REGULATORY ROLE OF MIRNAS DURING MORPHOGENESIS AND CELL SPECIFICATION IN SEA URCHIN EARLY DEVELOPMENT

by

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ABSTRACT

Development of complex multi-cellular organisms requires careful regulation at the transcriptional as well as post-transcriptional levels. Post-transcriptional gene regulation is in part mediated by a class of 21-25 nucleotides long non-coding RNAs known as microRNAs (miRNAs). While we have made great progress in understanding the gene regulatory network of the early developmental events in the purple sea urchin, *Strongylocentrotus purpuratus*, not much is known about the post-transcriptional regulation mediated by miRNAs. miRNAs are essential for sea urchin gastrulation, a developmental stage where regulation of cell movement, cell proliferation, and cell specification need to be carefully controlled to ensure a successful embryo (Song et al. 2012). The current study tests the hypothesis that miRNAs regulate genes involved in cell migration and cell specification during early development. We took a candidate approach in examining potential genes important during gastrulation that may be targets of miRNA regulation. Results indicate that both *cadherin* and *β-catenin* are translationally regulated by miRNAs. We identify miR-2007 and miRDeep 2-35240 to directly mediate the translational silencing of β-catenin. Our results suggest that while the regulatory miRNAs of *cadherin* and *β-catenin* may be different across species, the mechanism of their translational regulation by miRNAs is conserved.

Chapter 1

INTRODUCTION

1.1 Biogenesis and function of miRNAs

microRNAs (miRNAs) are encoded within the genome and the majority of miRNAs are transcribed by RNA polymerase II, but some miRNAs are transcribed by RNA polymerase III (Bartel, 2004). The first step in miRNA processing is the cleavage of the primary miRNA transcript by the RNAse III ribonuclease Drosha, giving rise to a 60-70 nt stem loop structure known as precursor miRNA (pre-miRNA) (Figure 1.1). This processed pre-miRNA is then transported from the animal nucleus into the cytoplasm by Ran-GTP and the nuclear export receptor Exportin-5. In the cytoplasm the pre-miRNA is further processed by another RNAses III endonuclease enzyme, Dicer, which recognizes the double stranded portion of the pre-miRNA. After the final processing steps mediated by Dicer, the mature miRNA is incorporated into the RNA Induced Silencing Complex (RISC) (Fig 1.1), where the mature miRNA strand is used as a guide to direct post-transcriptional regulation by the binding of miRNA 5' seed (nucleotides 2-8) and anchor nucleotides (nucleotides 13-16) to the 3'Untranslated Region (3'UTR) of target mRNAs. (Lee et al. 2002, Lund et al. 2004, Bartel 2007). The other strand, called the passenger/*strand, can either be degraded or can be functional and regulate target genes (Guo and Lu 2010, Okamura et al. 2008, Chi et al. 2009, Yang et al. 2011, Filipowicz, Bhattacharyya and Sonenberg 2008).

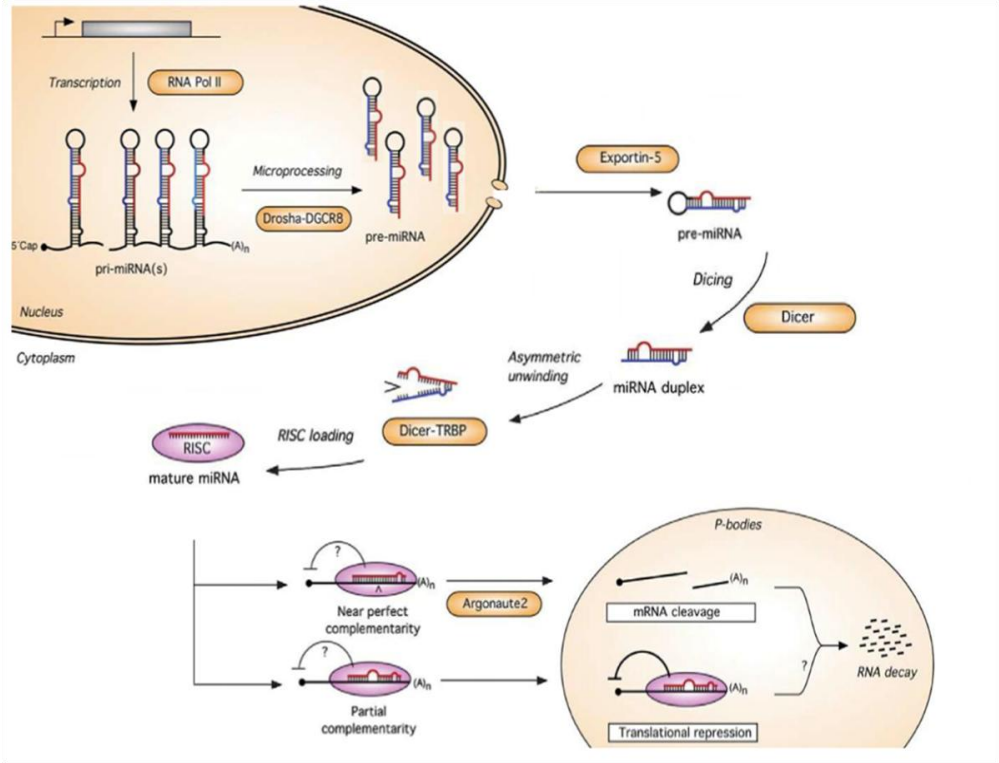


Figure 1.1: The Biogenesis of miRNAs. Most of the miRNAs are transcribed by RNA polymerase II and those primary miRNAs are sequentially processed by endonucleases Drosha and Dicer and their associated cofactors. Dicer and its cofactor TRBP will complete the processing of miRNA and incorporate the mature miRNA into the RNA Induced Silencing Complex, where target mRNAs are degraded or translationally silenced (Wienholds et al. 2005).

miRNAs can mediate transcriptional silencing through chromatin modifications such as histone methylation (Bartel 2007). The regulatory mechanism of the miRNA effect on target mRNAs depends on sequence complementarities between the target mRNA 3'UTR and its regulatory miRNA (Hutvagner and Zamore 2002, Zeng, Yi and Cullen 2003, Zeng, Wagner and Cullen 2002). Because near-perfect complementarity is required for RISC-mediated mRNA cleavage but not translational

repression, the lower degree of complementarity seen in animals suggests that translational repression is more prevalent in animals than in plants (Bartel 2007, Ambros 2004, Kim 2005). However, miRNAs can indirectly induce target mRNA degradation by directing the bound mRNA to Processing Bodies (P-bodies), where the targeted mRNAs are degraded by exonucleases (Liu et al. 2005, Selbach et al. 2008). Overexpression of a single miRNA has been shown to induce both mRNA and protein repression, although these changes are mostly mild (less than two fold) (Selbach et al. 2008). Among these proteins that undergo changes, the majority of them had changes in both mRNA and protein levels whereas some were exclusively repressed at the level of translation. The choice of down regulation at the mRNA or translational level depends on the individual mRNA-miRNA pair (Selbach et al. 2008).

1.2 Role of microRNAs during development

The regulatory role of miRNAs in early development was demonstrated by deleting Dicer, an enzyme required for the biogenesis of miRNAs, which causes either developmental defects or embryonic lethality in many animal systems (Bernstein et al. 2003, Chen et al. 2008, Mishima, Stahlhut and Giraldez 2007, Sokol 2012). Development of Dicer 1 mutant mice is arrested before the body plan is configured during gastrulation and 50% of embryos died by E7.5 (Bernstein et al. 2003). In zebrafish, Dicer null mutants had lethal effects and developmental growth arrest by 8 days post fertilization (d.p.f) and embryos died after 14-15 d.p.f (Wienholds et al. 2005). Global depletion of miRNAs with Dicer and Drosha knockdown in the sea urchin embryo resulted in early developmental defects, including gastrulation failure and embryonic lethality (Song et al. 2012). In Drosha knockdown sea urchin embryos,

genes that are involved in endomesodermal specification have increased accumulation of transcripts, suggesting that these molecules might be under miRNA regulation (Fig. 1.2) (Song et al. 2012).

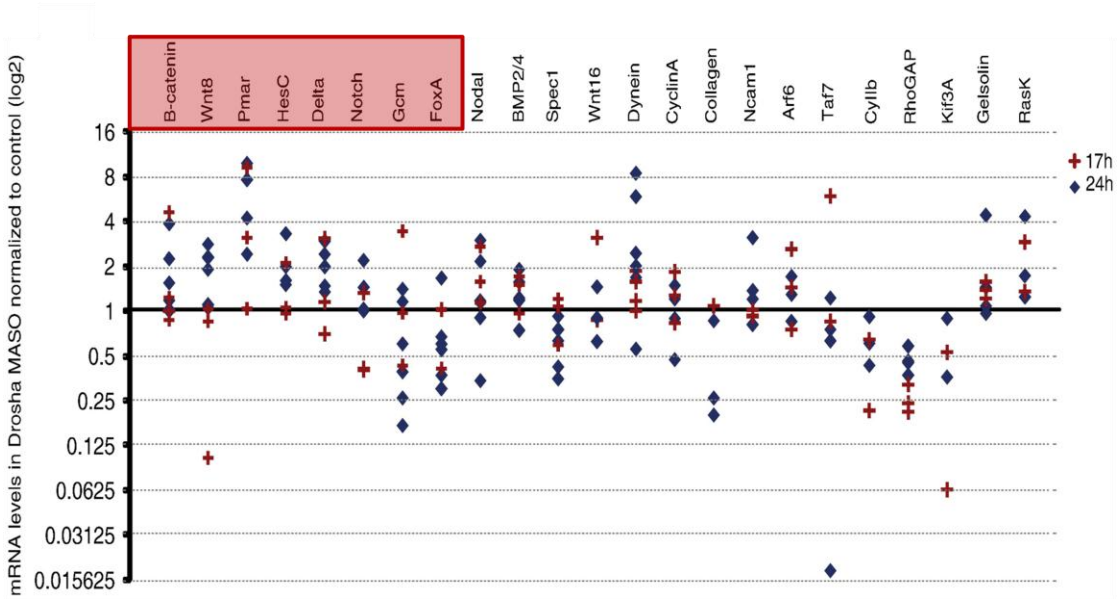


Figure 1.2: miRNAs regulate broad gene sets. mRNAs levels of genes involved in the specification of endoderm and mesoderm might be under miRNA regulation (highlighted in box) (Song et al. 2012).

Only limited specific miRNA knockout studies reveal the functions of miRNAs. Often such studies in whole animals are uninformative due to the redundancy of miRNA functions in vertebrates and/or embryonic lethality resulting from individual miRNA knockouts. For example, functional studies on the evolutionarily conserved miRNA, miR-1, found that deletions of miR-1-2 and miR-126 in mice resulted in 50% embryonic lethality (Zhao et al. 2007, Mishima et al.

2007, Divakaran and Mann 2008). Unlike vertebrates that have hundreds of miRNAs in multiple families, the sea urchin lacks multiple miRNA families, making it an attractive model to examine single miRNA function (Song et al. 2012).

1.3 Sea urchin development

Echinoderms branched from the chordate lineage, and they are the closest known relatives of the chordates (Sodergren et al. 2006) (Fig. 1.3). The echinodermata phylum includes sea stars (Asteroidea), sea urchin and sand dollars (Echinoidea), brittle stars (Ophiuroidea), sea cucumbers (Holothuroidea), and sea lilies (Crinoidea).

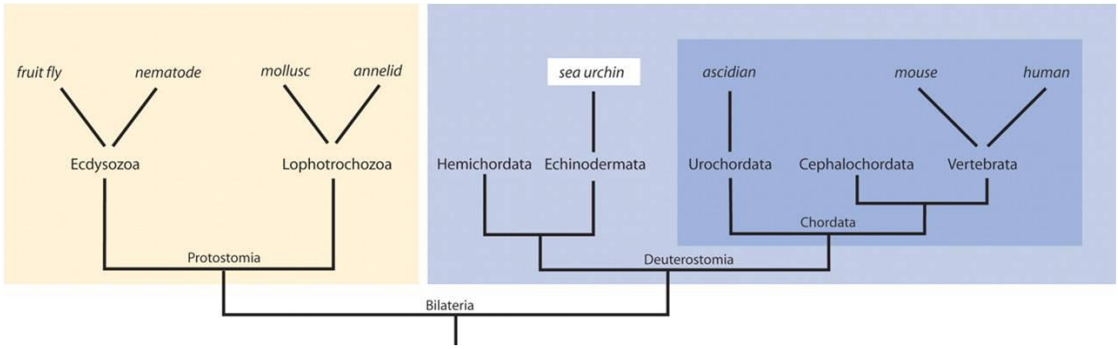


Figure 1.3: The phylogenetic position of the sea urchin relative to other model systems and humans. The chordates are shown on the darker blue background overlapping the deuterostomes as a whole on a lighter blue background (Modified from (Sodergren et al. 2006).

Echinoderms were included with the deuterostomes because of their shared character of the mouth as the second invagination during development, similar to

humans. Due to their relatively simple and rapid development and the ability to withstand experimental perturbations , the sea urchins and sea stars are used as model animals to understand early developmental processes. The fertilization of the sea urchin is external. By 24 hour post fertilization (hpf), embryos reach the blastula stage, and by 48 hpf, endomesodermal specifications and gut formation are completed in the gastrula. Sea urchin early development is rapid and embryos reach the free-swimming larval stage by 72 hpf (Fig. 1.4).

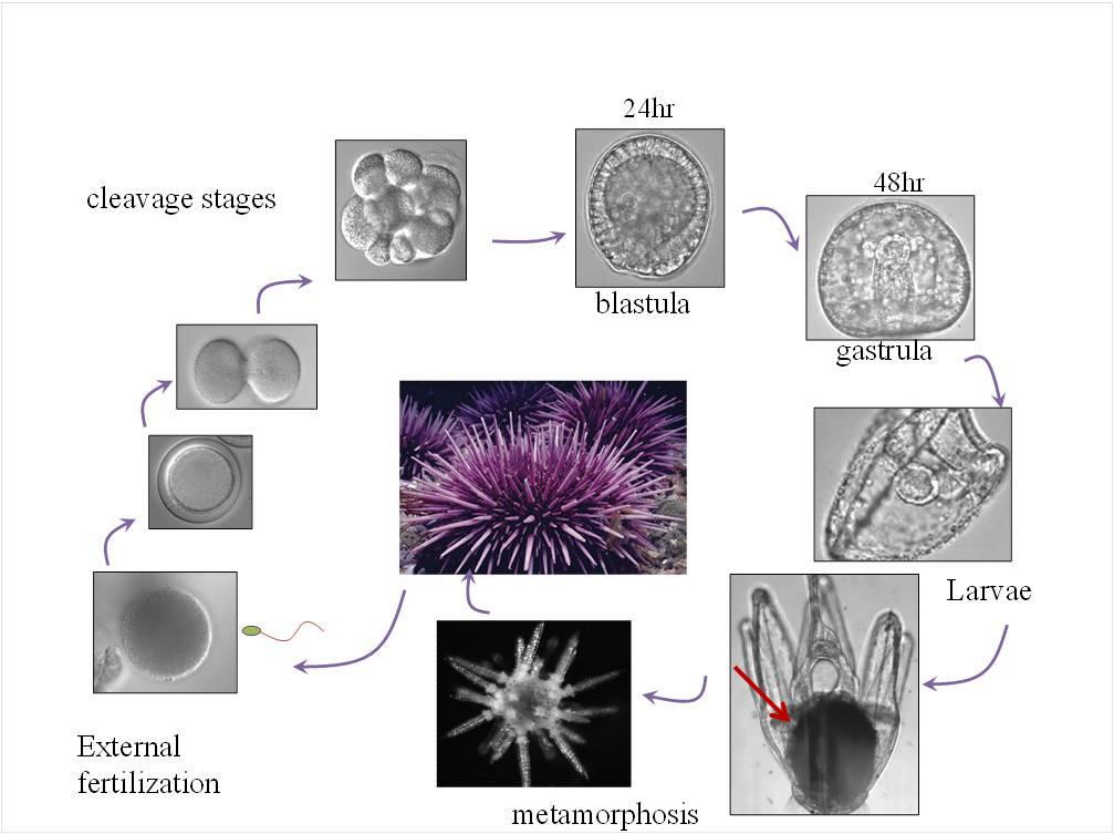


Figure 1.4: Purple sea urchin life cycle. Fertilization of the sea urchin is external. The embryo reaches the blastula stage by 24 hpf, the gastrula stage by 48 hpf, and the larval stage by 72 hpf. The left coelomic pouch, indicated by the arrow, of the larva undergoes metamorphosis to give rise to a juvenile sea urchin (Song, unpublished).

1.4 Specification and patterning in the early embryo

Cell specification, begins early in the sea urchin. Similar to the mouse, zygotic transcription starts shortly after fertilization in the sea urchin (Davidson, Cameron and Ransick 1998). Newly transcribed zygotic genes are responsible for the diverse specification activities that occur during early embryogenesis. In *Xenopus* and *Drosophila,* zygotic transcription appears later during the mid blastula transition between cleavage cycles 12 and 13 (Newport and Kirschner 1982, Tadros and Lipshitz

2009). In the sea urchin, the first unequal division occurs at the fourth division where the vegetal half of the embryo gives rise to the micromeres, macromeres and mesomeres. 5-6 hours into development, the cells in the vegetal pole are arranged in tiers, with four small micromeres fated to become coelomic pouches (contribute to the adult rudiment), four large micromeres become skeletogenic cells, and four macromeres give rise to the future endoderm (Fig. 1.5) (McClay 2011).

