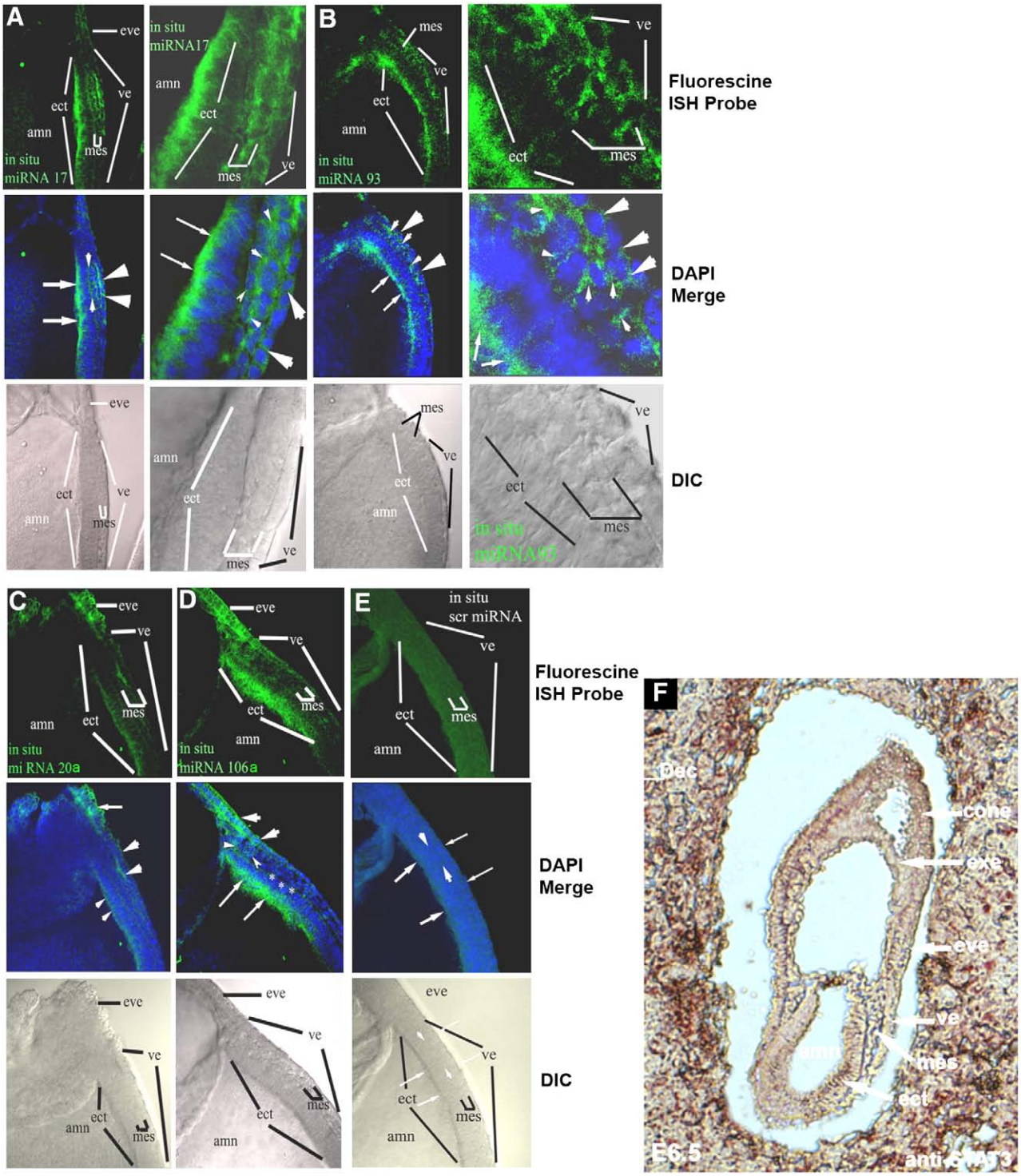


Fig. 4. (A–E) In gastrulation stage embryos (E6.5–E6.75) miR-17-5p (A), miR-20a (C), miR-93 (B) and miR-106a (D) are all localized to ectoderm cells one cell layer away from primitive streak ectoderm (white arrows). In addition, miR-17-5p and miR-93 showed specific upregulation in differentiating mesoderm cells (A, small arrowheads). In most cases miRNA expression was low in the visceral endoderm (A–D, large arrowheads). A few bright cells within the visceral endoderm are thought to be intercalating mesendoderm cells. Note: miR-93 is specifically found in differentiating cells of both blastocyst and gastrulation stage embryos (B and Fig. 2). (F) Immunohistochemistry of an E6.75, gastrulating embryo demonstrated elevated levels of STAT3 protein in ect (black arrowheads) not associated with the primitive streak. eve — extraembryonic visceral endoderm; ve — visceral endoderm; me — mesoderm; ect — ectoderm; amn — amniotic cavity; dec — deciduas; cone — ectoplacental cone.

