microRNAs Regulate Neural Crest Development and Epithelial-Mesenchymal Signaling in the First Pharyngeal Arch
microRNAs Regulate Neural Crest Development and Epithelial-Mesenchymal Signaling in the First Pharyngeal Arch

by

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ABSTRACT

Neural crest cells (NCCs) are a subset of multipotent, migratory stem cells that populate a large number of tissues during development and are important for craniofacial and cardiac morphogenesis. Although microRNAs (miRNAs) have emerged as important regulators of development and disease, little is known about their role in NCC development.

Here I show that a single miRNA, miR-145, when introduced in to multipotent, progenitor NCCs in vitro, induces the differentiation of these NCCs in to vascular smooth muscle cells (VSMCs). The fact that a single miRNA is capable of directing NCC fate down a specific differentiation path indicates a likely important role for miRNAs in directing the development of the neural crest. To expand on this idea, I go on to show that loss of miRNA biogenesis by NCC-specific disruption of Dicer results in embryos lacking craniofacial cartilaginous structures, cardiac outflow tract septation, and thymic and dorsal root ganglia development. Dicer mutant embryos had reduced expression of Dlx2, a transcriptional regulator of pharyngeal arch development, in the first pharyngeal arch (PA1). miR-452 was enriched in NCCs, was sufficient to rescue Dlx2 expression in Dicer mutant pharyngeal arches, and regulated non-cell-autonomous signaling involving Wnt5a, Shh, and Fgf8 that converged on Dlx2 regulation in PA1. Correspondingly, knockdown of miR-452 in vivo decreased Dlx2 expression in the mandibular component of PA1, leading to minor craniofacial defects. These results suggest that post-transcriptional regulation by miRNAs is required for differentiation of NCC-derived tissues and that miR-452 is involved in epithelial-mesenchymal signaling in the pharyngeal arch.
To further understand the mechanism by which Wnt5a inhibits Shh signaling, I present evidence that the inhibitory signal requires the activity of G-proteins. Downstream of G-protein activity, there is a Wnt5a-dependent increase in cyclic-AMP levels that induces an increase in PKA activity. Inhibiting PKA activity, even in the presence of Wnt5a, abolishes the downregulation of Shh-responsive genes suggesting that PKA activity is required for Wnt5a-mediated inhibition of Shh signaling. This work establishes a novel pathway connecting these two developmentally important signaling pathways and lays the groundwork for future studies that may shed light on the importance of this signaling interaction.
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INTRODUCTION

Description of the Neural Crest

Neural crest cells (NCCs) are a population of migratory, multipotent progenitor cells that are induced at the interface of the neural plate and the non-neural ectoderm of vertebrate embryos. As the neural plate closes, to form the primitive neural tube, the NCCs delaminate from the dorsal most portion of the neural tube and undergo an epithelial-to-mesenchymal transition (Sauka-Spengler and Bronner-Fraser, 2008). The mesenchymal NCCs then migrate ventrolaterally into the body along very stereotypical paths and contribute to the development of a number of tissues; including craniofacial, cardiac outflow tract, peripheral and enteric nervous system, melanocytes, and thymic tissues (Helms and Schneider, 2003, Jiang et al., 2000, Le Douarin et al., 2004 and Lee et al., 2004).

Much of our fundamental understanding of the neural crest came from significant contribution from Le Douarin in the 1970s. She utilized the fact that cells from chicken embryos could be morphologically distinguished from quail embryonic cells due to high amounts of heterochromatin in the quail cells (Le Douarin, 1974). Utilizing this difference in embryonic cells allowed for trans-species transplantation studies where portions of the embryonic chick neural tube prior to NCC delamination were replaced by segments of a developmentally matched quail neural tube. The transplanted quail cells would grow and thrive in the chick embryo and could be easily distinguished from the native cells of the chick. The beauty of this system was that by systematically replacing
sections of the neural tube along the anterioposterior axis, Le Douarin and colleagues were able to faithfully map the migratory patterns and developmental derivatives of the NCCs (Le Douarin and Jotereau, 1975, Le Lievre and Le Douarin, 1975 and Fontaine et al., 1977). These seminal experiments showed that the NCCs, in general, maintained their identity specified in the donor embryo despite being transplanted in to non-similar developmental environments. Meaning, the migratory route and developmental fate of the NCCs was determined by the anterioposterior position along the neural tube from the donor embryo, and not necessarily the local environment they were transplanted within (Le Lievre et al., 1980, Le Douarin, 1980 and Teillet et al., 1999). These experiments also identified that distinct populations of NCCs could be identified via their axial position, and thus be separated into three segments: the cranial, cardiac, and trunk NCCs. The NCCs within these different segments give rise to very specific cell types and contribute to the development of specific tissues and structures of the developing embryo (Le Douarin, 1980 and Teillet et al., 1999). As more advanced genetic and transgenic analyses have been developed, many groups have gone on to refine the characteristics of the various segments and have identified the genes contributing to the patterning, migration, and directed differentiation of the NCCs (Bronner-Fraser, 1995 and Gross and Hanken, 2008).