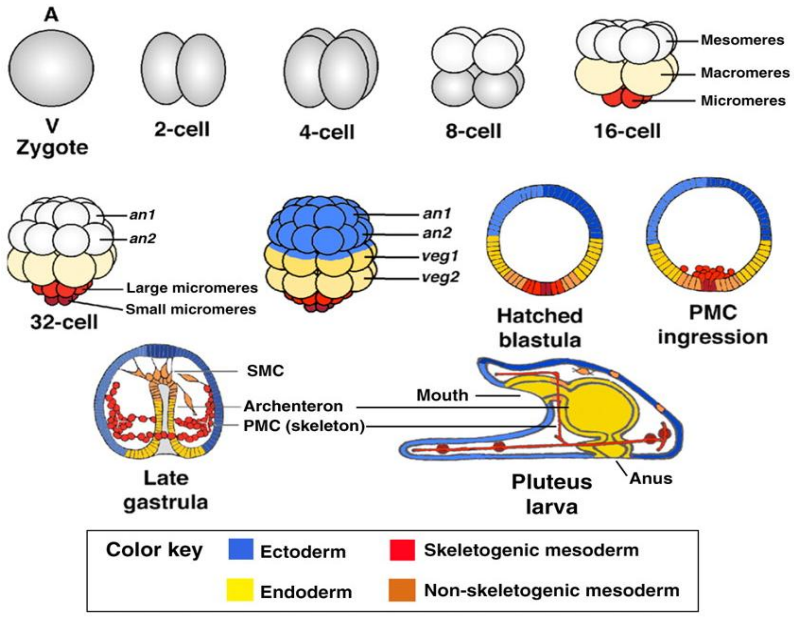


Figure 1.5: Early sea urchin development. Sequence of sea urchin development from the zygote to the pluteus larva stage. At the 16 cell stage there are four micromeres (red) at the vegetal (V) pole, four central macromeres (light yellow) and eight mesomeres (grey) at the animal (A) pole. From the hatched blastula stage onwards, the embryo is shown as a mid-sagittal section. The colors indicate when the cells begin to be specified toward ectoderm (blue), mesoderm (red) and endomesoderm (yellow). Later, the ectoderm becomes subdivided into oral and aboral ectoderm (as indicated by different shades of blue), and the mesoderm (orange) separates from endoderm (dark yellow) (McClay 2011).

At the molecular level, these tissues are specified and patterned by the coordinated actions of localized and maternally derived determinants and cell-cell signals (Davidson 1989, Oliveri and Davidson 2004). The sea urchin community has systematically tested and examined the gene regulatory network (GRN) to identify transcription factors (TFs) and signaling molecules that are involved in early development (until the larval phase) (Sodergren et al. 2006); http://SpBase.org). Early specifications are primarily carried out by transcription factors (TFs) such as, *β–catenin, paired-class micromere anti-repressor (*PMAR-1), and *hesC* (Fig. 1.6). In micromeres the accumulation of β–Catenin (that specifies endoderm and mesoderm) activates the transcription factor *pmar1*. *Pmar1* transcriptionally represses *hesC*, which is a ubiquitously expressed transcriptional repressor that normally represses transcription factors that activate skeletal forming genes. The expression of β-Catenin in the micromeres activates *pmar*, which repressed *hesC*, leading to the activation of skeletogenic genes such as *alx1, thr, ets* (Logan et al. 1999, Weitzel et al. 2004, Ettensohn 2006, Ettensohn et al. 2003b, Nishimura et al. 2004, Kurokawa et al. 1999, Fuchikami et al. 2002a, Oliveri, Tu and Davidson 2008, Oliveri, Carrick and Davidson 2002). In the rest of the embryo, the absence of β-catenin nuclear localization prevents the activation of *pmar*, leading to the activation of the ubiquitous repressor *hesC* and repression of skeletogenic fates (Fig 1.6).

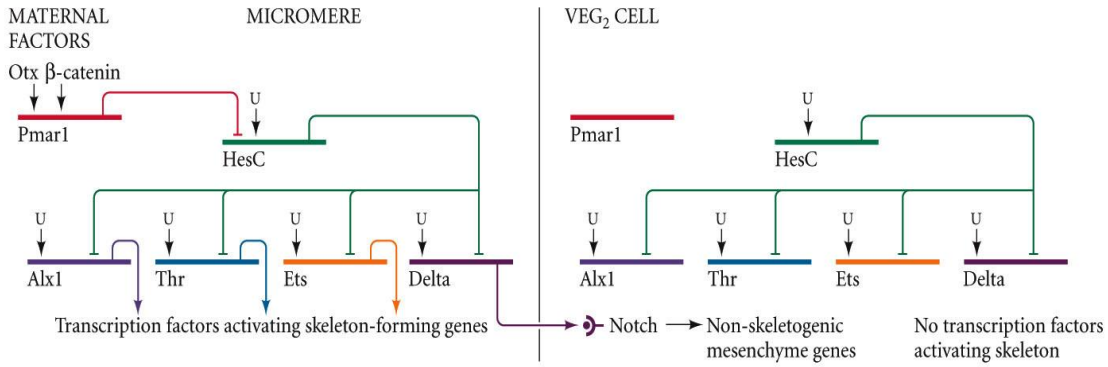


Figure 1.6: Overall current GRN for specification of the skeletogenic and non-skeletogenic micromere lineage. Activation of β-*catenin* in micromeres activates transcription factor (TF), *pmar1,* which represses the repressor *hesC*. This double repression leads to the activation of TFs that regulate skeletogenesis. At the vegetal cells (VEG2) layer, absence of nuclear localized β-*catenin* leads to the repression of *pmar1* and activation of ubiquitous repressor *hecC* and repression of skeletogenic fates (Modified from *Developmental Biology*, 9e, Figure 5.12). U=ubiquitous.

Cell-cell signaling plays a role in determining cell fates along the animal vegetal axis (Davidson 1989, Oliveri and Davidson 2004). An important molecule that is activated by β-catenin in the micromeres is the signaling molecule Delta. The endoderm and mesoderm lineages diverge under the primary control of Delta/Notch signaling pathway (Logan et al. 1999, Sherwood and McClay 2001, Ruffins and Ettensohn 1996). Delta is expressed by the micromere derivatives at the blastula stage and activates the Notch protein in neighboring cells to promote the specification of non-skeletogenic mesodermal cells such as pigment cells and blastocoelar cells at the vegetal pole region. By mesenchyme blastula stage, Delta expressed by this non-skeletogenic mesoderm activates Notch protein and promotes the mesodermal

specification (Sherwood and McClay 2001). Studies indicate that Delta signaling is also involved in the formation of foregut neurons in the sea urchin (Yaguchi et al. 2011, Wei et al. 2011). Signals derived from micromeres initiate a series of inductive interaction that position the ectoderm-endoderm boundary and regulate the diversification of cell types within mesodermal and endodermal tissues (Davidson 1989, McClay and Logan 1996).

1.5 Molecular mechanism of gastrulation

Morphogenic movements and the specification of the three germ layers are important cellular processes that occur during gastrulation. Epithelial cells undergo epithelial to mesenchymal transition (EMT) to migrate from the vegetal pole of the embryo into the blastocoel to form the primitive gut or the archenteron, giving rise to primary and secondary mesenchyme cells, or the skeletogenic and non-skeletogenic cells, respectively. The cells of the archenteron retain high levels of Cadherin, which mediates cell to cell adhesion, as the cells undergo convergent extension movements (Miller and McClay 1997b, Miller and McClay 1997a). The EMT of primary mesenchymal cells is due to the loss of membrane associated Cadherin as a result of endocytosis of the cadherin from the cell surface into intracellular compartments (Miller and McClay 1997b).

The Cadherin/Catenin-based adhesion system is the major pathway by which cells adhere to one another. Cadherin are members of calcium dependent adhesion receptors that have four extracellular repeats, a single transmembrane domain and a highly conserved intracellular domain (Takeichi 1988). Its adhesive activity depends

on the homophilic binding between two cadherin molecules and also depends on the intracellular domain interaction with the cytoplasmic proteins α-Catenin, β-Catenin, γ-Catenin/plakoglobin and p120 (Takeichi 1988, Gumbiner and McCrea 1993). Previous studies have shown that perturbations in *cadherin* function will result in the disruption of cell junctions and the loss of cell surface polarity (Behrens et al. 1985, Gumbiner and Simons 1986, Balda et al. 1993) and cell differentiation (Damsky et al. 1983).Cadherin protein was observed to be internalized in migrating PMCs in order to disassemble the adhesion junctions (Miller and McClay 1997b, Miller and McClay 1997a). In embryos treated with cordycepin, an inhibitor that blocks the disassembly of adhesion junctions in PMCs, Cadherin was detected at the adhesion junctions of PMCs, which makes PMCs unable to migrate. This suggests that changes in PMCs adhesion is due to and requires internalization of Cadherin (Miller and McClay 1997a) (Fig. 1.7).

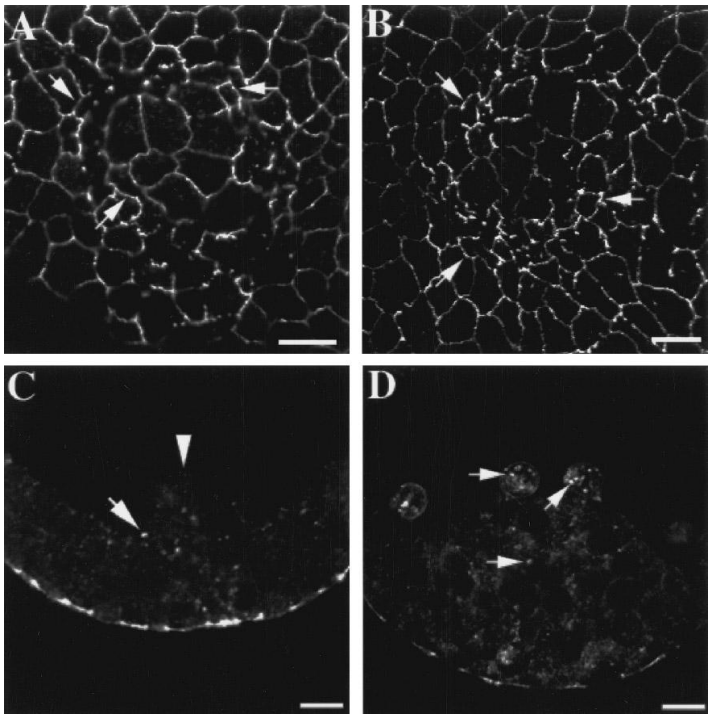


Figure 1.7: Treatment of Embryos with cordycepin blocks the cadherin from being internalized. (A,B) In embryos treated with cordycepin, PMCs retained Cadherin at adhesion junctions. (C) Intracellular Cadherin was decreased in PMCs compared to control embryos (D) (Modified from Miller et al., 1997a).

β-catenin, a central structural component of the Cadherin/Catenin adhesion complex, also acts as a transcriptional co-activator in the Wnt signaling pathway. Wnt signaling controls cell fate decisions and axial patterning during development (Willert et al. 2002). In the absence of Wnt ligands, β-catenin is recruited into a complex that contains adenomatous polyposis coli (APC) and Axin, which facilitate the phosphorylation of β-catenin by casein kinase 1 (CK1) and then glycogen synthase kinase 3 (GSK3). This leads to the ubiquitination and proteasomal degradation of β-

catenin. As a result, cells maintain low cytoplasmic and nuclear levels of β-catenin and its target genes will be kept in a repressed state. The binding of the Wnt ligand to its receptor Frizzled leads to activation of the phosphoprotein Dishevelled (Dsh or Dvl). The activation and membrane recruitment of Dsh probably recruits the destruction complex that consists of Axin, APC and GSK3β to the plasma membrane where Axin directly binds to the cytoplasmic tail of LRP5/6. Wnt initiates the destabilization of Axin by stimulating the interaction between LRP5 and Axin (Mao et al. 2001). Destabilization of Axin leads to a decreases in β-catenin degradation. The activation of Dsh also leads to the inhibition of GSK3, which further reduces the phosphorylation and degradation of β-catenin. As β-catenin level rises, it accumulates and reaches the nucleus, where it interacts with DNA-bound TCF and LEF family members to activate the transcription of target genes that influence cell proliferation, survival, as well as cell fate (Fig.1.8) (Nelson and Nusse 2004, Tolwinski and Wieschaus 2004). Dysregulation of the Wnt signaling pathway results in several diseases and syndromes. Constitutive activation of the Wnt/β-catenin pathway generally has deleterious consequences in humans such as cancer (Moon et al. 2004, Clevers 2006).

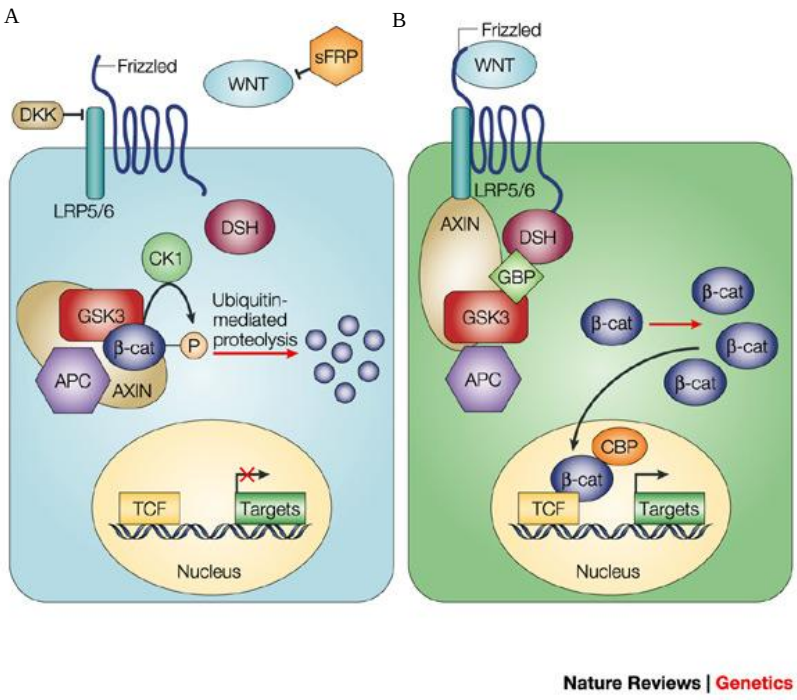


Figure 1.8: Wnt/β catenin pathway. (A) In the absence of active Wnt ligand (WNT) β-catenin is degraded, and prospective target genes are in a repressed state. (B) Upon WNT ligand binding, β-catenin degradation is reduced. β-catenin enters the nucleus and binds to T-cell factor (TCF) and lymphoid enhancer-binding protein (LEF)-family transcription factors to activate transcription of their target genes. APC, adenomatous polyposis coli; β-cat, β-catenin; CBP, CREB-binding protein; CK, casein kinase; DKK, Dickkopf; DSH, Dishevelled; GBP, GSK3-binding protein; GSK, glycogen synthase kinase; LRP, LDL-receptor-related protein; P, phosphorylation; sFRP, secreted Frizzled-related protein; TCF, T-cell factor (Moon et al., 2004).

Along with the cell-cell interactions, receptor-ligand combinations are also important during migration. During migration, the primary mesenchymal cells (PMCs) express β L and β G integrins that bind to the extracellular matrix (ECM) in the blastocoel (Marsden and Burke 1998, Marsden and Burke 1997, Ettensohn 1999). β-

Integrins bind to α integrins and form heterodimer in order to bind to ligands such as components of the extracellular matrix (Watt 2002). In *S. purpuratus*, β-Integrin has 4 subunits: β-C integrin, β-D integrin, β-G integrin, β-L Integrin. β-C and β-D integrins are expressed in blastodermal cells and β-G and β-L integrins are expressed and shown to be necessary during gastrulation (Marsden and Burke 1998, Marsden and Burke 1997, Whittaker et al. 2006). 11 α-integrins were identified in purple sea urchin (Sodergren et al. 2006). Nine out of eleven α integrins are expressed during gastrulation (Wei et al. 2006, Sodergren et al. 2006). Both migrating primary and secondary mesenchymal cells (PMCs and SMCs) are shown to extend filopodia and interact with each other to transfer pattern or lineage information to ensure their specific fates (Malinda et al. 1995, Miller et al. 1995).In purple sea urchin four different FGFR molecules have been identified, FGFR1, FGFR2, FGFRL, FGFRopL, and two different VEGFRs, VEGFR 7 and VEGFR 10 have been identified (Sodergren et al. 2006). Molecules such as Fibroblast Growth Factor A (FGFA) and Vesicular Growth factor (VEGF) expressed in the ectoderm act as chemoattractants for migrating PMCs that express receptors such as FGFRs and VEGFR (Duloquin et al. 2007, Rottinger et al. 2008).

