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microRNAs regulate β-catenin of the Wnt signaling pathway in early sea urchin development

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ABSTRACT

Development of complex multicellular organisms requires careful regulation at both transcriptional and post-transcriptional levels. Post-transcriptional gene regulation is in part mediated by a class of non-coding RNAs of 21–25 nucleotides in length known as microRNAs (miRNAs). β-catenin, regulated by the canonical Wnt signaling pathway, has a highly evolutionarily conserved function in patterning early metazoan embryos, in forming the Anterior–Posterior axis, and in establishing the endomesoderm. Using reporter constructs and site-directed mutagenesis, we identified at least three miRNA binding sites within the 3′ untranslated region (3′UTR) of the sea urchin *β-catenin*. Further, blocking these three miRNA binding sites within the *β-catenin* 3′UTR to prevent regulation of endogenous *β-catenin* by miRNAs resulted in a minor increase in β-catenin protein accumulation that is sufficient to induce aberrant gut morphology and circumesophageal musculature. These phenotypes are likely the result of increased transcript levels of Wnt responsive endomesodermal regulatory genes. This study demonstrates the importance of miRNA regulation of *β-catenin* in early development.

Introduction

β-catenin, the key effector molecule of the canonical Wnt pathway, has a highly conserved function in regulating cell proliferation, differentiation and fate decisions (Komiya and Habas, 2008; Moon, 2005). Aberrant Wnt signaling has been associated with many human diseases, including cancer, skeletal disorders, neuronal diseases, and cardiovascular diseases (Anastas and Moon, 2013; Clevers, 2006; Clevers and Nusse, 2012; Kim et al., 2013; MacDonald et al., 2009; Moon et al., 2004). In the absence of a Wnt ligand, β-catenin is phosphorylated at several N-terminal serine and threonine residues by casein kinase I and Glycogen Synthase Kinase 3β (GSK3β). Phosphorylation of β-catenin occurs in a multiprotein complex composed of GSK3β, a scaffolding protein Axin, and the tumor suppressor gene product Adenomatous Polyposis Coli protein (APC). Phosphorylation by GSK3β leads to the ubiquitination and proteasome-mediated degradation of β-catenin. Upon binding of the Wnt ligand to the Frizzled and LRP5/6 receptors, activated Disheveled (Dvl) recruits Axin to cell membrane leading to the disassembly of the destruction complex and inhibition of the degradation of β-catenin (Logan and Nusse, 2004). Increased cytoplasmic levels of β-catenin leads to its accumulation in the nucleus, where β-catenin interacts with TCF/LEF transcription factors and activates the transcription of target genes that mediate body plan determination, tissue patterning, and endoderm specification (Komiya and Habas, 2008; Moon, 2005; Peter and Davidson, 2010).

In addition to its essential role as a transcription coactivator, β-catenin is also a central structural component of the Cadherin/Catenin adhesion complex (Aberle et al., 1996; Nelson and Nusse, 2004). The Cadherin/Catenin-based adhesion system is the major mechanism by which cells adhere to one another. The abundance of β-catenin in the adhesion complex at the plasma membrane affects its accumulation and function with the signaling complex in the nucleus. This is demonstrated with experiments in which perturbation of cadherin complexes has an effect on Wnt/β-catenin regulated processes. For example, overexpression of cadherins in *Xenopus* embryos inhibited dorsal axis formation which is known to be dependent on canonical Wnt signaling (Heasman et al., 1994). E-cadherin knockout embryonic stem cells showed accumulation of β-catenin/Lef1 in the nucleus and activation of a Wnt reporter, which could be reversed by expression of E-cadherin (Orsulic et al., 1999).

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The initial regionalization of β-catenin in the early embryo contributes to polarity establishment, patterning, and germ layer specification (Logan et al., 1999; Petersen and Reddien, 2009). In numerous deuterostome embryos, including amphibians, fish, chicks, ascidians and sea urchins, β-catenin becomes localized in the nuclei of blastomeres at one pole of the cleavage stage embryo (Imai et al., 2000; Larabell et al., 1997; Logan et al., 1999; Roeser et al., 1999; Rowning et al., 1997; Schneider et al., 1996). In general, the pole of the embryo in which β-catenin is detected in the nucleus gives rise to endodermal and mesodermal tissues. Similar to many deuterostomes, the sea urchin β-catenin is required for the specification of the endoderm and mesoderm. (Logan et al., 1999; Wikramanayake et al., 1998). Overexpression of proteins that interfere with nuclear localization and/or function of β-catenin such as cadherins, GSK3β, and dominant forms of TCF/LEF, lead to embryos with excess ectodermal tissues and a lack of mesenchyme cells and gut (Emily-Fenouil et al., 1998; Logan et al., 1999; Vonica et al., 2000; Wikramanayake et al., 1998). Conversely, overexpression of β-catenin leads to embryos deprived of ectodermal tissue, consisting of mainly endodermal and mesodermal derivatives (Wikramanayake et al., 1998).

While the Wnt signaling pathway has been examined in the sea urchin (Emily-Fenouil et al., 1998; Logan et al., 1999; Vonica et al., 2000; Wikramanayake et al., 1998), the regulatory roles of microRNAs (miRNAs) in this developmental pathway have not been examined. miRNAs are a relatively novel class of 22-bp non-coding RNA molecules that fine tune gene expression by pairing to the 3′ untranslated region (3′UTR) of protein coding mRNAs to repress their translation and/or induce mRNA degradation (Bartel, 2004; Rajewsky, 2006). They are pivotal regulators of nearly all biological processes, including cell fate specification and differentiation (Bartel, 2004; Mukherji et al., 2011).