The Cranial, Cardiac, and Trunk Neural Crest and their Derivatives

The cranial and cardiac neural crest populations (together they are referred to as the cephalic neural crest) arise from the forebrain, midbrain, and hindbrain regions of the neural tube. These NCCs will migrate away from the neural tube and populate transient
outgrowths of the embryonic head-and-neck region known as the pharyngeal arches (PAs). The cranial NCCs populate the frontonasal process (FNP), PA1, and PA2; whereas, the cardiac NCCs populate PA3, PA4, and PA6 (Dupin et al., 2010) (Figure 1). Based on their distinct local environments along the neural tube axis and the variety of distinct instructive signals from the pharyngeal epithelia (i.e., endoderm and ectoderm), the populations of NCCs within the various PAs will differentiate into distinct cellular lineages (Betancur et al., 2010). The cranial NCCs differentiate into the cartilage, membraneous bone, soft tissue, and proximal ganglia of the craniofacial region. The specific structures, especially the bone and cartilage structures, are determined by where in the PA regions that they reside. For example, the NCCs that migrate into PA1 will form the bones and cartilages of the jaw. PA1 is also segmented into a maxillary component (mxPA1), which is located just posterior to the optic vesicle region, and the mandibular component (mdPA1) comprising what is morphologically identified as the first pouch (Minoux and Rijli, 2010). As their names suggest, the NCCs in mxPA1 will form the bones and cartilages of the upper jaw and palate region, while the NCCs in mdPA1 will form the bones and cartilages of the lower jaw. Additionally, the NCCs within PA2 have some skeletal contributions, namely the hyoid bone of the neck and the stapes bone within the middle ear. This indicates strong environmental cues within both the local neural tube region where these cells originated and within the PAs themselves which instruct each population of cells to form specific structures (Minoux and Rijli, 2010).
Directly posterior to PA1 and 2, the cardiac NCCs respond to a different set of signals and cues. This population of NCCs is responsible for the smooth muscle cells lining the cardiac outflow tract, connective tissue of the developing thymus, thyroid, and parathyroid glands, and parasympathetic innervations of the heart. In addition, cardiac NCCs are required to establish the aorticopulmonary septum that separates the aortic and pulmonary arteries (Kirby and Waldo, 1995). The cardiac NCCs that migrate into PA 3-6 will ultimately migrate down into the outflow tract cushions of the heart. Ablation of these cells results in defective patterning of the outflow tract, most commonly associated with persistant truncus arteriosus and overriding aorta (Kirby, 1990). It is interesting that the cardiac NCCs are not required to establish the initial endothelial tubes of the outflow tract forming a rudimentary vessel network. However, if the cardiac NCCs fail to develop properly, these endothelial-only vessels will regress and retract unpredictably suggesting that the NCCs are required to instruct the proper patterning of the outflow tract (de la Pompa and Epstein, 2012).

While the cranial and cardiac NCCs have very specific migrational paths and form very specific structures, the trunk NCCs tend to be slightly more plastic and will differentiate into various cell types based on the distance which they migrated from the neural tube. The trunk NCC segment begins just posterior to the hindbrain (below rhombomere 8) and continue the length of the embryo (Bronner-Fraser, 1994). The trunk NCCs will migrate between the somites of the developing embryo, specifically avoiding the posterior side of the somite. Some of these trunk NCCs will remain close to the dorsal neural tube and will develop into the dorsal root ganglia containing the sensory
neurons. Other NCCs will migrate more ventrally from the neural tube and will eventually form the sympathetic ganglia. Additionally, another subset of trunk NCCs will migrate dorsolaterally into the ectodermal tissue and will form the pigmented melanocyties found in the skin (Gammill and Roffers-Agarwal, 2010 and Dupin et al., 2006).