Our laboratory has previously demonstrated that knockdowns of key enzymes in the miRNA biogenesis pathway lead to gastrulation failure and embryonic lethality (Fig 1.9) (Song et al. 2012).

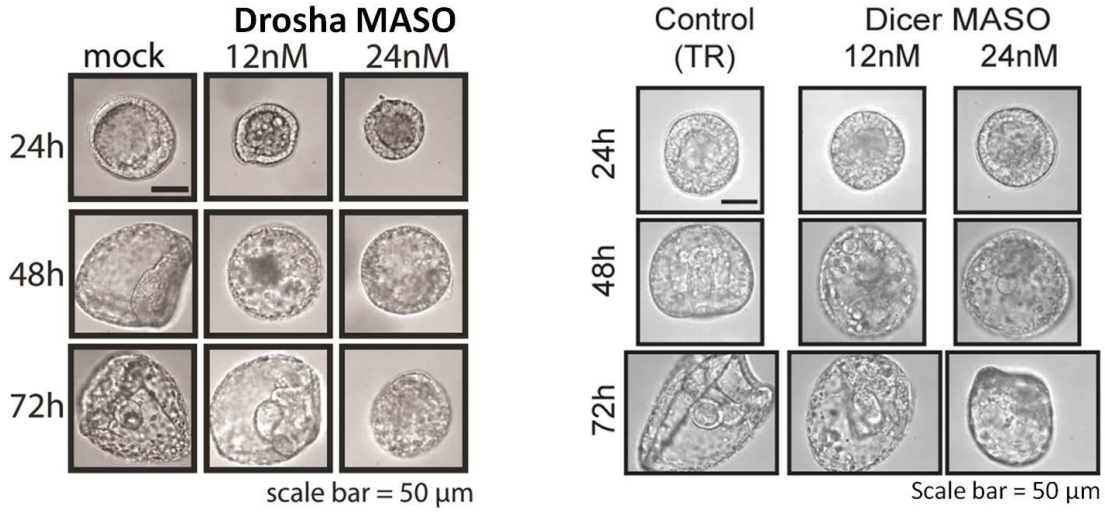


Figure 1.9: Drosha and Dicer are required for sea urchin early development. Dicer and Drosha morpholino antisense oligonuclotide (MASO) injected embryos have dose dependent developmental defects that ranged from developmental delay to embryonic lethality (Song et al. 2012).

In order to further understand the regulatory role of miRNAs on early development, we test the hypothesis that miRNAs regulate cell migration and cell specification during gastrulation. We examine the regulatory role of miRNAs on molecules that are critical for cell movement, cell proliferation, and cell specification, such as β L Integrin, FGFRs, Cadherin, and β-catenin.

Chapter 2

MATERIALS and METHODS

2.1 Animals

Adult *Strongylocentrotus purpuratus* were obtained from California (Point Loma Marine Company). Gametes were obtained by intracoelomic injection of 0.5M KCl and embryos were cultured in filtered natural sea water collected from the Indian River Inlet; University of Delaware Lewes campus) at 15°C.

2.2 Real time, quantitative PCR (qPCR)

100 embryos were collected at each of the following time points the egg, 12, 18, 24, 32, 36, 42, 48 hours post fertilization (hpf) for endogenous expression profile analysis. Drosha knockdown and Texas Red mock injected embryos were collected at 19 hpf, 24 hpf, and 30 hpf. Total RNA was collected using Qiagen microRNeasy kit according to manufacturer's instructions (Qiagen Inc., Valencia, CA). cDNA was synthesized using TaqMan Reverse Transcription Reagents kit (Applied Bio systems, Foster city, CA). qPCR was performed using two embryos equivalent for each reaction with the Applied Biosystem power SYBER Green PCR Master Mix (Invitrogen) in the 7000

Real-Time PCR cycler system (Applied Biosystems, Foster City, CA) with the following conditions: step 1, 50°C for 2 minutes, step 2, 95°C for 10 seconds, step 3, 95°C for 15sec for 40 times and step 4, 60°C for 1 minute. Results were normalized to the mRNA expression of housekeeping gene ubiquitin and showed as fold changes compared to control embryos that were injected with the Texas red (the ΔΔCt method). Three biological replicates were conducted. Statistical significance was calculated using the 2 tailed unpaired Student T-test.

Table 1: **Primer sequences used for qPCR**

Gene	**Forward primer 5'-3'**	**Reverse primer 5'-3'**
Ubiquitin	CACAGGCAAGACCATCACAC	GAGAGAGTGCGACCATCCTC
Cadherin (SPU_015210)	CAGGTCTTCGCACAAGATCA	GATAGGACCGTTGACCGAGA
β L integrin (SPU_004038)	CGGTCATCTCAGCGCCAAGCTA	AAGCGTCCCGACTGTCGCTC
β catenin (SPU_009155)	GACATCAACGTGGTGACCTG	GCTGGCTCTGTGATTTCCTC
FGFRL (SPU_020680)	GGAGTTTGGAGGCACAACAT	TCTCGATGGTTGCATTGGTA
Fgfr2 (SPU_016375)	ACGGTACATCGATGGCTCTC	ACCAGGATACGGTTGAGCAC
FGFRop L (SPU_007579)	GTGTGGACTTGGGAGAGAGG	CGTGAATGACAGACGTGTCC
FGFR1 (SPU_020677)	TCTATTGCTCCCCTGTTTGG	TTACCAAACGCACCTTCTCC

2.3 Immunolabeling

Immunolabeling with β-catenin antibody was carried out as previously described with the following modifications (Miller and McClay 1997a). Drosha knockdown embryos were fixed with 90% ice cold methanol for 1 h at -20°C followed by 5 five minute PBS (Sigma, St. Louis, MO, catalog#P3813) washes containing 0.05% Tween (PBST) . Fixed embryos were blocked with 4% sheep serum (Sigma, St. Louis, MO) for 3 hours at RT and then incubated with the sea urchin β-Catenin polyclonal antibodies (gift from Dr. David McClay, Duke University) at 1:50 in blocking buffer (4% sheep serum in PBST) overnight at 4°C. Embryos were washed 4 times with PBST and incubated with goat anti-rabbit Alexa Fluor 488 conjugated antibody at 1:3000 (Invitrogen, Carlsbald, product code A10520) in blocking buffer for 1 h at RT. The embryos were washed 4 times with PBST and stained with Hoechst nuclear stain for 5-10min followed by 4 PBST washes. Embryos were mounted using mounting medium (0.02 M Trizma hydrochloride solution at pH 8, 0.15 M of sodium chloride, 9.25 mM of p-phenylenediamine and 90% glycerol) and imaged on a LSM 780 (Carl Zeiss, Inc.; Thornwood, NY).

2.4 Western blotting

200 each of normal and Drosha knockdown embryos were pelleted by spinning the embryos at 15,000 RPM for 30 s and resuspended in 15 µl of 4X sample buffer (1 mM

Tris HCl, 0.1 mM sucrose, 4% SDS and 10 mM EDTA) and vortexed for 30 s and heated at 100°C for 10 min. Samples were stored at -80°C until later use. 1 mM DTT was added to the protein sample and heated for 10 min at 100°C immediately prior to loading samples onto Tris-Glycine 4-20% gradient gel (Bio-Rad, Hercules, CA) and ran at 120 V for 1 h in Tris-glycine electrophoresis buffer (25 mM Tris, 250 mM glycine at pH 8.3, 0.1% SDS). Proteins were transferred onto PVDF membranes (Bio-Rad,catalog #D109618). PVDF membranes were pretreated with 100% methanol for 1 min followed by 3 min incubation in transfer buffer (39 mM glycine, 48 mM Tris base at pH 7.5 and 0.037% SDS) prior to use. Transfer conditions were at 250 AMP for 2 h at 4°C. Membranes were blocked with Blotto (0.05 mM Tris at pH 7.5, 0.18 M NaCl, 3% dry milk and 0.05% Tween 20) for 2 hours and then incubated with β-Catenin antibody at1:250 (gift from Dr. Athula Wikramanyake, University of Miami) in Blotto overnight at 4°C, followed by three TBST (0.05 M Tris, 0.18 M NaCl, 0.05%Tween) washes. The blot was incubated with secondary antibody goat anti-rabbit HRP (Jackson Immuno Research, West Grove, PA) diluted to 1:3000 in Blotto for 45 min at RT. The blots were washed 3 times with TBST and signals were detected using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific, Rockford, IL) according to the manufacturer's instructions and exposed for 9 s using Kodak CareStream Imager 4000R system. Quantification of bands was done using the Alpha view–FluorChem QSA, version 3.2.2.0.

2.5 3’ Rapid Amplification of cDNA Ends (RACE)

The first round of PCR was carried out using 1 µl of Reverse Transcription reaction from cDNA generated by using 2 µg of RNA from 48hours embryos. Primers used for this reaction are gene specific Outer Primer 5’ AGTGAGTGTCCGGGAGAACA 3’ and outer 3’ RLM RACE PCR primers provided by the Ambion First choice RLM-RACE kit (Invitrogen, Carlsbald). The PCR conditions are step 1, 94°C for 3 min, step 2, 94°C for 30 s for 35 times, step 3, 60°C for 30 s, step 4, 72 for 1 min, step 6, 72°C for 7 min. 1 µl of the first PCR reaction was amplified a second round with gene specific β-Catenin inner primer 5’ TAATGCACAGGTGAGGACCA 3’ and the 3’RACE inner primer provided by the RLM-RACE kit under the same PCR reaction as described above.

2.6 Cloning of the reporter constructs

In order to test miRNA regulation on the translation of *cadherin* and *β-catenin*, their 3’UTRs were fused with the GFP coding sequence and GFP fluorescence was quantified in control and Drosha knockdown embryos. 3’UTR of the *cadherin* and *β-catenin* genes were PCR amplified and cloned downstream of an eGFP reporter construct. Primers were designed using the Primer 3 program (Rozen and Skaletsky, 2002). Primers used for *cadherin* were Cad 3’UTR For-5’-ATA TCTCGAGAGGGCTACAGATCCAGA TGC-3’and Cad 3’UTR Rev-5’-AGAGGCGGCCGCGGATCCGAATTAATACGACTCACT-3’ and for *β-catenin*, 5’ –AACGCTCGAGATCCAGATGTACCAAGCCAA-3’ and 5’-GGCACTAGTAGGAACTACAAGAAAGTCTC-3’. Restriction sites used for subcloning are underlined. 24 hpf and 48 hpf cDNA derived from total RNA were

synthesized using TaqMan Reverse Transcription Reagents kit (Applied Biosystems, Foster City) and used as template for this PCR reaction (0.2 mM of both forward and reverse primers, 1X Green Flexi buffer, 2 mM $MgCl_2$, 0.2 mM dNTP, 0.025 units of Go Taq Hot Start polymerase (Promega, Madison, WI). PCR conditions used were step 1, 95°C for 2 min, step 2, 95°C for 1 min, step 3, 60°C for 1 min, step 4, 72°C for 1 min(steps 2-4 repeated 35 times) Strep 5, 72°C for 5 min. PCR products were purified with Promega Wizard SVgel and PCR cleanup kit (Promega, catalog # A9282) prior to ligation. For cloning into TOPO TA cloning kit dual promoter (with PCR II –TOPO) (Invitrogen, Carlsbald), 1:3 vector to insert ratio was setup for ligations overnight at 14°C and transformed into DH5-α cells (Invitrogen, catalog# 18265-017). White colonies were picked for plasmid purification and potential clones were tested with EcoR1 restriction digestion followed by DNA sequencing (Genewiz Services). 3'UTR inserts from positive clones were treated with restriction enzymes and cloned into the eGFP reporter construct in the pGEMT plasmid backbone. 3'UTR inserts in PCRII vector were digested with XhoI and NotI for cadherin and XhoI and SpeI for β-catenin and gel purified (Promega Wizard SVgel and PCR cleanup kit, catalog # A9282) and ligated with pGEMT-eGFP vector. The pGEMT-eGFP vector was treated with restriction enzyme XhoI at 37°C overnight followed by purification with the PB buffer (QIAprep Spin Miniprep Kit, catalog#27106) at 5X the volume as the restriction digestion volume. Contents were transferred onto spin columns to get rid of salts and enzymes. The contents were spun at 15,000 RPM for 1 min and the flow through was discarded. 500 µl PE Buffer (From QIAprep Spin Miniprep Kit, catalog#27106) was added and spun at 15,000 RPM for 1 min and flow through was discarded. Columns were spun for additional 1 min and DNA was eluted with 30 µl of

molecular grade water. 5 µg of digested DNA with Not I at 37°C overnight. The double digested vector is then phosphatase-treated with TSAP (Promega) for 45 min at 37°C followed by TSAP inactivation at 74°C for 15 min. Products were gel purified to remove the pre-existing Vasa 3'UTR insert. A ligation was set up with 100 ng of the phosphatased vector and the 3'UTR inserts at 1:1 and 1:3 vector to insert ratio and transformed into DH5-α cells. Potential positive clones were sequenced (Genewiz Services).

2.7 *In vitro* Transcription.

GFP and mCherry reporter constructs were linearized before *in vitro* transcription (Cadherin was linearized with NotI, β catenin was linearized with SpeI and mCherry was linearized with SalI). Linearized GFP and mCherry reporter constructs were *in vitro* transcribed using mMessage machine kit (Ambion). The T7 RNA polymerase was used for generating the *cadherin* and *β-catenin* mRNAs, and the Sp6 RNA polymerase was used for generating the mCherry mRNA according to the manufacturer's instructions with the following modifications: DNAse treatment step during *in vitro* transcription reaction was carried out for 40 min at 37°C instead of 15 min. mRNAs were purified by using Qiagen microRNeasy kit according to manufacturer's instructions (Qiagen Inc., Valencia, CA) and purified mRNAs were loaded onto the Millipore spin columns to further clean the mRNAs (Millipore ULTRAFREE-MC filter units, catalog #UFC30GVOS). 2.2 µg of either *cadherin* or *β-catenin* mRNA and 1.8 µg of mCherry mRNA was lyophilized with control morpholino and 3.3 µg of either *cadherin* or *β-catenin* mRNA and 2.7 µg of mCherry mRNA was lyophilized with the Drosha morpholino.

2.8 Microinjections:

Morpholino antisense oligonucleotides (MASO) for *drosha* were ordered from GeneTools (Philomath, OR). MASO sequences for *drosha* was 5′ ACACGGTATGGCAGCCACTGGAACA 3′. Microinjections were performed as previously described (Song et al. 2012, Cheers and Ettensohn 2004) with modifications. MASO oligos were resuspended in sterile water and heated for 10 min at 60 °C prior to use. Injection solutions contain 20% sterile glycerol, 2 mg/ml 10,000 MW Texas Red lysine charged dextran (Molecular Probes, Carlsbad, CA) and 24 nM of *drosha* MASO. Eggs from *S. purpuratus* were collected and dejellied in acidic sea water (pH 5.15) for 10 min on ice, followed by two sea water washes. Dejellied eggs were rowed onto 60 × 15 mm petri dishes that were previously coated with protamine sulfate. Eggs were fertilized with sperm in the presence of 1 mM 3-amino-triazol (Sigma, St. Louis, MO). Injections were performed using the Femto Jet injection system (Eppendorf; Hamberg, Germany). Vertical needle puller PL-10 (Narishige) was used to pull the injection needles 1x90 mm glass capillaries with filaments (Narashige; Tokyo, Japan) The volume of injected solution was calculated based on the size of the injection bolus at about 1/5 of the egg diameter and determined the μmoles of injected morpholino. The final concentration of the injected morpholino was determined by dividing μmol of injected morpholino with the volume of the egg calculated with a radius of 40 μm (Song et al. 2012).

2.9 Quantitative analysis of GFP signals

Injected embryos were first treated with 2X sea water for 2 min to induce a temporary shedding of the cilia to immobilize the swimming embryos (Ettensohn C.A, 2004, *Methods in cell biology,* Volume 74, Acadamic press). They were washed with 1X sea water twice and imaged with Zeiss Observer Z1 (Carl Zeiss Incorporation, Thorwood, NY) as living embryos at low magnification (100x) to maximally capture fluorescent pixels. The imaging settings were determined with all sets of embryos to ensure that the setting captures the GFP and the mCherry fluorescence within the linear range. All embryos within an experiment were imaged under the exact same conditions. The GFP and mCherry fluorescence signals were quantified using Zeiss software (Axio vision SE 64 Rel.4.8). The GFP signal in each embryo was normalized to the mCherry signal to account for injection volume differences. Unpaired student T-test was used to determine statistical significance.

2.10 Site directed mutagenesis

The miR-2007 seed sequence was identified to have two binding sites within the *β-catenin* 3'UTR. The miR-Deep 2-35240 seed sequence was identified once within the *β-catenin* 3'UTR. All seed sequences were mutated at positions 3 and 5 within the miRNA seed sequence. miR-2007 seed sequence 5'CTGAAAT 3' was modified to 5' CTCACAT 3' and miRDeep2-35240 was modified from 5' GTGCAAT 3' to 5' GTCCTAT 3'.