In addition to the in situ studies, we performed immunohistochemistry for STAT3 expression on paraffin embedded sections of E6.5 embryos (Fig. 4F). While these sections showed high levels of specific nuclear STAT3 staining in the ectoderm (ect), cells near the posterior primitive streak appear devoid of STAT3, suggesting that STAT3 is inactive in this area. We detected little or no STAT3 in nuclei of visceral endoderm and low levels in the extraembryonic ectoderm.

miRNAs -17, 20a, -93 and 106a are expressed in blastomere and ES cells

To test our hypothesis that miR-17-5p, miR-20a, miR-93 and miR-106a are involved in regulation of differentiation during embryonic development we used two model systems; isolated cell-types from blastocysts and ES cells. By conducting our studies in vitro we could easily manipulate individual miRNAs and assess their effects on cell

Table 1
Expression of miRNAs and STAT3 in developing embryos

	Blastocyst (E4.0)			E5.5			E6.5			
	ICM	PE	TE	PEct	Exe	VE	PS	Ect	Mes	VE
miR-17-5p	+	+	++	+	++++	++++	–	+++	+++	+++
miR-20a	+	+	++	–	+	–	–	++	+	+
miR-93	++	++++	++++	–	+++	++	–	+++	+++	+++
miR-106a	++	++	++	–	++++	++++	–	+++	+	+
Differentiating cell type	N	Y	Y	N	Y	Y	N	Y	Y	Y
STAT3	++++	–	+	++++	++	–	++++	+++	–	+

This table summarizes the expression levels of miR-17-5p, miR-20a, miR-93, miR-106a, and STAT3 during early embryonic development as determined by in situ hybridization and immunostaining (Figs. 2, 3, and S1).
(–) below level of detection; (+) low level of expression; (++) level of expression slightly higher than (+); (+++) strong expression; (++++) highest expression; N, No; Y, Yes.

fate. We first employed qRT-PCR to quantify mature miRNA expression in blastocysts from isolated trophoblast cells, pluripotent ICM, and ICM destined to become primitive endoderm. We also used qRT-PCR to analyze miRNA expression in ES cells (Fig. 5). Using this approach, we were able to quantitatively compare changes in miRNA expression as cells of the blastocyst differentiated into primitive endoderm and trophectoderm.

It is important to note how these data were controlled for this set of experiments. All qRT-PCR samples were normalized to qRT-PCR of U6 snRNA using the delta-delta Ct method. Then, to make the data easier to interpret, one sample (generally the one with the lowest overall value) was picked and set to 1 serving as the baseline. The other samples were then compared to the baseline sample for each graph in Fig. 5. In Fig. 5A, miR-20 was set as the baseline and expression of the other miRNAs is compared to miR-20. In Fig. 5B, miRNA-106a was set as the baseline and expression of the other miRNAs is compared to miRNA-106a. The baselines were set differently because miRNA expression was different when comparing ES cells to blastocysts (see below).

Within the blastocyst, miR-93 was expressed at levels higher than any other tested miRNA (Fig. 5A). Interestingly upon extraction and analyzing three cells per experiment from the three areas within the blastocyst, miR-93 was the only miRNA that was significantly upregulated in the putative primitive endoderm and trophectoderm. Thus, these qRT-PCR data supported the results previously found using in situ hybridization (Fig. 2). To this end, miR-17 and miR-20 expression levels were relatively consistent across cell types, while miR-106a was highest in the ICM. Since these data matched our in situ hybridization data (Fig. 2), we determined that qRT-PCR would be the best method for measuring miRNA expression in ES cells.

Surprisingly the miRNA expression profile of ES cells was different than that of blastocyst ICM cells. In cultured ES cells, miR-20 expression was higher than any other tested miRNA, miR-17-5p and miR-93 displayed equal expression levels, and miR-106a was the lowest (Fig. 5B). Despite the differences between baseline miRNA expression in ES cells and ICM cells, the upregulation of these miRNAs during differentiation was consistent (Fig. 5C). Specifically, when compared to miR-93 expression in undifferentiated ES cells, miR-93 expression nearly doubled during the first three days of ES cell differentiation. While miR-106a expression also increased, this occurred approximately 2 days later than the miR-93 increase, suggesting miR-106 may play a different role in differentiation. Both miR-20 and miR-17 exhibited slight increases over the course of differentiation, thus supporting the blastocyst qRT-PCR and in situ data.

Differentiation of individual blastomeres within blastocysts occurs more synchronously than differentiation of individual cells within an EB. Therefore, we surmise that the lower fold increase in miRNA expression in ES cells when compared to blastomeres is due to decreased temporal regulation of differentiation. Based on this comparison of ES and blastocyst differentiation, we have demonstrated that differences between the in vivo niche and cell culture conditions may alter basal miRNA expression levels. This could be due to the mix of growth factors present in the fetal bovine serum used to culture ES cells. It is also possible that in ES cell culture Drosha functions differently than in embryos, and thus changes in miRNA expression may be due to differential post-transcriptional processing