**Instructive Signals of the Neural Crest**

The neural crest is an amazing collection of cells due to the vast array of different cell types that they are capable of forming and the distinct segmentation of different NCC-subtypes that arise based on their axial positioning. Over the last few decades, it has been appreciated that NCCs from any region of the neural tube are fairly capable of forming any of the known NCC-derivatives in vitro. However, this is not completely true in vivo where environmental cues restrict certain NCC-derivatives while pushing the cells down specific differentiation paths (Sandell and Trainor, 2006 and Le Douarin et al., 2004). As mentioned, these cues come not only from their environment within the neural tube but also the local environment within the different PAs and regions of the trunk. For the remainder of this section I will focus on the main instructive signals found that effect the cranial and cardiac NCCs, however for more detailed review of the literature on trunk NCC signaling pathways see the following reviews (Adams and Bronner-Fraser, 2009, Gammill and Roffers-Agarwal, 2010, Kalcheim, 2011 and Kuo and Erickson, 2011).

At the level of the neural tube, the NCCs begin being patterned prior to their delamination and migration. One of the major instructive signals that initially pattern the
NCCs is from a specific family of homeodomain-containing genes known as the Hox genes (Duboule and Dolle, 1989, Kessel and Gruss, 1991 and McGinnis and Krumlauf, 1992). The Hox family of genes are a highly diverse and highly conserved family of transcription factors that were initially discovered in Drosophila, called the HOM-C family, and their role in anterioposterior patterning has been intensively studied (Fjose et al., 1985, Graham et al., 1989, Scott and O'Farrell, 1986 and Mallo et al., 2010). In mice, the Hox family members are clustered into four distinct genomic clusters (HoxA, B, C, D). These clusters are further organized into 13 paralogous (or homology) groups (Krumlauf et al., 1993). The genomic clustering of the different family members is considered to contribute to their distinct spatial expression patterns (McGinnis and Krumlauf, 1992). This non-homogeneous expression pattern along the anterioposterior axis of the developing embryo sets up a kind of “code” such that different populations of NCCs express/interact with very specific Hox genes. The heterogeneous Hox expression along the anterioposterior axis of the embryo sets up the initial patterning of the NCCs.

The ordered and partially overlapping expression of the various Hox gene members gives rise to the well-defined segmentations of the hindbrain known as the rhombomeres (r). For example, the NCCs that ultimately will populate PA1 are mainly derived from the Hox-negative r1 and r2. Whereas, the NCCs that populate PA2 have high expression of Hoxa2 and Hoxb2 and are derived from r4. Moving more posterior, r6 and r7 derived NCCs upregulate Hoxa3, Hoxb3, Hoxd3, and Hoxd4 and will populate PA3-6 (Figure 2). What becomes interesting is that the NCCs responsible for the entire craniofacial skeleton originate solely from the Hox-negative region (Hunt et al., 1991 and
Trainor, 2005). Experimentally, if Hox-positive regions of the neural tube are transplanted to replace the Hox-negative region, the facial skeleton will fail to form properly (Couly et al., 1998). Additionally, if Hox genes are experimentally forced to be expressed in the Hox-negative region, the facial skeleton also fails to form (Creuzet et al., 2002). This indicates that, the absence of Hox gene expression in the anterior rhombomeres is required for proper craniofacial skeleton development. Building upon this, if Hoxa2 expression is experimentally knocked out in mice, structures normally derived from PA2 will be lost and replaced by structures that are normally formed by PA1 ectopically. The PA2 NCCs, which lack Hox expression in these mutant mice, will now change identity and take on a more Hox-negative/PA1 identity (Gendron-Maguire et al., 1993). Therefore, not only is the absence of Hox genes necessary for the craniofacial skeleton, but it is critical that Hox gene expression begins precisely at r3 in order for PA2 structures to form.

Initially, if the converse of the transplantion experiment is performed where Hox-negative NCCs are transplanted to replace the Hox-positive region between r4-r6, Noden and colleagues noticed the generation of a second jaw forming, suggesting that the NCCs were “hardwired” to maintain PA1 identity (Noden, 1983). However, what Noden and colleagues did not appreciate at the time was their experimental setup included transplanting both NCCs and surrounding non-NCC neural tube tissue. This experiment was repeated by Couly and colleagues over a decade later, where they were very meticulous in only transplanting the NCCs from the dorsal-most neural tube. With this more specific NCC transplantation methodology, the previously Hox-negative NCCs
upregulated Hox gene expression and developed normally as if they were always r4-r6 NCCs (Couly et al., 1993). What Couly and colleagues were able to show was that although some aspects of NCC gene expression programs are intrinsic, there are certain gene expression programs that are established via environmental cues.