Mutageneic primers were designed according to the primer design program at www.agilent.com/genomics/qcpd. Mutations in the miR-71* seed regions within the *cadherin* 3'UTR and miR-2007 (single and double mutations) and miR Deep 2-35240 seed regions within the *β- catenin* 3'UTRs were generated using the QuikChange Multi site-directed mutagenesis kit according to manufacturer's instructions (Stratagene, catelog#210518). Positive clones were sequenced (Genewiz Services).

Table 2: Primer sequences used for site directed mutagenesis

miRNA	**Sense primer 5'-3'**	**Antisense primer 5'-3'**
miR-2007 at 928bp position	caggcctcgtcaagattatatctcacatagatatctcatgattggctac	gtagccaatcatgagatatctatgtgagatataatcttgacgaggcctg
miR-2007 at 2598bp position	aatctaagctacttccatttttcatgtctcacataaactgaatacattcttttaagattgc	gcaatcttaaaagaatgtattcagtttatgtgagacatgaaaaatggaagtagcttagatt
miR Deep 2 35240 at position 2352 bp position	Gcatattgatgcctcttaaaaaggtcctataaagagtacaatatgcaacaatc	gattgttgcatattgtactctttataggacctttttaagaggcatcaatatgc
miR-71* at position 400 bp position	Aggaaatgagcccaaaaaaaaccctatactcagtcgtattaattcgg	ccgaattaatacgactgagtataggggttttttttgggctcatttcct

Underlined are the seed sequences.

Chapter 3

RESULTS

3.1 Genes involved in morphogenesis during gastrulation are expressed.

To test if these molecules are expressed and potentially regulated by miRNAs during gastrulation, we synthesized cDNAs from 100 embryos at 12, 18, 24, 30, 36 and 48 hours post fertilization (hpf). *Cadherin, β- integrin, FGFRs, VEGFRs* mRNA levels were measured by qPCR. For all these genes except *FGFR2* (1 out of 3 FGFRs) and both *VEGFRs,* transcript levels were increased gradually during blastula to gastrula stages as compared to the egg, suggesting that they are expressed at a time when they may be utilized for morphogenesis and cell specifications during gastrulation (Figs. 3.1-3.4).

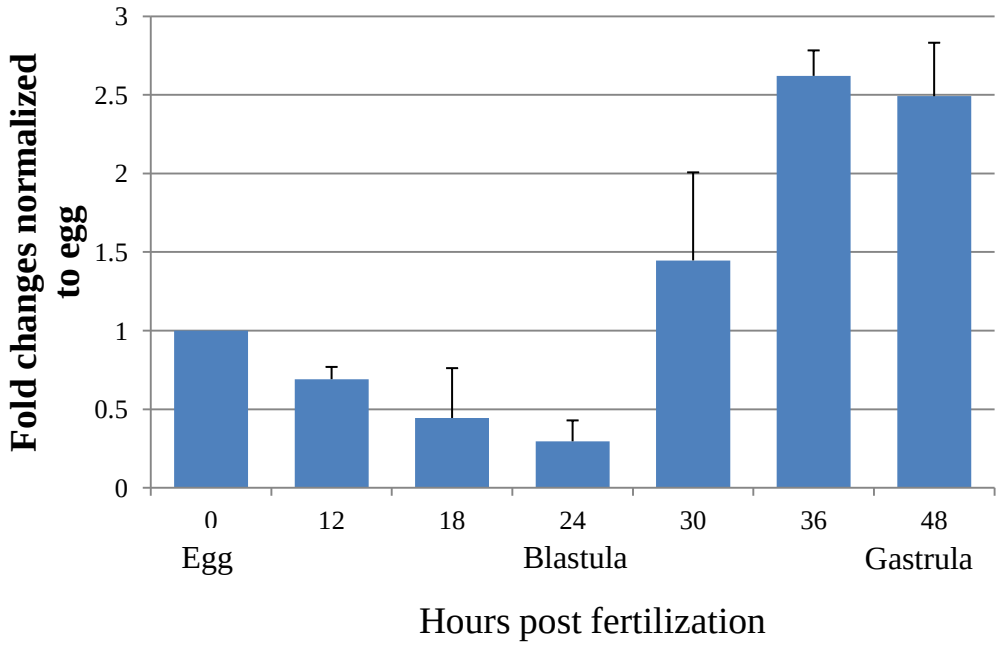


Figure 3.1: Expression of *β L Integrin* during early development. *β L Integrin* (closest homolog is *β 1 integrin* in vertebrates) expression is measured by qPCR. Its expression increased during gastrulation (24hpf to 48hpf). Error bars represent 3 technical repeats.

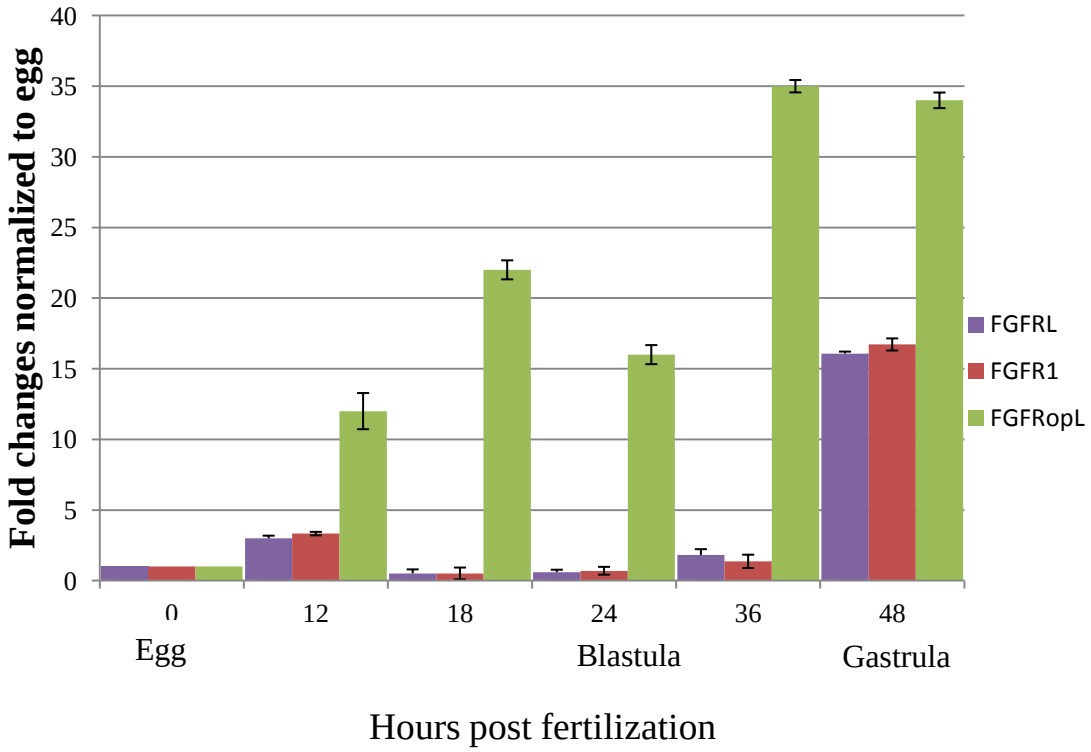


Figure 3.2: Expression of *FGFRs* during early development. Transcript levels are measured with qPCR. *FGFRL, FGFR1* and *FGFRopL* increased their mRNA levels between blastula and the gastrula stages. Error bars represent 3 technical repeats.

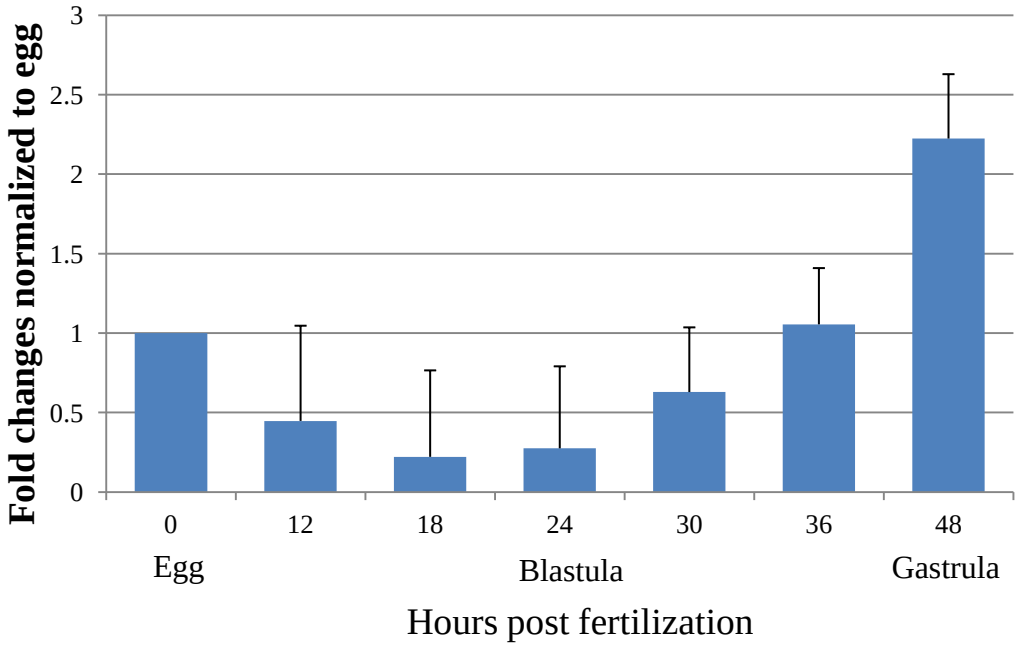


Figure 3.3: *Cadherin* expression during morphogenesis. Transcript levels were measured with qPCR. *Cadherin* mRNA increased from early blastula to gastrula stages. Error bars represents 3 technical replicates.

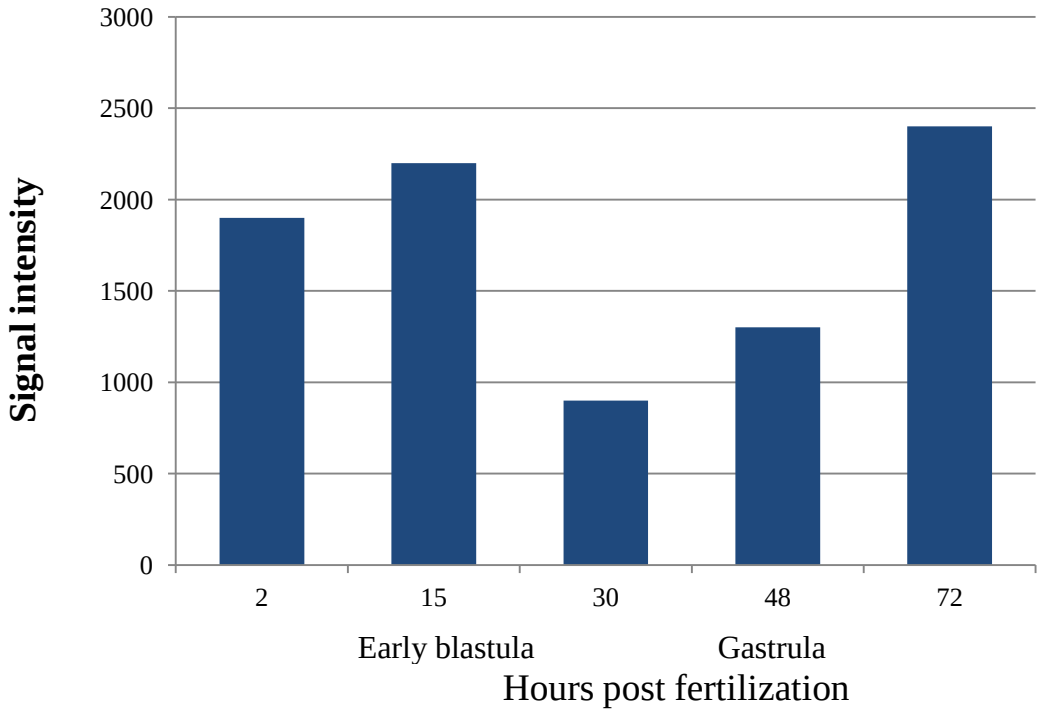


Figure 3.4: Expression of *β-catenin* during early development. Data are adapted from the microarray analysis of *β-catenin*. (http://urchin.nidcr.nih.gov/blast/exp.html and (Wei et al. 2006).

3.2 *FGFRL, cadherin*, and *β-catenin* are potentially regulated by miRNAs.

miRNAs in animal cells indirectly destabilize target transcripts as well as mediate direct translational silencing of their target genes (Selbach et al., 2008). To determine if any of the genes involved in gastrulation, we examined their transcript levels in wild type and miRNA-depleted embryos. Newly fertilized eggs were microinjected with Texas Red as a mock control set or the Drosha morpholino (MASO) to perturb the miRNA biogenesis pathway, leading to global miRNA depletion (Song et al. 2012). Embryos were collected at the early blastula (19 hpf), blastula (24 hpf), and late blastula (30hpf) stages.

For *β L integrin*, which is necessary for proper gastrulation (Burke et al., 1998), transcript levels increased in Drosha knockdown embryos compared to the control embryos, but the difference was not statistically significant (Fig 3.5).

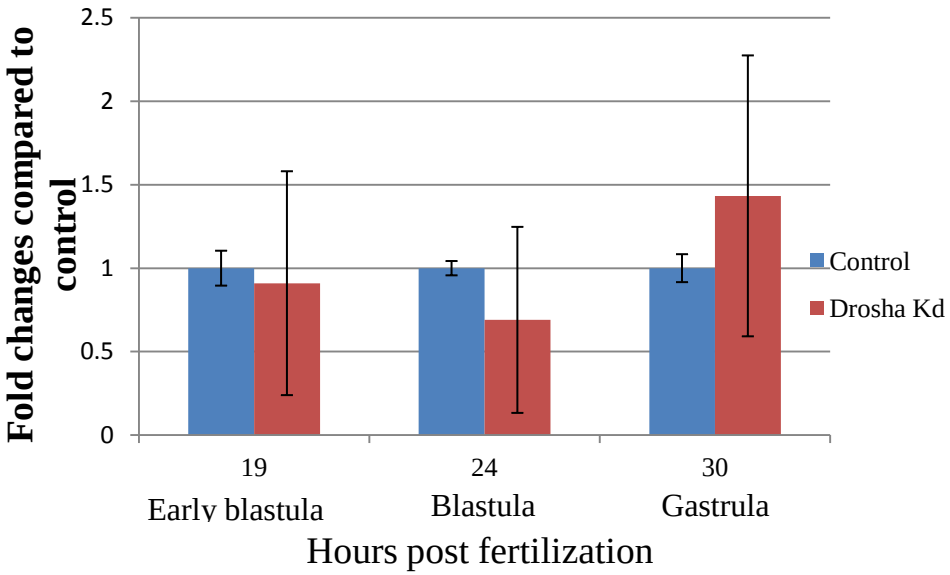
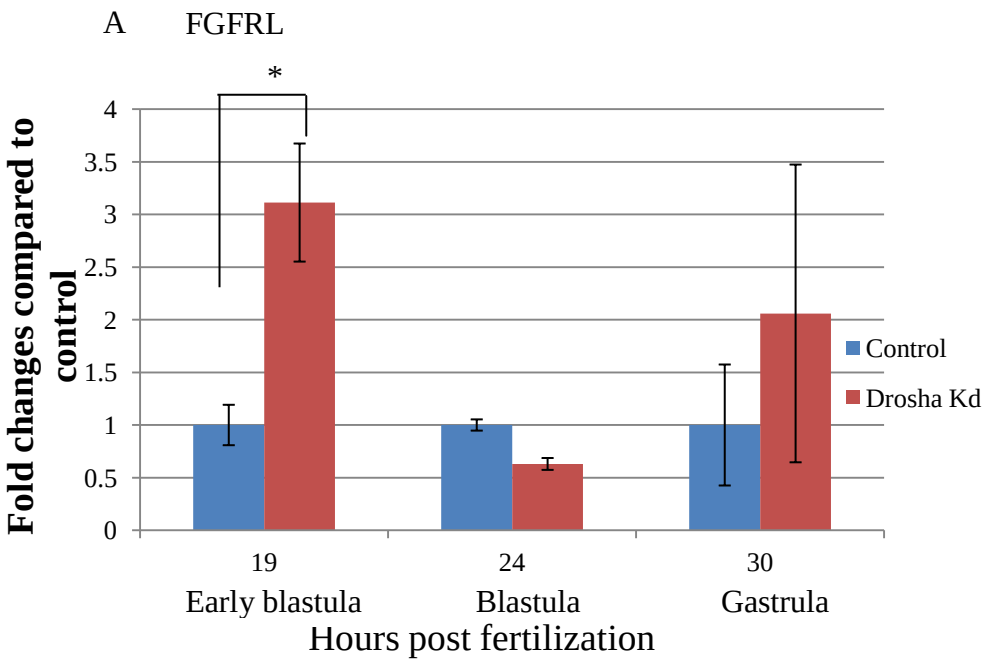
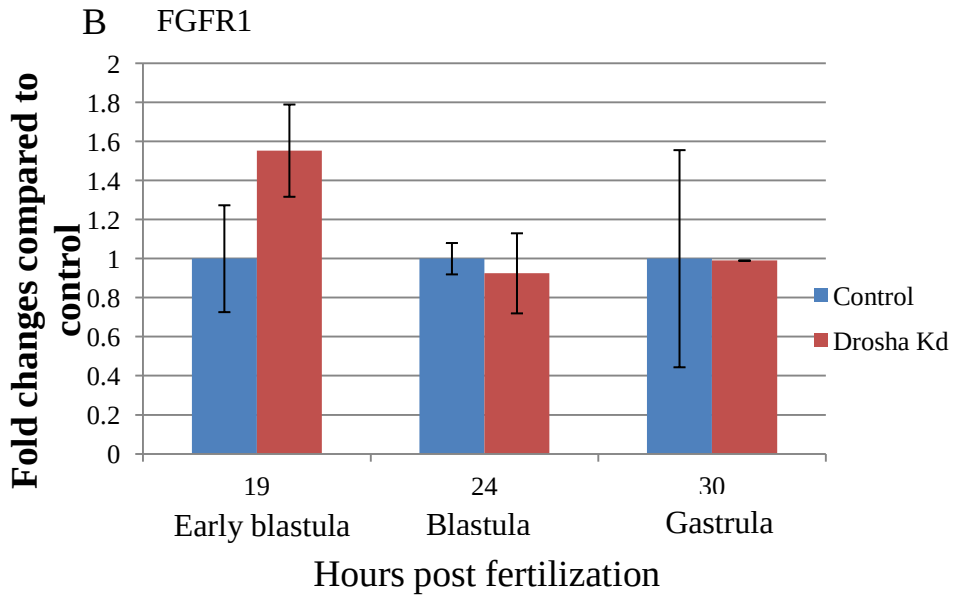


Figure 3.5: Real time, quantitative PCR of *β L Integrin* transcript levels in Drosha knockdown embryos. The transcript level of *β L integrin* was not significantly increased in Drosha knockdown (Kd) embryos compared to control embryos.