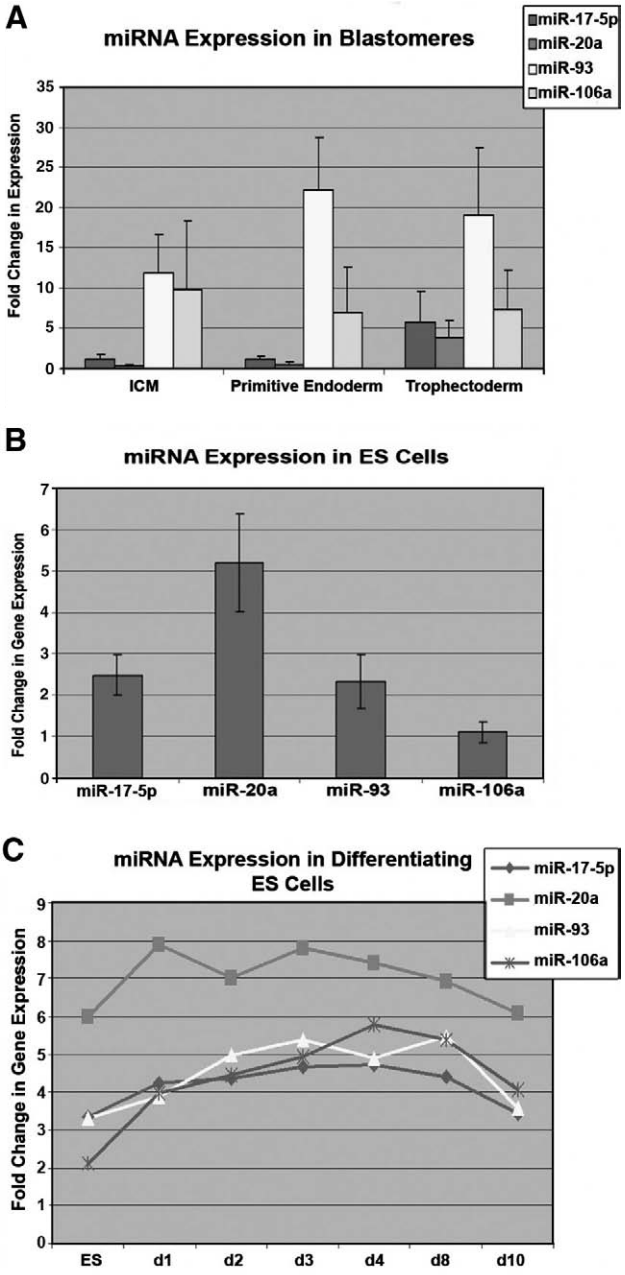


Fig. 5. Change in miRNA expression after differentiation. (A) Mature miRNA expression in E4.0 blastocyst cell types was examined by qPCR. miR-93 expression was highest throughout the blastocyst, but showed a 10-fold increase when cells differentiated from the ICM to the primitive endoderm or trophectoderm. In general these data correlate to expression profiles determined by in situ hybridization. (B) miRNA expression differences between ES cells and ICM. In ES cells miR-20 was expressed at higher levels than the other miR-17 family miRNAs. (C) Expression analysis of the miR-17 family members over the course of ES cell differentiation revealed distinct patterns directly related to differentiation. In most cases, (e.g., miR-93, miR-106a) expression virtually doubled within the first 3 days of ES cell differentiation. (A–C) Each RT reaction was performed three times, and each qPCR sample was run in triplicate. Relative gene expression was calculated using the $2^{-\Delta\Delta C_T}$ method using U6 snRNA for normalization (Livak and Schmittgen, 2001). Data are presented as the mean ± the SEM.

(Thomson et al., 2006). Alternatively, differences in basal miRNA expression could be attributed to the fact that ES cells in culture do not interact with a supportive trophectodermal niche.

Although we found differences between miRNA expression in vivo and in vitro, we demonstrated a consistent upregulation of miR-93 upon initiation of differentiation in both cultured ES cells and in developing blastocysts. These data suggest that miR-93 is an important miR-17 family member for regulating cellular differentiation.

Changes in miRNA expression can alter cell fate during ES cell differentiation

To test the hypothesis that miR-17 family miRNAs affect cell fate during differentiation, we transfected mimics or inhibitors of miR-93 and miR-20 into undifferentiated ES cells (a non-specific inhibitor/mimic was used as a negative control). This transient transfection was followed by a second transfection once differentiation had begun (day 2; 72 h after first transfection). The cells were allowed to differentiate as EBs in suspension culture for up to 7 days (day 7). RNA was harvested at days 0, 3 and 7 and was then used for qRT-PCR analysis of germ layer specific differentiation markers (Fig. 6). Levels of mature miRNA within the cell were also measured by qRT-PCR in an effort to demonstrate the effectiveness of the transfection (Fig. S1). Because of the large scale of this experiment, we chose only to use inhibitors to miR-20 and miR-93. Our rationale stemmed from the strong in vivo data suggesting that miR-93 was the most likely candidate regulator of stem cell differentiation. As expression of the other miRNAs seemed less specific to differentiating cells, we chose to examine only miR-20, which we felt was representative of the other 3 miRNAs.

Upon transfection of inhibitors we saw a statistically significant decrease in Fgf-5 expression when compared to controls only at d0. These data suggest that ectodermal differentiation was not affected by the miRNA inhibitors (Fig. 6A). However, miR-93 inhibitors disrupted endodermal differentiation. In these cells Hnf4a levels were significantly lower at the onset of differentiation (24 h post transfection) and remained low even after several days of culture in differentiating conditions (Fig. 6B). Interestingly, the inhibitors also delayed the expression of the mesodermal differentiation marker, Brachyury, (Fig. 6C, d3). Based on these data we suggest that miRNAs exhibit different effects on different germ layers. As evidenced from our in vivo ISH data, these miRNAs are quite low in the ectoderm or epiblast (Fig. 3) and therefore their inhibition may not drastically effect differentiation. Additionally, our in vitro data support our in vivo data and suggest that miR-93 is, in fact, a more potent inducer of differentiation than miR-20 or other miR-17 family members.

When the opposite experiment was conducted, and miR-93 mimics were transfected, we saw a significant increase in Brachyury expression (Fig. 6F). However, this increase was not observed upon transfection of miR-20 mimics. Again, these data support a functional role for miR-93 in regulation of ES cell differentiation. Additionally, these data suggest that upregulation of miR-93 may promote mesodermal differentiation. This idea is supported by the strong mesodermal expression of miR-93 in gastrulating embryos (Fig. 4B). Taken together, our data demonstrate the first functionally significant role for miRNAs in regulation of ES cell differentiation in vitro.