These paradoxical results can be partially explained by the presence of FGF8 in the anterior endoderm near the Hox-negative NCCs. Fgf8 expression enforces specific gene programs to keep the Hox genes silenced in r1 and r2 and can rescue the craniofacial defects seen in Hox-positive transplanted PA1 NCCs (Creuzet et al., 2004). However, it is insufficient in shutting down Hox gene expression in NCCs whose Hox gene expression program has already been activated (Trainor et al., 2002). Taken another step further, a bead soaked in Fgf8 protein placed near Hox-negative NCCs which have been transplanted into the r4-r6 region, the NCCs will remain Hox-negative and fail to form normal PA2 structures. However, there is no affect on the Hox-positive regions near the Fgf8-soaked bead in embryos that have not undergone the transplantation (Couly et al., 1998). The experiments enforce the idea that the NCCs have a certain level of pre-patterning at the level of the neural tube and rhombomeres, but require specific signals which arise from non-NCC-derived tissue.

**NCC Patterning and Tissue-Tissue Interactions in PAs**

The pharyngeal arch is an embryonic structure where specific interaction between various tissues is critical for proper development (Wood et al., 1991, Marshall et al., 1996, Clouthier et al., 1998 and Vitelli et al., 2006). The pharyngeal arch is made up of
four different tissues. It consists of a core of mesoderm surrounded by the neural crest-derived mesenchyme. This mass of cells (mesoderm and NCCs) is then covered by the pharyngeal epithelium, namely the ectodermal and endodermal tissue (Graham, 2001). Each of these tissues relies on specific patterns of gene expression for proper development. For example, there are a number of transcription factor families that play an important role in patterning and development of the NCCs, such as Dlx, Msx, Otx, Hox, Fox, and Tbx, among others (Qiu et al., 1995, Foerst-Potts and Sadler, 1997, Wurst and Bally-Cuif, 2001, Couly et al., 1993, Jeong et al., 2004, Vitelli et al., 2002, Clouthier et al., 1998 and Francis-West et al., 2003). It is crucial that the precise patterns and levels of these transcription factors are regulated correctly in order to properly develop. As mentioned, one of the major patterning gene expression programs that instruct pharyngeal development is the Dlx family of genes. This family of genes is mainly responsible for properly patterning the NCCs within the PAs. In mice, the Dlx family is comprised of 6 family members: Dlx 1-6. Much like the Hox gene clusters, the Dlx genes are expressed in distinct and overlapping expression domains. In PAs 1 and 2, Dlx1/2 are expressed throughout the NCC-derived mesenchyme of the arches. Dlx5/6 are expressed more distal within the arches and are notably absent from mxPA1. Finally, Dlx3/4 are expressed at the most distal tips of PA1 and 2 (Kraus and Lufkin, 2006) (Figure 3). This nested expression pattern is established based on the fact that these genes are genetically arranged as tightly-linked bigene pairs. Consequently, Dlx1/2 are linked and share similar regulatory regions (McGuinness et al., 1996). This holds true for the two other bigene clusters of Dlx5/6 and Dlx3/4 (Ruest et al., 2003). This nested expression of the
different Dlx genes is paramount for the proper development of the NCCs, especially of the craniofacial skeleton.