Among *FGFR*s, which are expressed by migrating primary mesenchymal cells during gastrulation (Rottinger et al., 2008), *FGFRL* transcript levels significantly increased in Drosha knockdown embryos compared to the control at 19 hpf (Fig 3.6) but not at 24 and 30 hpf.


A
FGFRL
*
Fold changes compared to control
4
3.5
3
2.5
2
1.5
1
0.5
0
19
24
30
Early blastula
Blastula
Gastrula
Hours post fertilization
Control
Drosha Kd


B
FGFR1
Fold changes compared to control
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
19
24
30
Early blastula
Blastula
Gastrula
Hours post fertilization
Control
Drosha Kd

C FGFRopL

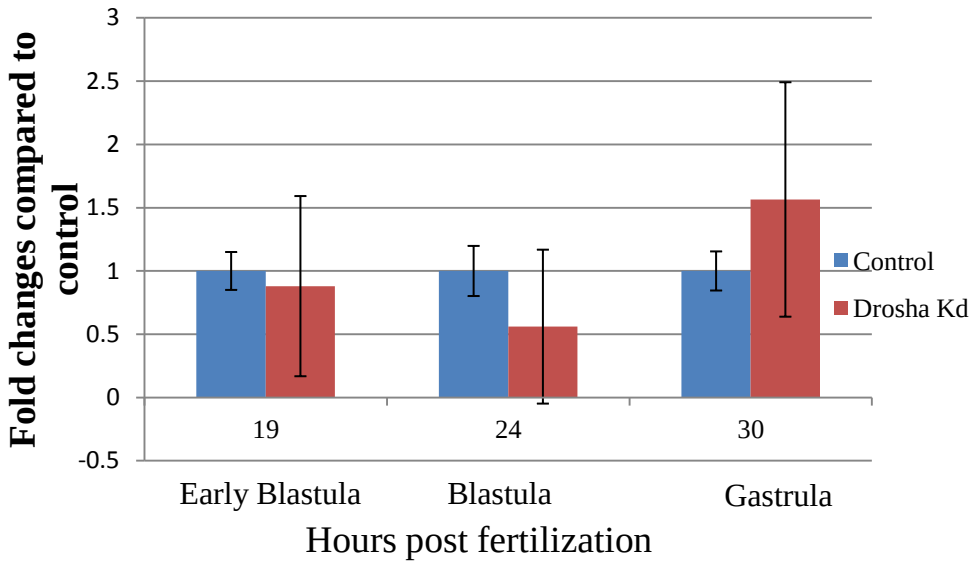


Figure 3.6: Real time, quantitative PCR of transcript levels of FGFRs in Drosha knockdown embryos. (A) The transcript level of *FGFRL* was significantly increased in Drosha knockdown (Kd) embryos compared to control embryos at 19 hpf. (B) *FGFR1* transcript levels in mock and Drosha morpholino-injected embryos are not significantly different. (C) *FGFRopL* transcript levels in mock and Drosha morpholino-injected embryos are not significantly different. * indicates statistical significance (unpaired two tailed Student T-test; p-value =0.02).

We next tested if transcripts of components of the adherens junctions are altered in a miRNA depleted background. *Cadherin* transcripts at the early blastula (19 hpf) and blastula (24 hpf) stages were increased significantly compared to control embryos, suggesting that miRNAs may potentially regulate *cadherin* (Fig. 3.7).

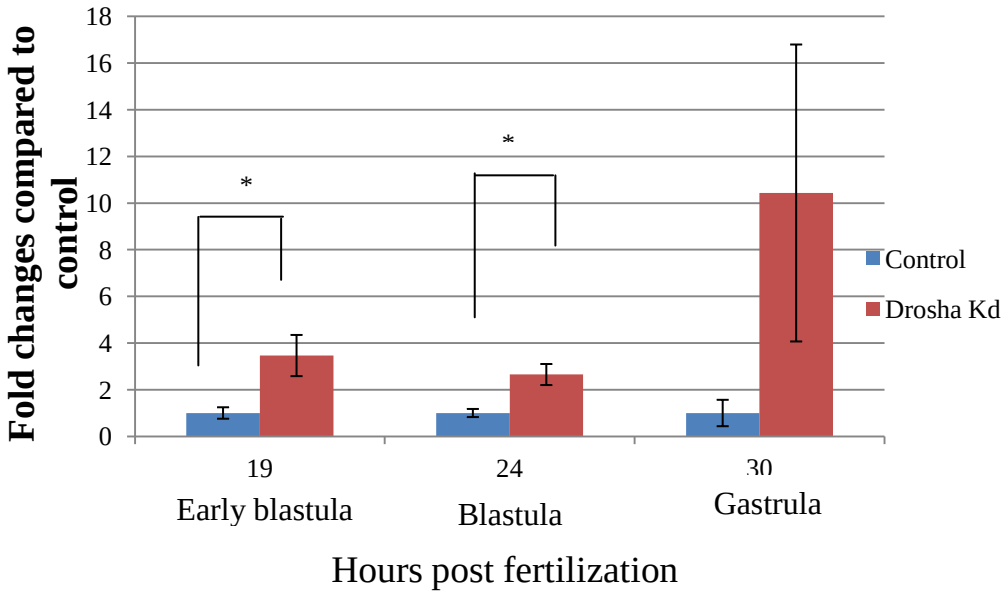


Figure 3.7: Real time, quantitative PCR of *cadherin* transcript levels in Drosha knockdown embryos. At the early blastula stage (19 hpf) and the blastula stage (24 hpf), the transcript level of *cadherin* was significantly increased (p-value=0.04 and 0.02, respectively) in Drosha knockdown (Kd) embryos compared to control embryos. However at late blastula (30hpf) the observed transcript increase was not statistically significant (p-value=0.25). * indicates statistical significance (2 tailed Student T-test).

B-catenin is a key molecule in the adhesion junction complex along with *cadherin* and is also a conserved transcription factor that regulates the specification of the endoderm and mesoderm during early development (Takeichi, 1988 ,Gumbiner and McCrea, 1993 and Willert et al., 2002). Transcript levels of *β-catenin* were

increased slightly in the Drosha knockdown embryos compared to control embryos (Fig. 3.8).

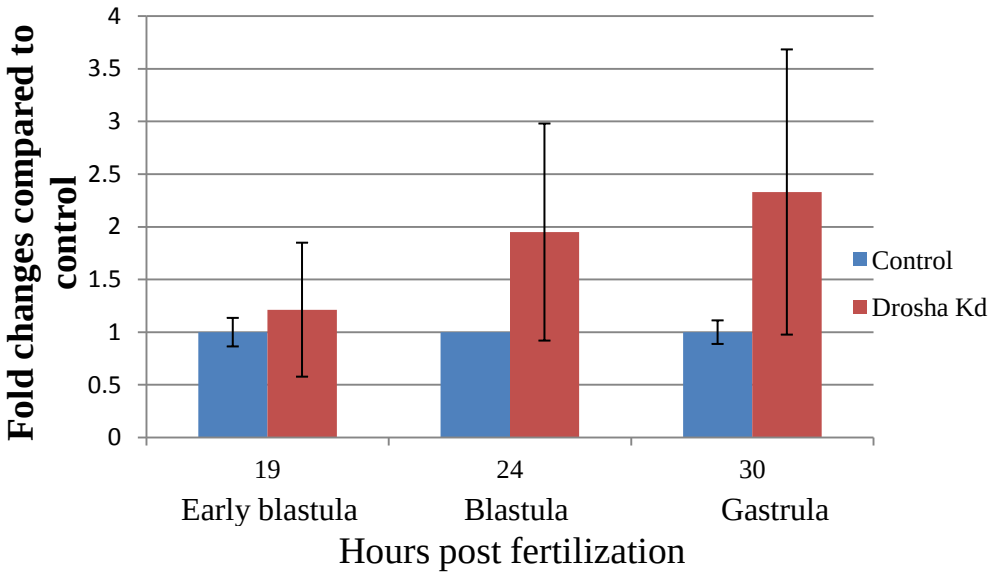


Figure 3.8: Real time, quantitative PCR of transcript levels of *β-catenin* in Drosha knockdown embryos. The transcript level of *β-catenin* was measured in both mock injected and Drosha knockdown (Kd) embryos. The transcript level of *β-catenin* was increased slightly, but not significantly in Drosha knockdown embryos compared to control embryos.

Since miRNA targets are mostly regulated at the translational level in animal cells and that *cadherin* may be a potential miRNA target, we tested if its associated *β-catenin* may be regulated by miRNAs at the translational level. Antibody against sea urchin β-Catenin was used to detect its protein levels in Drosha morpholino and control morpholino-injected embryos (Fig. 3.9). Results indicated 1.29 times more β-

Catenin protein was observed compared to control embryos, suggesting that it may be regulated by miRNAs at the post-transcriptional level (Fig. 3.9).

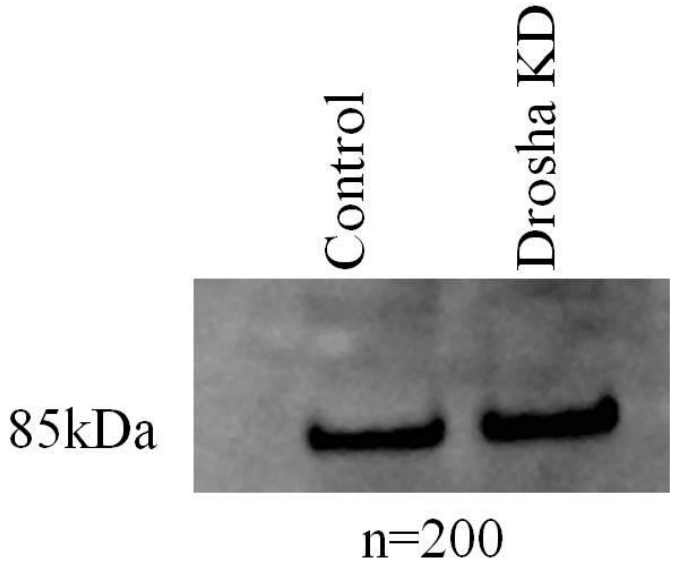


Figure 3.9: Western blot of β-Catenin in Drosha knockdown embryos. Embryos injected with control and Drosha morpholinos were collected at early blastula stage at 17 hpf and assayed for β-catenin protein expression. Compared to control embryos, Drosha knockdown (KD) embryos accumulated 1.29 times more β-catenin protein. Each lane contains 200 injected embryos and a representative blot from three biological replicates (with an average of 1.22 $\pm$ 0.042) is shown.

Since β-catenin has a dual function as a component of the adherens junction and as a transcription factor, we examined if its localization within embryos was altered by miRNA depletion. In comparison to the control embryos at the 32-cell stages, 48% (12

out of 25) of Drosha knockdown embryos lack or have minimal β-catenin present at the adherens junctions shown by immunolabeling (Fig. 3.10).

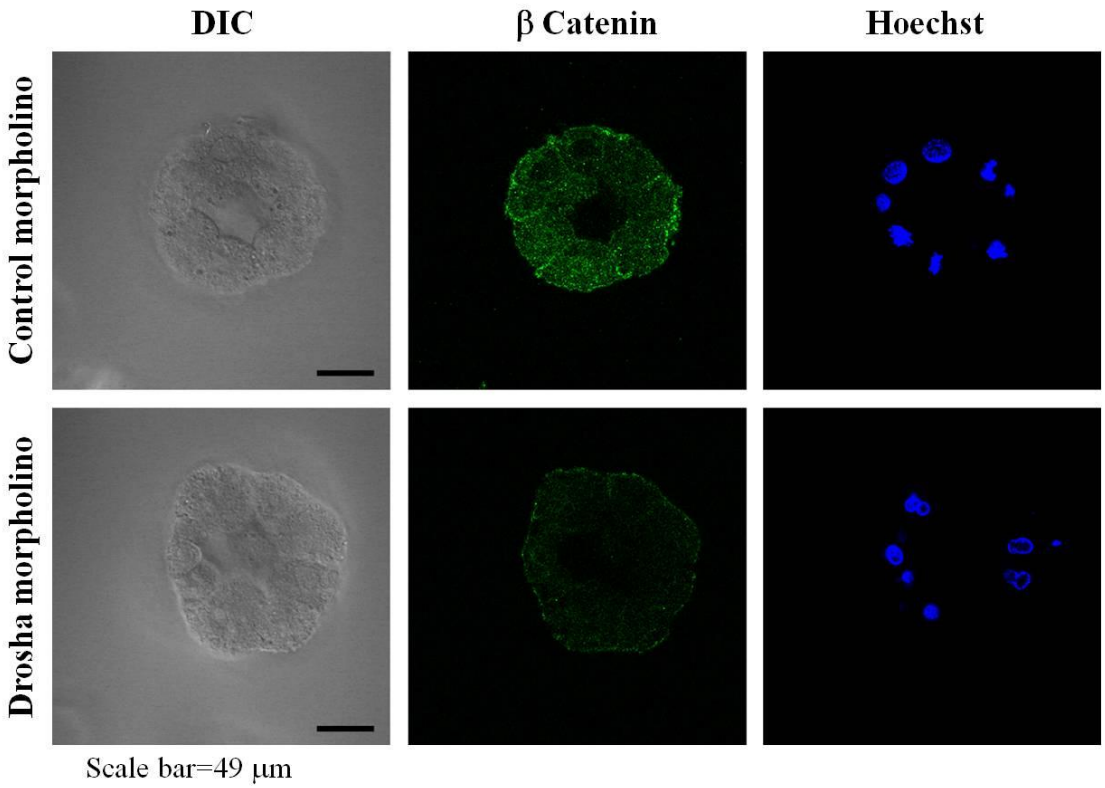


Figure 3.10: β-catenin is mislocalized in Drosha knockdown embryos. Embryos were collected at the 32-cell stage (5-6 hpf) and immunolabeled with β-Catenin shown in green. In the Drosha knockdown embryos (bottom panel), β-Catenin is decreased in abundance from the adhesion junctions compared to the control embryos (upper panel) where β-catenin is in the adhesion junctions and cytoplasm. Hoechst dye stains for nuclear DNA. Scale bar = 49 μm.

The immunolocalization result suggests that depletion of miRNAs leads to decreased level of β-catenin at the adherens junction and may potentially disrupt adherens junctions, which are important during early development to enable cell-to-cell attachment (Fig. 3.10).

3.3 Aim 2: Analysis of the direct regulation of target genes by miRNAs.

3.3.1 Cadherin 3'UTR is extended using Rapid Amplification of cDNA Ends (RACE).

miRNAs bind to target genes within their 3'UTRs and mediate translational repression or/and target mRNA degradation (Valencia-Sanchez et al. 2006). To examine the potential regulation of target genes by miRNAs, the 3'UTRs of *cadherin* and *β-catenin* were obtained. The *cadherin* 3'UTR was obtained by a PCR-based protocol, and the *β-catenin* 3'UTR was available from previous analyses (Sodergren et al. 2006, Samanta et al. 2006).