In addition to qRT-PCR analysis, we observed the effects of miR-93 mimics and inhibitors on EBs using H&E staining (Figs. 6G–I). Following the same procedure outlined above, transfected cells were allowed to differentiate for up to 10 days in culture. EBs were harvested, fixed, and sectioned for staining. EBs transfected with miR-93 inhibitors were comprised of homogeneous cells, as compared to controls or miR-93 mimic transfected cells. In addition, EBs transfected with miR-93 inhibitors lacked the outer layer of visceral endoderm that is commonly found in differentiating EBs (Fig. 6, black arrows). These histological data agree with our qRT-PCR data and support the conclusion that inhibition of miR-93 prevents differentiation of ES cells.

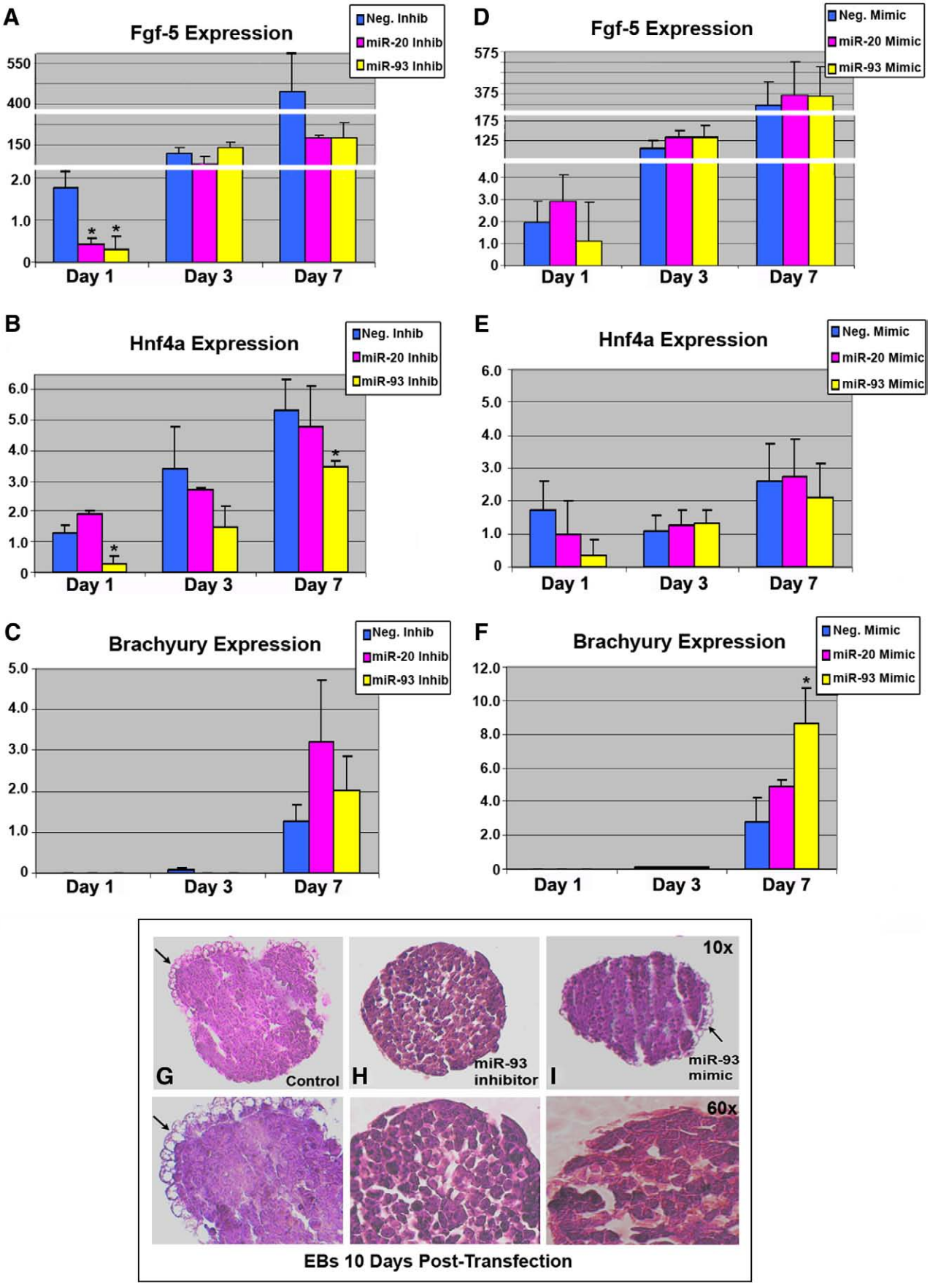
miR-20 and miR-93 can bind the STAT3 3′UTR and decrease STAT3 expression

Data from our previous studies demonstrated that STAT3 is important not only for pluripotency, but also for differentiation of mesodermally and ectodermally derived cells (Foshay and Gallicano, 2008; Foshay et al., 2005). Additionally, miR-93 and other miR-17 family members are predicted to target the 3′UTR of STAT3 (Fig. 1B). The results of the mimic and inhibitor transfection experiments could be explained by miRNA mediated regulation of STAT3, and therefore lend support to our hypothesis that STAT3 is a functional miR-17 family target.