The vast majority of miRNAs are transcribed by RNA polymerase II and initially processed by the enzyme Drosha and its cofactor DGCR8 into stem-loop structures which get transported out from the animal nucleus to the cytoplasm (Lee et al., 2003). This stem-loop precursor is further processed into mature miRNAs by the riboendonuclease Dicer. The mature miRNA is loaded onto the RNA Induced Silencing Complex (RISC) and used as a guide to direct the binding of miRNA 5′ seed (nucleotides 2–8) and anchor nucleotides to the 3′UTR of target mRNAs to mediate translational silencing and promote targeted mRNA degradation (Baek et al., 2008; Bartel, 2009; Ghildiyal and Zamore, 2009; Guo et al., 2010; Hendrickson et al., 2009; Liu, 2008; Selbach et al., 2008). The regulatory role of miRNAs in early development was demonstrated by deleting or knocking down Dicer, an essential enzyme in miRNA processing, which causes either developmental defects or embryonic lethality in many animal systems (Bernstein et al., 2003; Giraldez et al., 2005; Saurat et al., 2013; Song et al., 2012).

Our laboratory has previously demonstrated that knockdowns of key enzymes in the miRNA biogenesis pathway in the sea urchin embryos lead to gastrulation failure and embryonic lethality (Song et al., 2012). Dicer knockdown embryos at the gastrula stage express significantly reduced endodermal and mesodermal antigens, suggesting a failure to properly specify these cell types. This current study tests the hypothesis that the highly conserved canonical Wnt/β-catenin pathway which is required for endomesodermal specification is regulated by miRNAs and may in part contribute to the previous Dicer knockdown phenotype. The strength of our study is to test miRNA regulation of *β-catenin* in the context of a developing embryo at the systems level. Our results indicate that *β-catenin* is post-transcriptionally regulated by at least two miRNAs at three binding sites. Removal of miRNA regulation using miRNA target protector morpholinos (miRNA TPs) specific to the *β-catenin* 3′UTR resulted in a minor increase of the β-catenin protein level that led to aberrant gut morphology, endodermal differentiation, and less developed circumpharyngeal musculature. Further, at the molecular level, a greater than 2-fold increase of Wnt responsive gene transcript levels, including endodermal regulatory genes *Krl* (Howard et al., 2001), *FoxA* (de-Leon and Davidson, 2010; Oliveri et al., 2006), *Eve* (Peter and Davidson, 2010, 2011), *Wnt8* (Wikramanayake et al., 2004), *Bra* (Gross and McClay, 2001), and ectodermal regulatory gene *Nodal* (Duboc et al., 2010; Yaguchi et al., 2008) was observed in *β-catenin* miRNA TP-treated embryos. These results demonstrate the importance of miRNA regulation on *β-catenin* in early development.

Materials and methods

Animals

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

Microinjections

Control morpholino antisense oligonucleotides (MASO) 5′ CCTCTTACCTCAGTTACAATTTATA 3′, *dicer* MASO 5′ GGACTCGATGGTGGCTCATCCATTC 3′ (Song et al., 2012), and miRNA target protector MASOs (miRNA TPs) were ordered from GeneTools (Philomath, OR). 1.5 mM of Dicer MASO stock injection solution was used. Three miRNA TPs were designed to protect one miRDeep2-30364-35240 binding site and two miR-2007 binding sites from respective miRNA binding to the *β-catenin* 3′UTR. The morpholino that blocks miR-2007 at position +922 bp is 5′ CTATTTCAGATATAATCTTGACGAG 3′; the morpholino that blocks miR-2007 at position +2647 bp is 5′ TTATTTCAGACATGAAAAATGGAAG 3′ and the morpholino that blocks miRDeep2-30364-35240 is 5′ TTATTGCACCTTTTTAAGAGGCATC 3′. These miRNA target protectors were diluted to various stock injection solutions (3 nM to 3 mM). The estimated injected miRNA TPs were the following: from the 30 μM stock of injection solution, approximately 35 attomoles (35×10^{-18}) was injected. From the 300 μM stock of injection solution, approximately 350 attomoles (350×10^{-18}) was injected. The injected negative control morpholino contained the same molar concentration as the corresponding sum of injected miRNA TPs. 300 μM stock of each of the *β-catenin* miRNA TPs were used in all real time, quantitative PCR, dual luciferase, Western blotting and phenotyping experiments.

Microinjections were performed as previously described with modifications (Cheers and Ettensohn, 2004; Song et al., 2012; Stepicheva and Song, 2014). MASO oligonucleotides were resuspended in sterile water and heated for 10 min at 60 °C prior to use. Injection solutions contained 20% sterile glycerol, 2 mg/ml 10,000 MW Texas Red lysine charged dextran (Molecular Probes, Carlsbad, CA, USA) and various concentrations of MASO. Approximately 1–2 pl were injected into each newly fertilized egg. 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 (1% w/v). Eggs were fertilized with sperm in the presence of 1 mM 3-amino-1,2,4 triazole (Sigma, St. Louis, MO). Injections were performed using the Femto Jet injection system (Eppendorf; Hamberg, Germany) (Stepicheva and Song, 2014). A vertical needle puller PL-10 (Narishige, Tokyo, Japan) was used to pull the injection needles