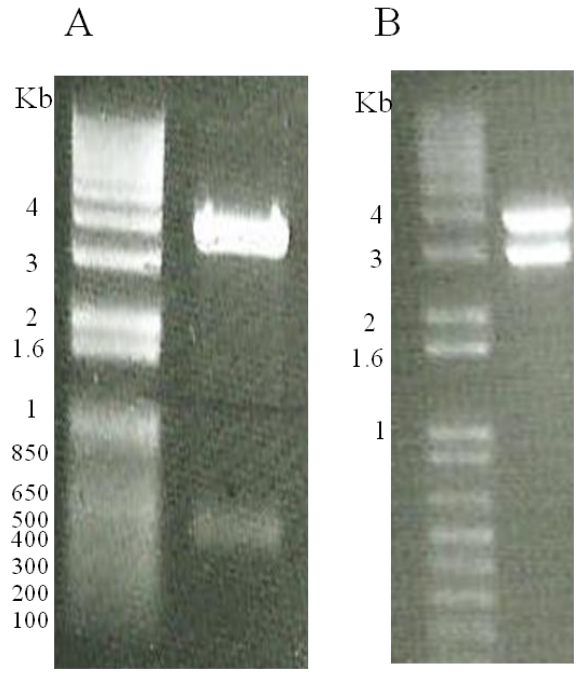


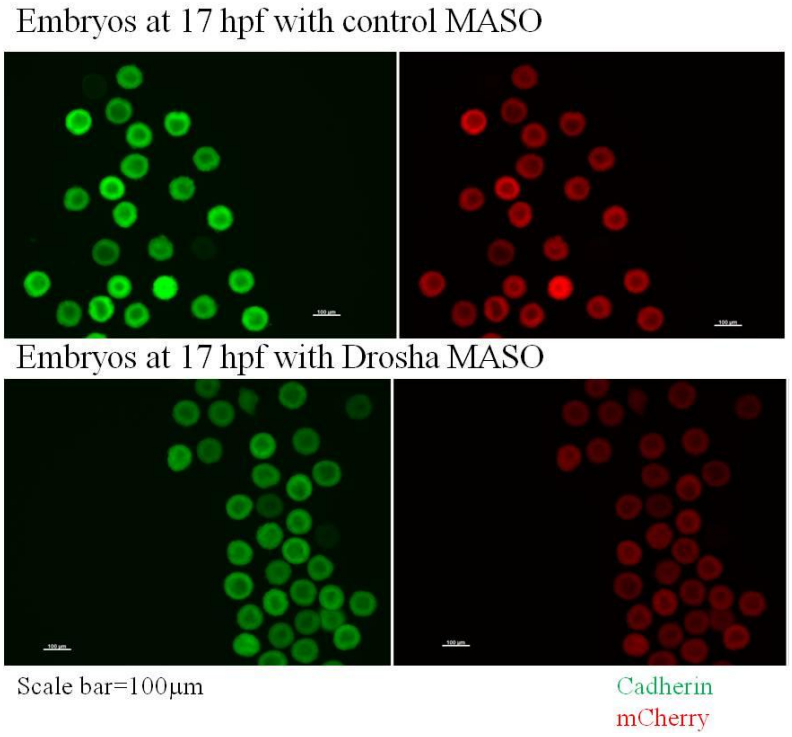
Figure 3.11: Positive clones of *cadherin* and *β-catenin* 3'UTRs in reporter constructs. 3'UTRs of *cadherin* and *β- catenin* were cloned into eGFP reporter construct in the pGEMT plasmid backbone. (A) *Cadherin* 3'UTR is 434 base pairs in length. (B) *β-catenin* 3'UTR is 3 kilobases (Kb) in length.

To examine the potential miRNA regulation of *cadherin* and *β-catenin*, we cloned their 3'UTRs downstream of the GFP reporter gene (Fig. 3.11). Newly fertilized eggs were coinjected with *in vitro* transcribed GFP reporter constructs fused with either the *cadherin* or *β -catenin* 3'UTRs, loading control mCherry reporter construct, and with or without Drosha morpholino. The mCherry reporter construct serves as a control to adjust for injection volume differences (Fig. 3.12).

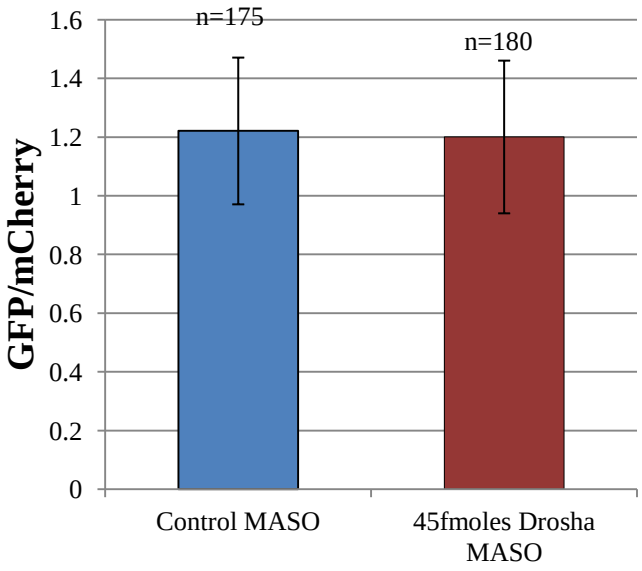
Figure 3.12: Schematic of GFP and mCherry reporter constructs. GFP reporter constructs flanked with *cadherin* or *β-catenin* 3'UTRs. mCherry flanked with *β-globin* UTRs.

If these two genes were regulated by miRNAs, the global depletion of miRNA pool in Drosha knockdown embryos would result in an accumulation of translated GFP compare to embryos without the Drosha morpholino. The fluorescent signals of GFP were normalized to the fluorescent signals of mCherry. Embryos were assayed at both early blastula (17 hpf) and blastula (24 hpf) (Fig. 3.13A, C). The *cadherin* reporter construct in embryos injected with Drosha morpholino (45 fmoles) did not show significant difference in GFP/mcherry signal at the early blastula stage (17 hpf) (Fig. 3.13B). However, these embryos had significantly higher GFP/mCherry fluorescence signal by the time they are blastula (24 hpf) in miRNA depleted embryos compared to the control morpholino-injected embryos, suggesting that cadherin is translationally regulated by miRNAs (Fig. 3.13D).

A

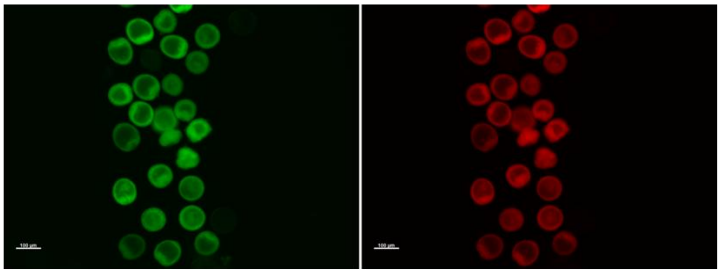


B Early blastula (17hpf)

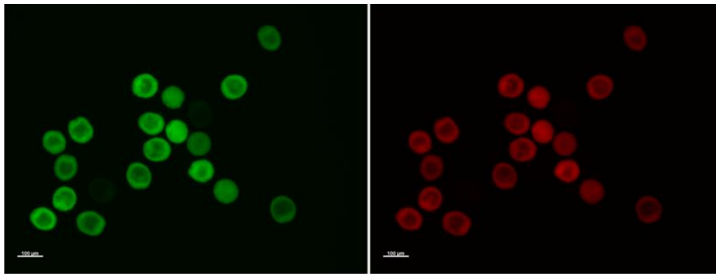


C

Embryos at 24 hpf with control MASO



Embryos at 24 hpf with Drosha MASO



Scale bar=100μm

Cadherin
mCherry

D Blastula (24hpf)

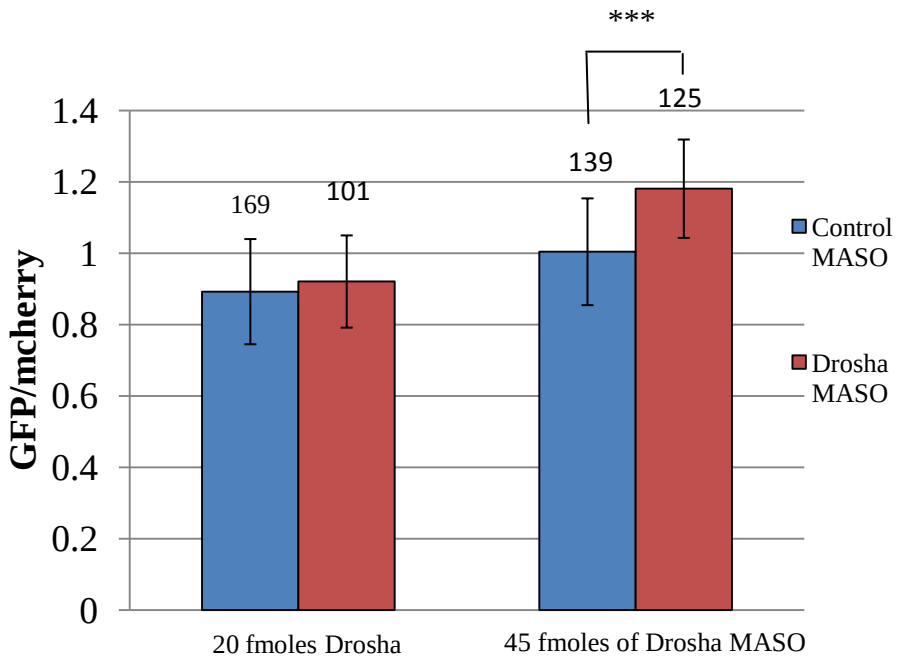


Figure 3.13: miRNAs regulate *cadherin* by translational repression. (A) GFP and mCherry expressing embryos at the early blastula stage (17 hpf). Embryos were coinjected with eGFP that was fused with *cadherin* 3'UTR and mCherry reporter constructs fused with *β-globin* 3'UTR with control morpholino or Drosha morpholino. (B) The GFP/mCherry signals did not change significantly in embryos injected with 45 fmoles of Drosha MASO compared to control MASO when assayed at 17 hpf. (2 biological replications) (C) eGFP and mCherry expressing embryos at the blastula stage (24 hpf). Embryos coinjected with eGFP that was fused with *cadherin* 3'UTR and mCherry reporter constructs that was fused to *β-globin* 3'UTR with or without Drosha MASO (D) The GFP/mCherry signals were significantly increased (unpaired two tailed student T-test; p=0.0001) when embryos were assayed at the blastula stage (24 hpf) (3 biological replications for the 20 fmoles data and 6 biological replications for the 45 fmoles data).

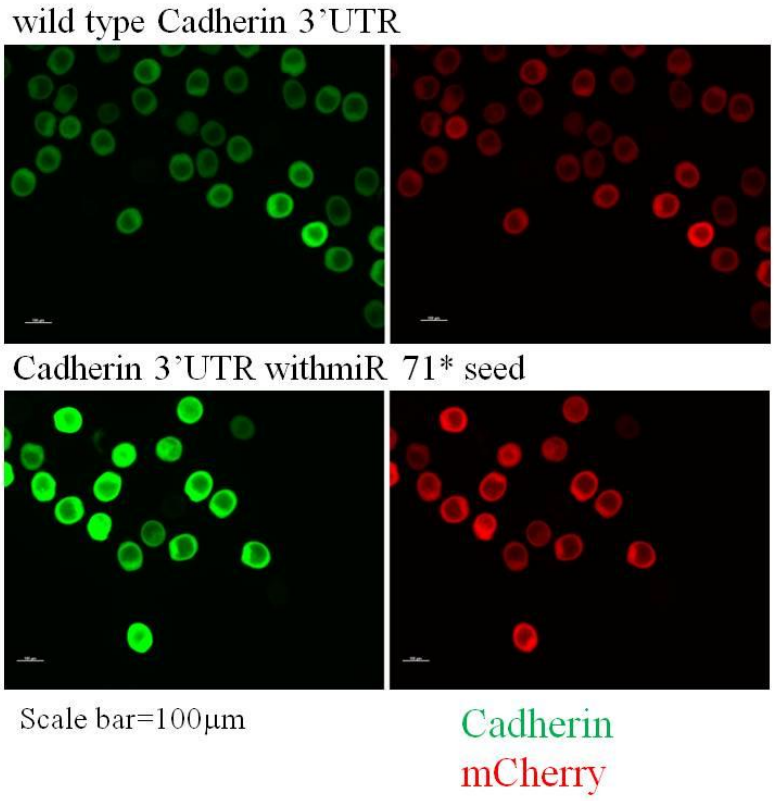
3.3.2 miRNAs directly regulate Cadherin by repressing its translation

qPCR and reporter construct results suggest that *cadherin* may be potentially regulated by miRNAs. We therefore used bioinformatics analysis to search miRNA seed sequences within the *cadherin* 3'UTR (434 bp in length) and identified one potential miR-71* binding site. Studies indicated that in many cases, the passenger strand of the miRNA is degraded in the cytoplasm (Filipowicz et al. 2008, Guo and Lu 2010, Okamura et al. 2008).

To test if the translational regulation of the *cadherin* 3'UTR is directly regulated by miR-71*, we used site-directed mutagenesis to alter the potential miRNA binding site. miR-71* has a binding sites at position +400 downstream of the stop codon) *cadherin* 3'UTR. The third and fifth base pairs of the seed sequence were mutated to disrupt the miRNA to target recognition (Gregory et al. 2008).

Newly fertilized eggs were injected with *in vitro* transcribed wild type or mutated *cadherin* 3'UTRs fused to the GFP constructs along with the loading control mCherry construct. Results indicated that the GFP construct fused with the mutated miR-71* site had significantly increased GFP fluorescence compared to the wild type *cadherin* 3'UTR, indicating that this is a functional miRNA regulatory site.

A


wild type Cadherin 3'UTR
Cadherin 3'UTR withmiR 71* seed
Scale bar=100μm
Cadherin
mCherry

B

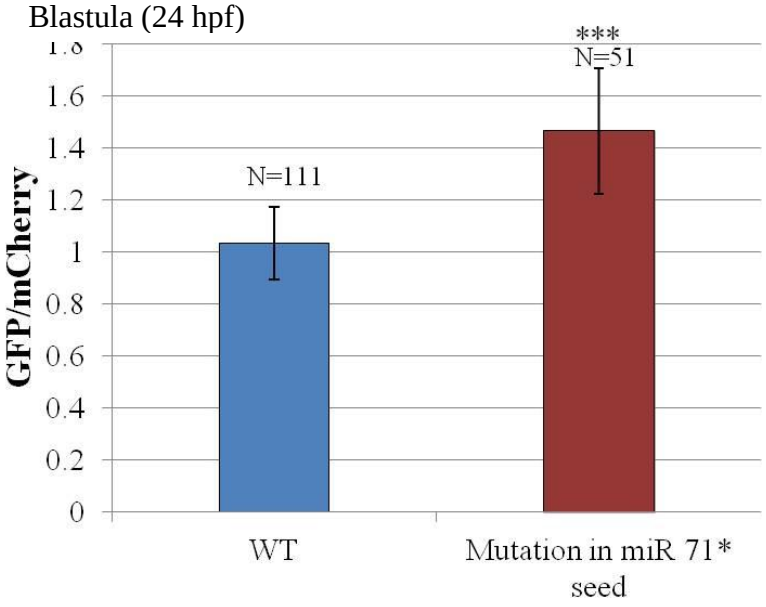
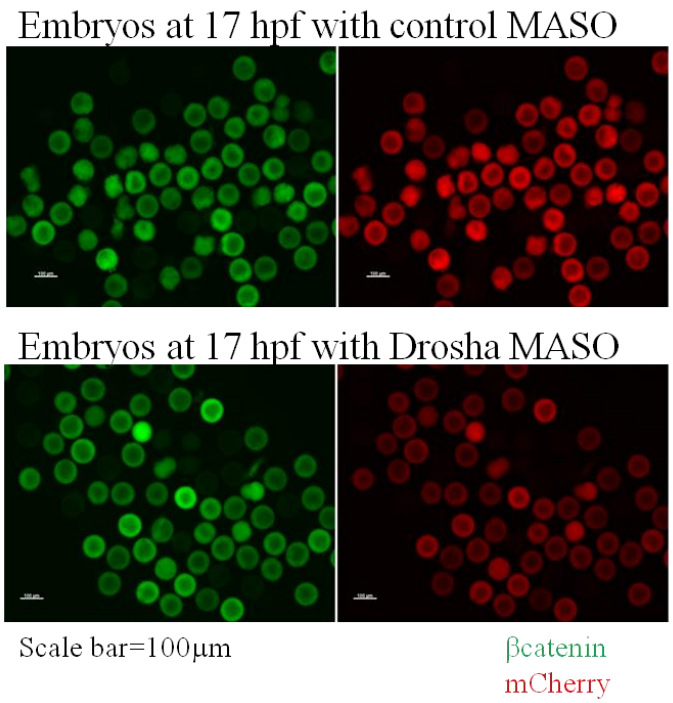

Blastula (24 hpf)
GFP/mCherry
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
N=111
***
N=51
WT
Mutation in miR 71* seed

Figure 3.14: Mutagenesis analysis of miR 71* binding site within the *Cadherin* 3'UTR. (A) Newly fertilized eggs were coinjected with either GFP fused with the wild type *Cadherin* 3'UTR or GFP fused with the mutated miR 71* site within the *Cadherin* 3'UTR at position +400 and mCherry flanked with *β-globin* UTRs. (B) The GFP construct with miR 71* mutation showed a significant increase in GFP fluorescence compared to the wildtype *Cadherin* 3'UTR at the blastula stage (24 hpf) (one biological replication) (unpaired two tailed Student T-test; p=0.0001).