To further test our hypothesis that miR-17 family miRNAs can target STAT3, we constructed reporter vectors containing a portion of the STAT3 3′UTR downstream of a CMV promoter-driven luciferase gene (Fig. 7A). When the miRNAs of interest are present, they bind to the cloned 3′UTR and silence luciferase expression. Our positive control vector, pMIR-miR20, contains two synthesized binding sites that are complementary to the mature miR-20 sequences. The negative control vector, pMIR-Hoxa11, contains the 3′UTR of the Hoxa11 gene, which is not predicted to bind miR-17 family miRNAs and is specifically silenced by miR-181 during mammalian myoblast differentiation (Naguibneva et al., 2006). The experimental vectors used in our studies contain either wild type (pMIR-S3) or mutant versions of one (pMIR-S3st1mt and pMIR-S3st2mt) or both (pMIR-S32xmt) of the two putative miRNA binding sites within the STAT3 3′UTR.

We assayed the luciferase activity of our reporter constructs in response to endogenous miRNAs over the course of ES cell differentiation. As hypothesized, the pMIR-S3 construct was silenced between days 1 and 3 at the onset of ES cell differentiation (Fig. 7B). The pattern of luciferase activity corresponds to the period of differentiation during which STAT3 activity is normally downregulated and miR-93 is upregulated (Fig. 5C). Moreover, in Fig. 7B, pMIR-S3 is highest at d4, suggesting that once ES cells have initiated differentiation, miRNAs are no longer regulating the STAT3 UTR. This is supported by Fig. 5 which shows miRNA levels peaking between days 2 and 3 and beginning to show a decrease by d4. To ensure that this pattern of luciferase activity was actually due to endogenous miRNAs binding the STAT3 3′UTR and not just an artifact of our system, we performed the same timecourse experiment using STAT3 3′UTR mutant constructs (Figs. 7B and S2). The construct containing mutations in the second miRNA binding site (pMIR-S3st2mt) displayed some responsiveness to miRNAs at the onset of differentiation but not later in the process (Fig. S2A). In contrast, the site one mutant (pMIR-S3st1mt) and double mutant (pMIR-S32xmt) constructs had an entirely different pattern of luciferase activity when compared to the wild type STAT3 construct over the course of differentiation (Figs. 7B and S2A). These two mutant constructs

Fig. 6. (A–F) Transfection of miR-20 or miR-93 inhibitors or mimics affects fate commitment in differentiating ES cells. Cells were transfected in the undifferentiated state and put into suspension culture without LIF 24 h post-transfection (d0). RNA was collected for qRT-PCR on days 1, 3, and 7. Transfection of either inhibitor caused a delay in ectodermal differentiation, as evidenced by significantly lower levels of Fgf-5 expression. The miR-93 inhibitor also blocked endodermal differentiation, resulting in decreased Hnf4a expression both at the onset of differentiation and after 7 days under differentiating conditions. Neither inhibitor seemed to affect mesodermal differentiation. However, transfection of the mimic to miR-93 did cause a significant upregulation in brachyury, a mesoderm marker. (G–I) Using the same transfection procedure as above, EBs were cultured for 10 days and then embedded in paraffin for H&E staining. In controls or EBs transfected with miR-93 mimics (G, I) a clear outer layer of visceral endoderm, as distinguished by the large vacuoles, is apparent. In EBs transfected with miR-93 inhibitors, these cells are absent.


A
Fgf-5 Expression
Neg. Inhib
miR-20 Inhib
miR-93 Inhib
550
400
150
2.0
1.0
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D
Fgf-5 Expression
Neg. Mimic
miR-20 Mimic
miR-93 Mimic
575
375
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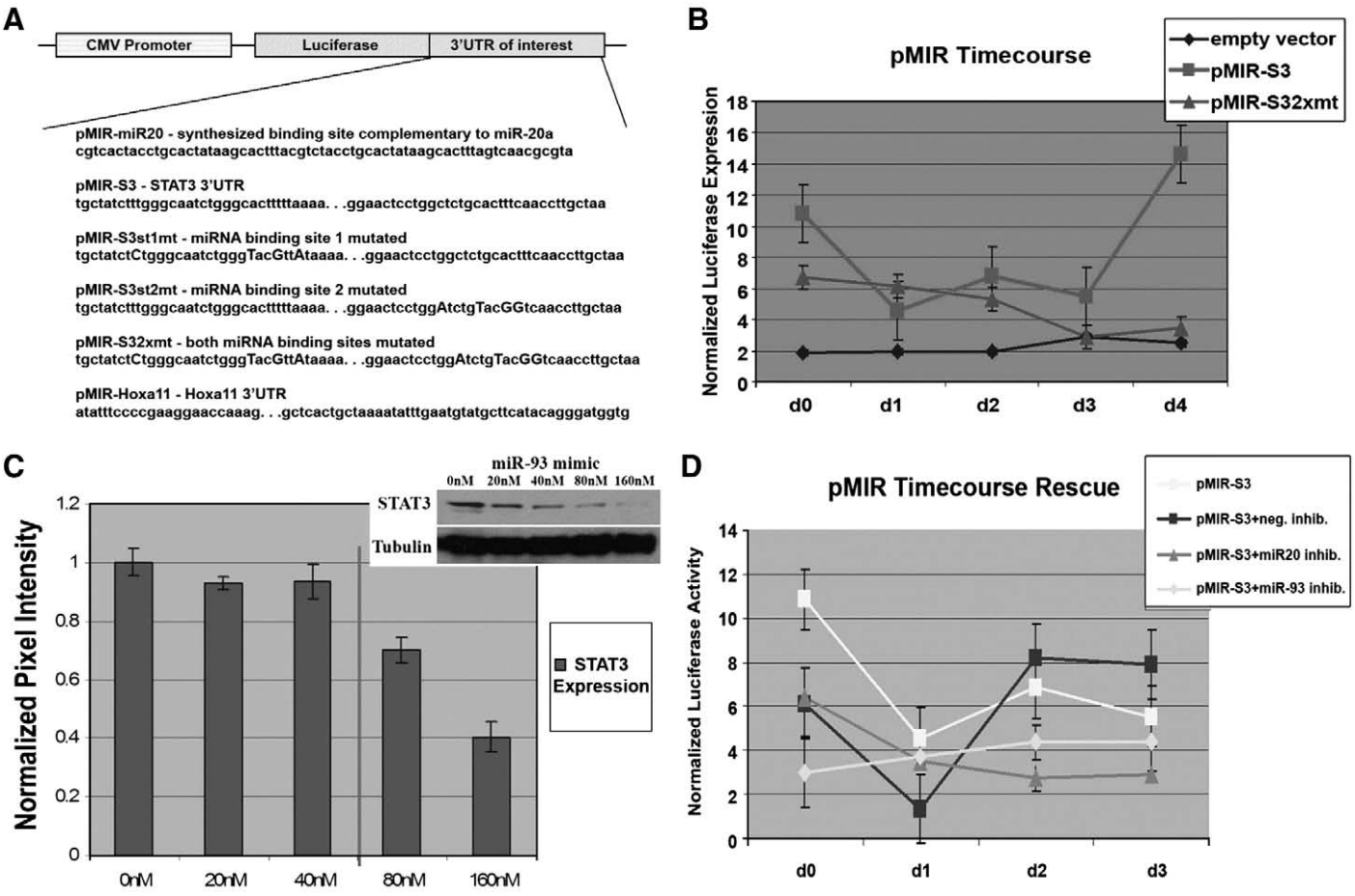

Fig. 7. (A) A schematic representation of the pMIR-luciferase reporter constructs used in this study. (B) Transfection of pMIR-S3 (vector containing the STAT3 3′UTR) into ES cells results in a rapid decrease in luciferase activity once differentiation begins. The decrease in luciferase lasts approximately 2 days and correlates to a period in which STAT3 activity is known to be reduced (Foshay and Gallicano, 2008; Foshay et al., 2005). Mutation of the miR-17 family binding sites on the STAT3 3′UTR completely changes the pattern of luciferase expression, suggesting that these are functional miRNA binding sites. All luciferase values are normalized to *Renilla* luciferase. (C) Dose response curve of STAT3 expression 48 h after transfection with miR-93 mimic. Inset Western blot shows representative results. Values for STAT3 expression were generated by calculation of the pixel intensity of each band using Adobe Photoshop CS, and were normalized to tubulin expression. Data are represented the mean ± the SEM ($n = 3$). A reduction in STAT3 expression is seen when concentrations of greater than 80 nM are used. (D) Luciferase activity of the pMIR-S3 construct alone or co-transfected with inhibitors to miR-20 or miR-93 was analyzed during ES cell differentiation. Only inhibitors to miR-93 could prevent the decrease in pMIR-S3 luciferase activity at the onset of differentiation. Neg. Inhib. is a non-specific inhibitor used as a control. All values are normalized to *Renilla* luciferase and presented as the mean ± the SEM.

showed no decrease in luciferase activity at the onset of differentiation. Together these data suggest that while both miRNA binding sites on the STAT3 3′UTR are functional, the first site is more important for miRNA binding at the onset of differentiation. Moreover, another important aspect of these data that must be stressed is that there is very little change in luc expression in the mutant construct while the wild-type construct shows dramatic changes in luc expression that virtually mirror when STAT3 protein is reduced during the initial stages of ES cell differentiation.

We also tested the effectiveness of these constructs by co-transfecting them along with mimics or inhibitors to miR-20 or miR-93. As expected, miR-20 mimics could successfully decrease the luciferase activity of our positive control vector ($p < .02$) but not our negative control vector (Fig. S2B). The miR-20 mimic also induced a modest but statistically significant 25% decrease in luciferase activity of the pMIR-S3 construct. These data suggest that miR-20 can both bind to and silence the STAT3 3′UTR. More surprising results were seen when co-transfecting these same constructs with miR-93 mimics. Specifically, the miR-93 mimics could not silence the pMIR-miR20 positive control vector. However, as expected, miR-93 mimics induced a drastic 50% reduction in luciferase activity from the pMIR-S3 construct (Fig. S2C). This interesting result suggests, as previously hypothesized, that miR-93 can more efficiently bind to and silence STAT3 expression. Additionally, these novel data suggest that factors other than the seed sequence, which is identical in miR-20 and miR-93, may regulate binding of miRNAs to target UTRs.

Of note is the increase in pMIR-miR20 luciferase activity in response to the miR-93 inhibitor (Fig. S2C). While the miRNA mimics work in a physiological manner (being loaded into the miRNA RISC silencing complex) the inhibitors simply bind complimentary miRNAs. We suggest that the miR-93 inhibitor is capable of binding to and blocking endogenous miR-20 or other miR-17 family members, resulting in increased luciferase expression of pMIR-miR20. We see this effect only when using the positive control vector (which was designed to bind multiple endogenous miR-17 miRNAs) but not when we transfect the pMIR-S3 construct. This suggests that binding of endogenous miR-17 miRNAs to the STAT3 3′UTR is more restricted than binding to the control sequence.