For *β-catenin*, both concentrations of the Drosha morpholino resulted in a significant increase in GFP/mCherry signals in the Drosha knockdown embryos in comparison to the control MASO-injected embryos at the early blastula stage (17 hpf) (Fig. 3.15). These results suggest that both *cadherin* and *β-catenin* are regulated by miRNAs at the translational level, with *β-catenin* more sensitive than *cadherin* to the depletion of miRNAs.

A


Embryos at 17 hpf with control MASO
Embryos at 17 hpf with Drosha MASO
Scale bar=100μm
βcatenin
mCherry

B Early Blastula (17 hpf)

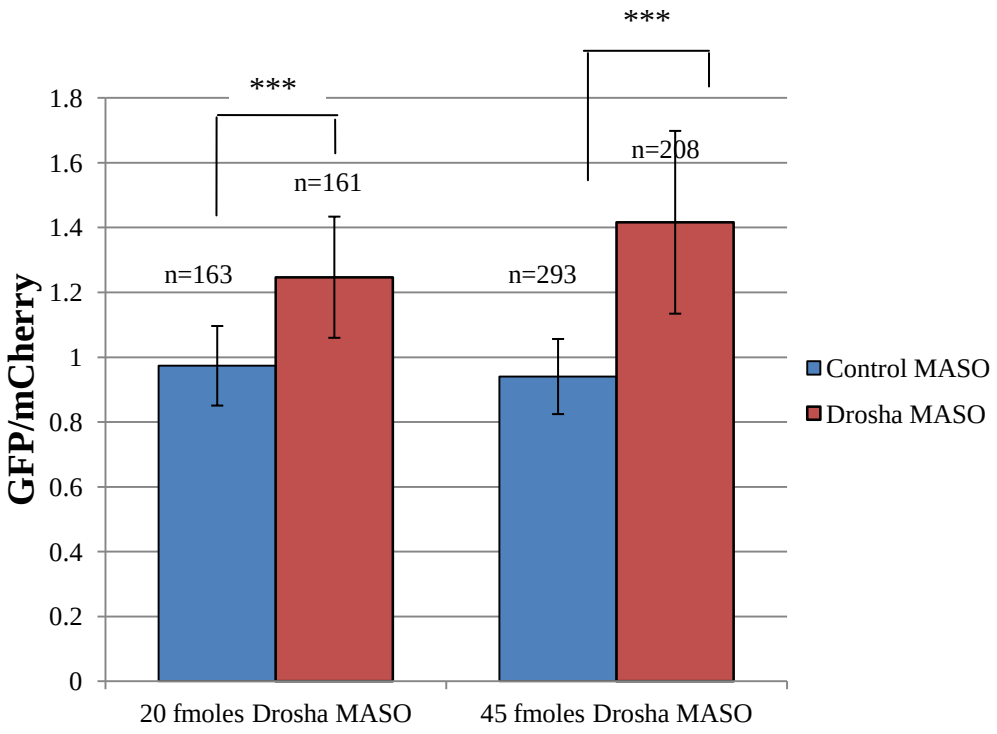


Figure 3.15: miRNAs regulate *β-catenin* by translational repression. (A) GFP and mCherry expressing embryos. Embryos were coinjected with eGFP that was fused with *β-catenin* 3'UTR and mCherry reporter constructs fused with β-globin 3'UTR with or without Drosha MASO. They were assayed at 17 hpf. (B) The GFP/mCherry signals were increased significantly (unpaired two tailed student T-test; p=0.0001) in embryos that were injected with 20 fmoles and 45 fmoles Drosha MASO compared to control embryos (4 biological replications for the 20 fmoles data and 3 biological replications for the 45 fmoles data).

3.3.3 miRNAs directly regulate β-catenin by repressing its translation

β- catenin did not have any significant difference in the amount of accumulated transcript in Drosha knockdown embryos compared to the control in the qPCR test; however, its 3'UTR (3 Kb in length) contains several potential miRNA and miRNA* binding sites (Table 1). Of the 16 miRNA predicted sites, only spu-miRDeep2-30364-35240 and spu-miR-2007 have greater than 100 sequence reads during early developmental stages of the sea urchin, and the rest are potential miRNA passenger strand binding sites (Table 1) (Song et al. 2012).

Table 3: **Deep sequence reads of miRNAs that have binding sites within the 3'UTR of *β-catenin*.**

microRNA	Seed sequence	Frequency of Seed sequences	Ovaries	Egg	32 Cell	24hours	48hours	72hours
spu-miRDeep2-30364 spu-35240	gtgcaat	1	2003	1794	591	907	1875	2390
spu-miR-2007	ctgaaat	2	2255	3441	789	1648	9380	8290
spu-miR-2007*	caacagg	1	0	1	17	43	11	72
spu-miR-252b*	tgtacag	1	0	0	33	8	3	44
spu-miR-2011*	agtgact	1	1	0	10	30	47	90
spu-miR-34*	aagctgg	1	4	0	27	31	21	83
spu-miR-2009*	caaaaac	1	1	1	51	139	50	245
spu-miR-2002*	ttggtct	1	2	0	14	28	3	47
spu-miR-124*	tgaacac	1	0	0	0	5	0	5
spu-miR-200*	cagtatt	1	147	37	40	70	59	503
spu-miR-9*	agcttta	1	2	0	13	33	11	63
spu-miR-137*	gaatacc	1	0	0	0	1	0	1
spu-let-7*	ctgtata	2	0	0	0	0	0	0
spu-miR-153*	aaaaatg	2	0	0	0	0	0	0
spu-miR-2004*	acagaaa	1	0	0	0	0	0	0
spu-miR-1*	aagtatg	2	57	5	1	51	225	104

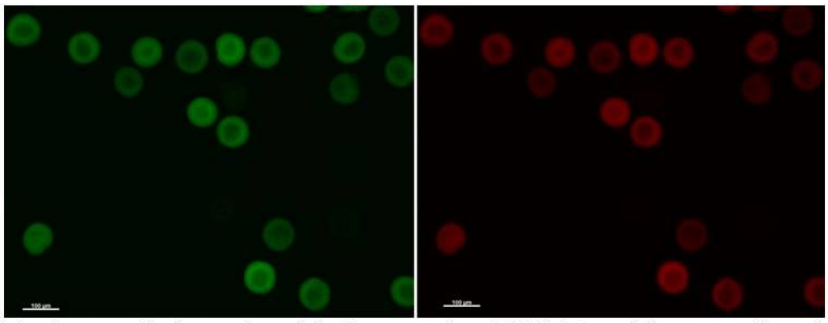
* Passenger strand. During miRNAs biogenesis, while the guide strand in the duplex will be incorporated into the RNA Induced Silencing Complex (RISC), the passenger either can be degraded or can be functional (Filipowicz et al. 2008, Guo and Lu 2010, Okamura et al. 2008).

To test if the translational regulation of the *β-catenin* 3'UTR is directly regulated by miRNAs, we used site-directed mutagenesis to alter the potential miRNA binding sites for two miRNAs. miR-2007 has two binding sites (at positions +928 and +2598 downstream of the stop codon) and miRDeep-2-35240 has one binding site (at position +2352 downstream of the stop codon) within the *β -catenin* 3'UTR. The third and fifth base pairs of the seed sequence were mutated to disrupt the miRNA to target recognition (Gregory et al. 2008). Embryos were injected as described above for miR-71* mutant construct injections.

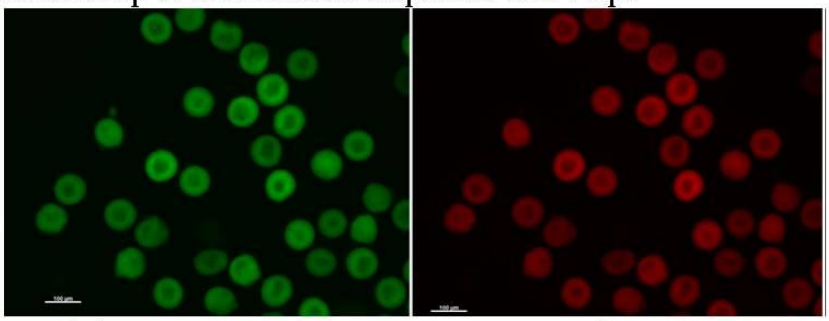
Results indicated that the GFP construct fused with the mutated miR-Deep2-35240 site had significantly increased GFP fluorescence compared to the wild type *β-catenin* 3'UTR, indicating that this is a functional miRNA regulatory site (Fig. 3.16).

A

Embryos injected with wild type β-catenin 3'UTR at 17 hpf



Embryos injected with β-catenin 3'UTR with mutation in miR Deep 2-35240 seed sequence at 17 hpf



Scale bar=100μm

β-catenin
mCherry

B Early Blastula (17 hpf)

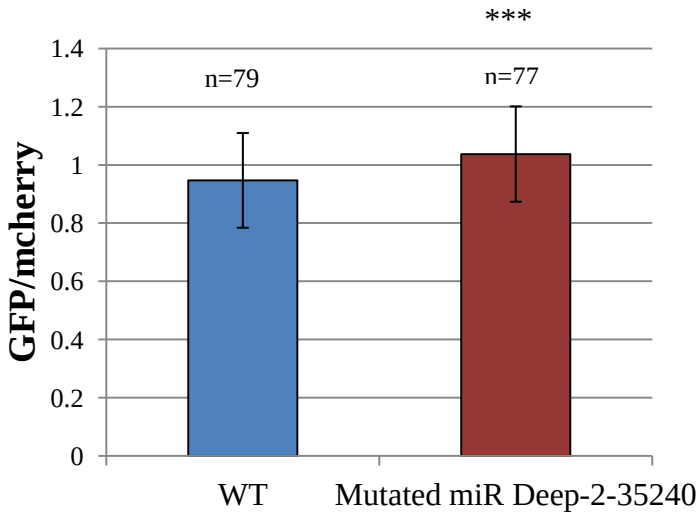
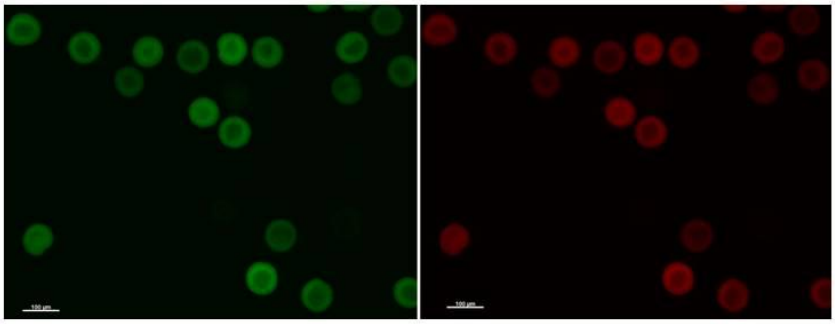


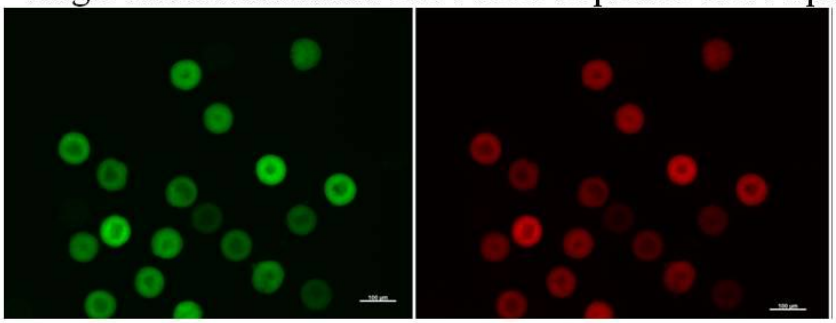
Figure 3.16: Mutagenesis analysis of miRDeep 2-35240 binding site within the *β-catenin* 3'UTR. (A) Newly fertilized eggs were coinjected with either GFP fused with the wild type *β-catenin* 3'UTR or GFP fused with the mutated miRDeep 2-35240 site within the *β-catenin* 3'UTR at position +2352 and mCherry flanked with *β-globin* UTRs. (B) The GFP construct with miRDeep2-35240 mutation showed a significant increase in GFP fluorescence compared to the wild type *β-catenin* 3'UTR at the early blastula stage (17 hpf) (2 biological replications) (unpaired two tailed Student T-test; p=0.0001).

GFP construct fused to the single (at position +928 from the stop codon) and double mutated miR-2007 sites had significantly increased GFP fluorescence compared to the GFP construct fused to the wild type *β-catenin* 3'UTR. These results indicate that miR-2007 directly regulates *β-catenin* at the tranlslational level and these miR-2007 binding sites are *bona fide* miRNA binding sites (Fig. 3.17).

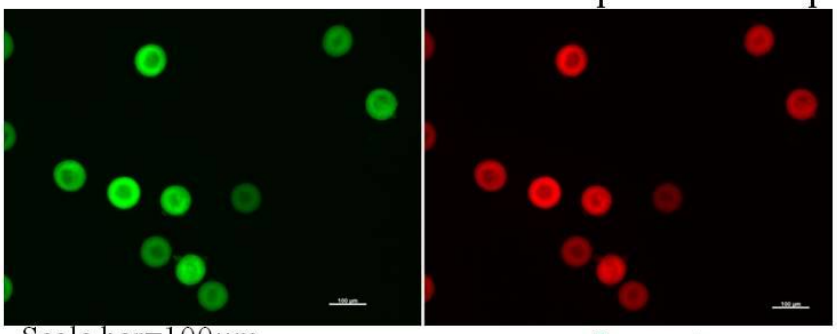
A Embryos injected with wild type β-catenin 3'UTR at 17 hpf



Embryos injected with β-catenin 3'UTR with single mutation in miR 2007 seed sequence at 17 hpf



Embryos injected with β-catenin 3'UTR with double mutation in miR 2007 seed sequence at 17 hpf



Scale bar=100μm

β-catenin
mCherry

B

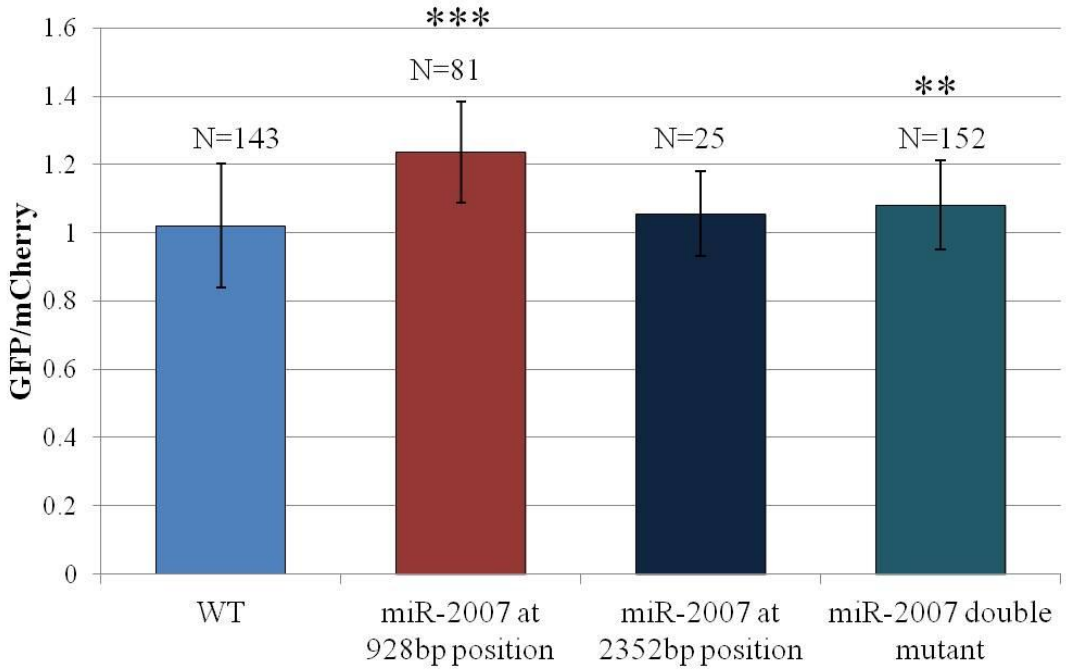


Figure 3.17: Mutagenesis analysis of miR-2007 binding site within the *β-catenin* 3'UTR. (A) Newly fertilized eggs were coinjected with either GFP fused with the wild type *β-catenin* 3'UTR or GFP fused with the *β-catenin* 3'UTR with single (at position +928) or double mutated miR2007 sites (at positions +929 and at position +2352) and the mCherry flanked with *β-globin* UTRs. (B) The GFP constructs with miR-2007 single at 928 bp and double mutations showed significantly higher GFP fluorescence level than the wild type *β-catenin* 3'UTR at the early blastula stage at 17 hpf (3 biological replications for wild type, 2 biological replications for miR-2007 single mutation at 928 bp, 1 biological replication for miR-2007 single mutation at 2352 bp and 3 biological replications for double mutants) (unpaired two tailed Student T-test; p=0.0001 for miR-2007 at 928 bp and p=0.001 for miR-2007 at 2352 bp).

Chapter 4

DISCUSSION

Our findings indicate that miRNAs may regulate cell migration and cell specification by modulating components of the adherens junction, including *cadherin* and the transcription factor *β-catenin* (Figs. 3.9, 3.13, and 3.14). qPCR analysis indicated that *cadherin* had a significantly higher level of transcripts in Drosha knockdown embryos compared to the control embryos (Fig. 3.7). However, we do not know if this increase in *cadherin* transcript level would result in an increase of the Cadherin protein. We do not have an antibody against Cadherin, hence we tested miRNA regulation of *cadherin* using eGFP reporter construct fused to the *cadherin* 3'UTR and discovered that miRNAs regulate the translation of *cadherin* (Fig. 3.13). The observed discrepancy of *cadherin* mRNA being significantly higher in Drosha knockdown at 19 hpf detected by qPCR (Fig. 3.7), but not translationally regulated by miRNAs at 17 hpf as shown by the GFP reporter data (Fig. 3.13B) may be due to assay differences where it takes time for post-translational modifications of the GFP molecule such as cyclization and oxidation and the folding of the eGFP molecules, resulting in a delay in GFP detection (Jackson, D Craggs and Huang 2006). By the blastula stage at 24 hpf, both the qPCR and GFP- *cadherin* 3'UTR studies indicated that *cadherin* is regulated by miRNAs. Our results are consistent with a previous study which identified miR-9 to regulate the human *E-cadherin*, suggesting post-transcriptional regulation by miRNAs is a conserved regulatory mechanism for *cadherin* (Ma et al. 2010).

Bioinformatics analysis identified miR-71* to have a potential binding site within the *cadherin* 3'UTR. Recent studies indicated that miRNA* strand can also be incorporated into the regulatory complexes and guide target repression (Okamura et al. 2008, Guo and Lu 2010). Factors that determine if a passenger strand is functional depend on its evolutionary conservation within the seed sequence and their abundance, as some miRNA* will degrade more slowly than others. (Guo and Lu 2010). With site directed mutagenesis we showed that miR-71* directly regulates Cadherin (Fig 3.14). The observed lack of gut formation in Drosha knockdown embryos in our previous study (Song et al. 2012) may be in part attribute to the accumulation of Cadherin that would potentially promote epithelial cell-to-cell attachment and disrupt the epithelial to mesenchymal transition that must occur for the PMCs and SMCs at the vegetal pole of the blastula embryo to migrate into the blastocoel during gastrulation.

β-catenin, as a transcription factor in the Wnt/ β-catenin signaling pathway, is regulated at transcriptional, translational, and post-translational levels (Huang et al. 2010, Zhao et al. 2011). We propose that the regulatory mechanism of β-catenin is through its post-translational modification by the GSK3β-mediated phosphorylation and/or by miRNA regulation , depending the the developmental stage(Figs. 4.1 and 4.2). This is supported by our observation that the level of β-catenin protein at the 32-cell stage is decreased in Drosha knockdown embryos, but later in the early blastula embryo, the level of β-catenin is increased in Drosha knockdown embryos (Figs. 3.9 and 3.15). The observed decreased amount of β-catenin at the 32-cell stage (5-6 hpf)

in Drosha knockdown embryos indicates that β-catenin may not only be regulated miRNAs (Fig. 3.10). We propose that in the early embryo at the 32-cell stage, miRNAs may regulate other molecules of the Wnt/β-catenin pathway such as APC/Axin. miR-135a/b and miR-315 targets APC and Axin, respectively, and suppress their expression which may result in the increase in β-catenin levels (Nagel et al. 2008, Silver et al. 2007, Huang et al. 2010). In Drosha knockdown embryos, depleted pools of miRNAs potentially result in increased APC and Axin of the destruction complex that phosphorylates β-catenin, leading to its proteosome-mediated degradation. Thus, at the 32-cell stage embryo, when β-catenin accumulates to activate the specification of endoderm and mesoderm, β-catenin is mainly regulated by post-transcriptional modification by GSK3β.

Based on previous deep sequencing results, the two miRNAs that we found to directly regulate β-catenin are zygotically activated between the 32-cell stage and the early blastula stage (Table 1) (Song et al. 2012), presumably at a time when they are needed for β-catenin regulation.

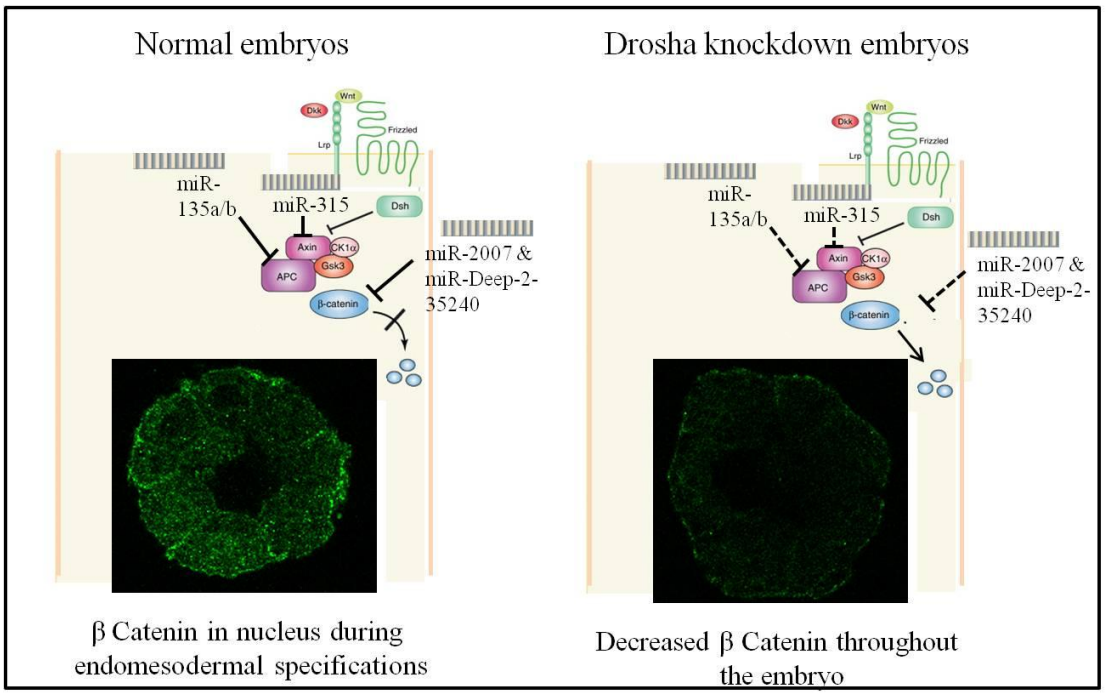


Figure 4.1: Proposed model of the regulatory mechanism of β-catenin at cleavage stages. At 32-cell stage (5-6 hpf), β-catenin is proposed to be mainly regulated by post-translational modifications. miRNAs, such as miR-315 and miR-135a/b, regulate components of the destruction complex, APC and Axin (Nagel et al. 2008, Silver et al. 2007, Huang et al. 2010). As a result, the destruction complex may not phosphorylate β-catenin for degradation, leading to β-catenin accumulation. In Drosha knockdown embryos, global depletion of miRNA pools results in reduced levels of miRNAs that normally inhibit the translation of APC and Axin, leading to accumulation of the destruction complex, which phosphorylates β-catenin and direct it to proteosome-mediated degradation pathway.

At the early blastula stage (17 hpf), a small but consistent level of β-catenin protein was increased in Drosha knockdown embryos compared to the control, using

both Western blotting and eGFP reporter construct containing the *β-catenin* 3'UTR (Figs. 3.9 and 3.14). One possible explanation for this may be that during early blastula stage, the dominant mechanism that regulates β-catenin is by miRNAs. Our results indicate that both miR-2007 and miR Deep 2-35240 directly regulate the translation of β-catenin. The observed increased β-catenin protein in Drosha knockdown embryos at early blastula supports this model (Fig 4.2).

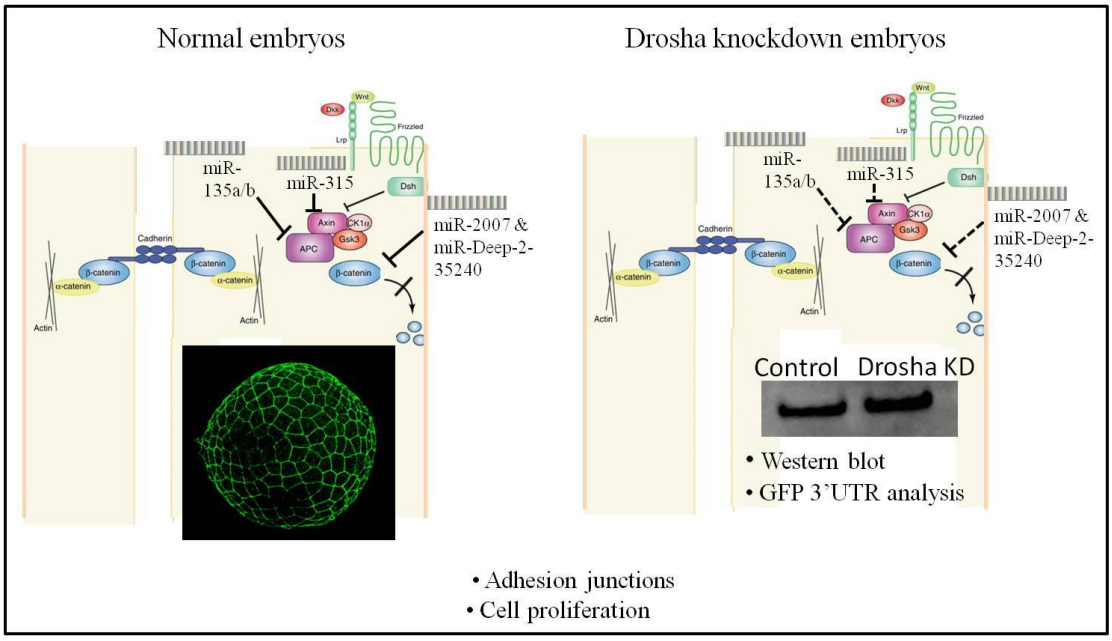


Figure 4.2: Proposed model of the regulatory mechanism of β-catenin at the early blastula stage. β-catenin is regulated by miRNAs during early blastula stage. miR-2007 and miR Deep 2-35240 directly bind to the 3'UTR of β-catenin and repress its translation . In Drosha knockdown background, depletion of these two miRNAs results in increase of β-catenin protein levels.

The observed 30% of the β-cateinin protein level may be explained by the Drosha knockdown background, where there will likely be residual miRNAs present

in the embryo. For example, in a Dicer knockdown background, the level of miRNAs decreases up to 40% compared to the wild type (Song et al. 2012). In addition, the endogenous function of miRNAs is to fine tune the level of translated proteins, resulting in protein level changes that are often less than a 2-fold (Selbach et al. 2008). Therefore, the actual level of β-catenin protein resulting from miRNA depletion is likely to be underestimated. Importantly, miRNA depletion with the Drosha morpholino already resulted in decreased level of β-catenin at the 32-cell stage, which is 5-6 hours into development of the embryo, indicating that Drosha knockdown is already in effect. At the early blastula stage (17 hpf), miR-2007 and miRDeep 2-35240 play a more significant role where they regulate the translation of β -catenin.

The entire Wnt/β-catenin pathway has been found to be regulated by miRNAs such as miR-200a, miR-155 miR-1826 and miR-25. (Huang et al. 2010, Burk et al. 2008, Gregory et al. 2008, Park et al. 2008, Zhang et al. 2012, Anton et al. 2011, Hirata et al. 2012). miR-1826 acts as a tumor suppressor by down regulating *β-catenin* (Hirata et al. 2012), whereas miR-25 has been shown to regulate *β-catenin* directly by binding to the coding sequence and not its 3′-UTR (Anton et al. 2011). We found *β-catenin* to be directly regulated by miR-2007 and miRDeep-2-35240 at the translational level in the early blastula stage.

However we observed a significant decrease in both miR-2007 at 2352 bp position and miR-2007 double mutant compared to the wild type *β-catenin* construct. The mutation at position 2352 may have potentially created a novel binding site for a RNA binding protein that negatively regulates the translation of *β-catenin.*However this needs to be further tested. Our overall results suggest that the exact regulatory

miRNAs of *β-catenin* may not be conserved across species, but that the mechanism of its translational regulation by miRNAs is conserved.

Regulation of the expression of components of the Wnt/β-catenin pathway by miRNAs revealed another layer of regulatory complexity of β-catenin.

β-catenin is an essential and conserved transcription factor that regulates specification of the endoderm and mesoderm in metazoans (Ettensohn 2006). In the sea urchin embryo, β-catenin regulates endomesodermal genes such as *pmar1, hecC, delta, alx1, ets, tbr* (Logan et al. 1999, Weitzel et al. 2004, Ettensohn 2006, Ettensohn et al. 2003a, Nishimura et al. 2004, Kurokawa et al. 1999, Fuchikami et al. 2002b, Oliveri et al. 2008, Oliveri et al. 2002). Some of these genes, *pmar1, hecC,* and *delta* have increased mRNA accumulation in Drosha knockdown embryos as previously shown, suggesting that the developmental pathway for endomesodermal specification may be under miRNA regulation (Song et al. 2012). Here we demonstrated that β-catenin is directly regulated by miR-2007 and miRDeep-2-35240 (Figs. 3.16 and 3.17). Whether other genes in the endomesodermal specification are regulated by miRNAs will need to be examined.

This study contributes to an improved understanding of the basic biology of β-catenin signaling pathway that is critical for proper specification of the endoderm and mesoderm in early development.

Chapter 5

FUTURE DIRECTIONS

5.1 Test the direct regulation of FGFRL with miRNAs

Studies from another sea urchin species, *Paracentrotus lividius,* indicated the important role of FGFRs in primary mesenchymal cell migration and in proper gut and skeletal formation (Rottinger et al., 2008). In purple sea urchin, *S. purpuratus*, one of the FGFR genes, FGFRL, had significant increase in transcript accumulation in Drosha knockdown embryos in the early blastula stage at 19 hpf, suggesting miRNAs might be regulating this gene. Bioinformatics analysis identified (Table 2) 16 miRNAs that has binding sites within 3'UTR. Among these miRNAs, miR-183, miR-2002, miR-2001, miR-2008, miR-2012, miR-7, miR-96 have a high numbers of deep sequencing reads (Song et al. 2012). With eGFP reporter constructs and mutagenesis studies, we can identify how many of those miRNAs regulate FGFRL directly.

Table 4: **Deep sequencing reads of miRNAs that have potential binding sites in the FGFRL 3'UTR**

microRNA	Frequency of Seed sequences	Ovaries	Egg	32 Cell	24hours	48hours	72hours
SPU-miR 183	1	12	115	22	228	576	373

SPU-miR-2002	3	567	87	13	222	880	530
SPU- miR-2004	1	676	266	56	44	76	68
SPU-miR-2001	1	7	16	1	1645	4680	2332
SPU-miR-2008	1	2	1	1	14	239	306
SPU-miR-2012	2	80649	198493	37044	131048	276925	224212
SPU-miR-7	1	30	17	1	80	597	905
SPU-miR-9	1	11	2	2	6	99	36
SPU-miR-96	1	22	85	16	226	1099	451
SPU- miR-200*	1	147	150	37	40	70	59
SPU- miR-7*	1	11	36	8	59	115	110

*=passenger strand. During miRNAs biogenesis, while the guide strand in the duplex will be incorporated into the RNA Induced Silencing Complex (RISC), the passenger strand is usually degraded or can be functional (Filipowicz et al. 2008, Okamura et al. 2008).

5.2 Test miRNA regulation of the endomesodermal specification

With the activation of *β–catenin* in micromeres, the specification of the endoderm and mesoderm begins. Genes that are involved in the specification of the endoderm and mesoderm include *paired-class micromere anti-repressor (Pmar-1*), *wnt8, hesC, alx1, thr, Ets* and *Delta* (Weitzel et al. 2004, Oliveri et al. 2008, Oliveri et al. 2002, Ettensohn et al. 2003a, Ettensohn 2006, Nishimura et al. 2004, Kurokawa et al. 1999, Fuchikami et al. 2002b, Logan et al. 1999)(Fig. 1.6). Transcript levels of some of these genes, *Pmar-1*, *wnt8*, *hesC* and *Delta,* increased in Drosha knockdown embryos and suggest that they may be regulated directly by miRNAs (Song et al. 2012). Future experiments with GFP reporter construct and mutagenesis studies will identify additional regulatory roles of miRNAs in endomesodermal specification. Our results will integrate post-transcriptional regulatory role of miRNAs to the gene regulatory network to achieve a comprehensive understanding of the specification of the germ layers in early development.

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