To test that the decreases in luciferase expression correlated to a decrease in STAT3 protein, mimics and inhibitors of miR-20 and miR-93 were transfected into undifferentiated ES cells. After 48 h, lysates were made and analyzed by Western blot for STAT3 expression. We constructed a dose response graph and demonstrated a significant decrease in STAT3 when ES cells were transfected with 80–160 nM of miR-93 mimic (Fig. 7C). Based on these results we chose to use 20 nM and 100 nM concentrations of miRNA mimics and inhibitors in a larger

scale experiment. We demonstrated that both miR-20 and miR-93 mimics, transfected at the 100 nM concentration, could significantly reduce STAT3 expression (Fig. S3A). Additionally, this decrease could be rescued by co-transfection of inhibitors along with mimics, suggesting that the effects on STAT3 were specifically caused by the activity of the miRNA mimics. The co-transfection of mimics to both miR-20a and miR-93 (each at 50 M) also resulted in a significant decrease in STAT3 expression. However, in this case the decrease was less than that seen when the miR-93 mimic was transfected alone. These data suggest that miR-93 may be more efficient at silencing STAT3 and that perhaps miR-93 and miR-20 do not bind the STAT3 3′ UTR with the same affinity.

To confirm that the decrease in STAT3 expression was due to downregulation by miRNAs and not a secondary effect of ES cell differentiation, we transfected negative control or miR-93 mimics (100 nM) into MDA-MB-231 breast cancer cells (Fig. S3B). In accordance with our expectations, the miR-93 mimic led to a decrease in STAT3 protein when compared to reagent only or negative mimic controls. As STAT3 has no pluripotency function in these cells, any decrease in STAT3 protein is likely due to miRNA downregulation and not indirect effects on cellular differentiation.

Finally, to determine which endogenous miRNAs were binding the STAT3 3′UTR during ES cell differentiation, we repeated our time-course experiments, this time co-transfecting the inhibitors to either miR-20 or miR-93 along with the pMIR-S3 construct (Fig. 7D). While transfection of the miR-20 inhibitor had a slight effect on the pattern of pMIR-S3 luciferase expression, a decrease in luciferase activity was still observed at the onset of differentiation. Only transfection of miR-93 inhibitors completely prevented the decrease of luciferase activity at the onset of differentiation, closely resembling the pattern of luciferase activity seen with the pMIR-S32xmt construct (Fig. 7B). These data strongly suggest that endogenous miR-93 binds to and silences the STAT3 during ES cell differentiation.

Discussion

While the current literature has demonstrated that ES cells express a unique set of miRNAs, few studies clearly define a functional consequence of miRNA expression in this model system. In this manuscript we have not only identified a family of miRNAs that are present in ES cells, but we have confirmed their existence in developing mammalian embryos and linked their expression to a functional role in the regulation of ES cell differentiation.

Although the knockout mouse has become the gold standard for identifying the effects of specific genes on embryonic development and cellular differentiation, several confounding factors decreased the usefulness of a knockout mouse model in this study. First, as the miR-17 family of miRNAs has been duplicated throughout evolution, many of these miRNAs exhibit high sequence homology and most likely overlapping functions. Due to this redundancy, the knockout of any single miR-17 family miRNA most likely would not provide clear insight into its role in development. A second option would be a knockout of the entire miR-17 family. Because this family exists as three clusters on three separate chromosomes, this endeavor would be technically challenging and may disguise any divergent miRNA functions. Thus, to elucidate both unique and redundant miRNA functions, knockouts of each miR-17 family member would have to be generated and screened for mutants that phenocopy one another. However, the creation of 14 individual knockouts would be an overly ambitious project. Taking all of these issues into consideration, we believe that the ES model system is currently the best system for examining the functions of individual miRNAs within miRNA families during embryonic development.

As the miR-17 family of miRNAs is expressed as polycistronic clusters, we were not surprised to find several members of this family expressed in both ES cells and embryos. However, within this family, individual miRNAs were clearly expressed at different levels in different cell types both in vivo and in vitro (Figs. 2–5). In addition, these miRNAs seemed to function differently in different germ layers (Fig. 6). These data suggest that miRNA expression is tightly regulated and simple transcription of the miRNA genes does not necessarily correlate to or predict expression and function of the mature form. This idea is supported by current investigations on post-transcriptional miRNA processing by Drosha in cancer cells (Thomson et al., 2006). Thus, we suggest that investigation into the varying mechanism that may control splicing of polycistronic transcripts and processing of precursor forms is necessary to completely understand the actions of miRNAs.

In addition to highlighting the tight regulation of miRNA expression during development, our data also allude to the fact that "seed sequence" recognition is not sufficient to predict miRNAs binding. Although several algorithms predicted binding of miR-17 family members to STAT3, differences in binding affinity were clear. Specifically, in light of the identical core and other sequence similarities between miR-93 and miR-20, it was surprising that miR-93 could not silence the pMIR-miR20 positive control vector. And yet, miR-93 was able to silence the STAT3 3′UTR more efficiently than miR-20. Based on these interesting data, it is apparent that better algorithms and a more detailed understanding of binding mechanisms are needed to predict functional miRNA:mRNA pairs.

Our data demonstrate the functionality of miR-93 and STAT3 binding as a mechanism for inducing murine ES cell differentiation. However, as the role of STAT3 in human and mouse ES cells varies, the question of the importance and conservation of this interaction in human development begs to be answered. Although the human ES cell experiments have not and cannot be conducted in our laboratory due to ethical policies at Georgetown University, we predict that the role of miRNA regulation of STAT3 in ES cell differentiation is conserved between species. First, it is important to note that we are not referring to promotion of self-renewal but to inhibition of differentiation. Although STAT3 is not necessary for self-renewal in mouse or human ES cells, it is still expressed at high levels (Darr and Benvenisty, 2006; Kristensen et al., 2005). Thus, it is completely probable that downregulation of STAT3 is necessary at the onset of both mouse and human ES cell differentiation. This idea is supported by the fact that STAT3 regulates differentiation of several different cell types, including hematopoietic stem cells and neural stem cells (Chung et al., 2006; Foshay and Gallicano, 2008; Foshay et al., 2005; Hevehan et al., 2002; Hirabayashi and Gotoh, 2005; Krichevsky et al., 2006; Smithgall et al., 2000). In addition, at least one member of the miR-17 family of miRNAs, miR-17-5p, is expressed in human ES cells (Suh et al., 2004) and several miR-17 family miRNAs are predicted to bind the human STAT3 3′UTR. Thus, as all of the players necessary for the STAT3-miRNA interaction are present, we believe that within

Table 2
ES cell associated targets of miR-17 family microRNAs

ES cell associated predicted targets of the miR-17 family	
mRNAs	Role in ES cells/development
LIF	Pluripotency and self-renewal in mouse ES (Cartwright et al., 2005)
STAT3	Pluripotency and self-renewal in mouse ES (Niwa et al., 1998; Raz et al., 1999); differentiation (Foshay and Gallicano, 2008; Foshay et al., 2005)
Myc	Pluripotency and self-renewal (Cartwright et al., 2005)
Frizzled-1, -4, -7	Pluripotency and self-renewal (Walsh and Andrews, 2003)
Bmp2	Self-renewal (Peerani et al., 2007) or differentiation (Gossrau et al., 2007; Pera et al., 2004)
Bmpr2	Self-renewal (Peerani et al., 2007) or differentiation (Gossrau et al., 2007; Pera et al., 2004)
Smad5, 7	Self-renewal (Suzuki et al., 2006; Ying et al., 2003)

This table lists other stem cell and/or differentiation associated mRNAs that are predicted targets of at least one miR-17 family miRNA. Like STAT3, several of these proteins are involved in both self-renewal and differentiation. This suggests that changes in miRNA expression could mediate a shift between self-renewal and differentiation by downregulating these target mRNAs.

human ES cells and developing human embryos, miRNAs bind to and downregulate STAT3 at the onset of differentiation. Further support of this hypothesis comes from Ventura et al. (2008) who demonstrated that mice deficient for miR-17–92 die shortly after birth with heart and lung defects. More importantly, double and triple knockouts (DKO or TKO) of paralogs die prior to E15, suggesting that mature miRNAs from these paralogs can compensate for one another. Since the major defects in the DKO and TKO embryos as shown by Ventura et al. (2008) were cardiac defects and our previous papers as well as others within the literature have established a role for STAT3 in heart development, we believe the phenotype observed in the Ventura paper supports the idea that this family of miRNAs maintains appropriate levels of STAT3 during differentiation.

Although our novel data clearly demonstrate that modulation of miR-93 and miR-20 expression can alter fate commitment during ES cell differentiation, several questions remain. One such question is whether STAT3 is the only target mRNA responsible for mediating the effects of miR-17 family miRNAs. As miRNAs exhibit promiscuous binding, it is likely that other stem cell or differentiation associated mRNAs are also functionally downregulated by these same miRNAs. Using a bioinformatics based approach, we generated a table of miR-17 family target mRNAs that could also be responsible for regulating the onset of ES cell differentiation (Table 2). Interestingly, this family of miRNAs seems to target players in all of the major stem cell self-renewal pathways, including c-Myc, Wnt, BMP, and STAT3 signaling.

The fact that c-Myc is a predicted target of this family of miRNAs may be highly important as c-Myc has recently been shown to be a factor controlling pluripotency and early differentiation. C-Myc was one of four genes capable of generating induced pluripotent stem cells (iPS cells) from various somatic cell types (Wernig et al., 2008; Takahashi and Yamanaka, 2006). We would expect that members of the miR-17 family upon increase in expression would quickly down regulate c-Myc and Wnt mRNAs resulting in inhibition of self-renewal (Kristensen et al., 2005). C-myc is also involved in many cancers (Soucek et al., 2008) suggesting that mis-regulation of miR-17 family of miRNAs may be involved in the etiology of c-Myc induced cancers. It is enticing to speculate that these small miRNA molecules could be used to down-regulate c-Myc in these cancers. In any event, this level of understanding miRNA function within each pathway will most likely be necessary for determining the control mechanism(s) that differentiate ES cells and/or iPS cells into desired cell types (i.e., cardiomyocytes, neurons, b-islet cells, etc.).

While studies of miRNA function in ES cell differentiation may yield interesting new ideas for stem cell therapeutics, the role of miRNAs in embryonic development is equally as important and interesting. Our in vivo mouse embryo studies suggest that miR-93 could play a role in STAT3 regulation during gastrulation. Recent studies in the zebrafish have revealed that STAT3 can regulate cell migration, polarity, and anterior–posterior axis formation during gastrulation of the zebrafish embryo (Miyagi et al., 2004; Sepich et al., 2005; Yamashita et al., 2002). Based on our in situ hybridization experiments and this current literature, we plan to further assess the function of miR-93 during formation of the primitive streak, gastrulation, and axis formation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2008.11.016.

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