Depew and colleagues have elegantly described the patterning programs established by the Dlx genes. By knocking out the different Dlx family members individually and in combination, they have uncovered the different craniofacial skeletal elements that require the different Dlx genes for proper development and have artfully named this the “Dlx code” (Depew et al., 2005). Loss of both Dlx1/2 leads to dramatic loss of most mxPA1 derived upper jaw structures. One might suspect this to occur based on the nested expression pattern of the Dlx genes where only Dlx1/2 are present in mxPA1. However, there was very little change in the lower jaw structures and development of these structures was relatively normal (Qiu et al., 1997). This points to either overlapping functions of Dlx5/6 in mdPA1 and their capability in compensating for the loss of Dlx1/2, or Dlx1/2 are not involved in development of mdPA1 and are dispensable. To elucidate the true role of the nested expression of Dlx5/6, another double mutant mouse model was developed where Dlx5/6 are lost. In the Dlx5/6 double mutant, the upper jaw develops as expected, considering there is no Dlx5/6 expression present in mxPA1. However, now the lower jaw no longer develops normal (Robledo et al., 2002). This result indicates that Dlx5/6 are responsible for patterning mdPA1 and Dlx1/2 are not directing development of the lower jaw. Interestingly, in the Dlx5/6 double mutants the lower jaw region show signs of developing upper jaw-like bones. This transformation of mdPA1 derived structures in to mxPA1-like structures indicates that the Dlx genes nested expression pattern is required to establish the proper genetic programs that instruct the
NCCs to form the proper structures. Disruption of this “Dlx code” leads to disruption and transformation of the craniofacial skeleton (Depew et al., 2005). These results point to an important role of the nested expression pattern of the Dlx genes; not only is this expression pattern necessary to establish different instructive regions controlling craniofacial development, but there is a combination of both instructive and repressive programs established in order to create the proper craniofacial structures.

It is well established that the nested expression patterns of the different Dlx family members arise in response to signals originating from the non-NCC-derived epithelia of the PAs. These non-NCC tissue layers are critical for properly patterning the NCC-derived mesenchyme and instructing proper differentiation of the NCCs. These signaling factors include members of the Bmp, Wnt, Fgf, Endothelin, and Shh families of signaling molecules (Graham et al., 1994, Ikeya et al., 1997, Tucker et al., 1999, Clouthier et al., 1998 and Helms et al., 1997). The following section will detail the roles that these factors play in setting up the Dlx expression patterns. Although proper Dlx expression is crucial for normal development of NCC-derived tissues, there are many other players that play equally as crucial roles, please refer to (Creuzet et al., 2005, Ramos and Robert, 2005, Walker and Trainor, 2006, Scholl and Kirby, 2009 and Clouthier et al., 2010) for more in depth reviews.

One of the strongest pieces of evidence showing the role that tissue-tissue interaction plays in establishing Dlx patterning gene expression comes from PA cultures where the mdPA1 region of mouse embryos were cultured ex vivo and stained for Dlx expression.
If the explanted mdPAs are cultured normally, they retain their normal nested expression pattern of the Dlx genes. However, if the ectodermal layer is cut away from the underlying NCC mesenchyme, the Dlx genes are no longer expressed, suggesting that some factor(s) expressed specifically in the pharyngeal ectoderm is required for proper Dlx gene expression (Thomas et al., 1997). Subsequently, it has been shown that in the explants cultures with the epithelium removed, implantation of a bead soaked in Fgf8 protein can restore Dlx gene expression to the mdPA explants. Conversely, in intact mdPA explants, beads soaked in Bmp4 protein can antagonize Dlx gene expression to some extent (Thomas et al., 2000). Based on the expression patterns of Fgf8 (more proximal) and Bmp4 (more distal), it has been theorized that Fgf8 and Bmp4 are key regulators in establishing the proximal-to-distal expression patterns of Dlx 1/2 and 5/6 (Thomas et al., 2000). Bmp4 and Fgf8 are both expressed within the anterior pharyngeal epithelium of mdPA1, however their expression domains do not overlap, but rather form a very distinct boundary. Fgf8 is restricted to the proximal to medial region of mdPA1 and abuts to Bmp4 expression which is restricted to the distal aspects of the pharyngeal epithelium. Directly opposing interactions between Fgf8 and Bmp4 initially establish their expression boundaries, while the distinct genetic programs induced by the underlying mesenchyme help reinforce the pattern (Haworth et al., 2004). The fact that these genes are not only restricted to the anterior side of mdPA1 but also are restricted along the proximodistal axis as well, allows for the establishment of strong spatial polarity in both anterioposterior and proximodistal axes. These data, along with data showing that Fgf8 expression from the endoderm is present prior to establishment of the PAs, suggest that the boundary between Fgf8 and Bmp4 within the epithelia directly
regulates the establishment of the proximodistal nested expression of Dlx1/2 and Dlx5/6 (Thomas et al., 2000, Shigetani et al., 2000 and Hilliard et al., 2005).

In addition to the signaling molecules directly regulating and patterning the NCC-derived mesenchyme of the PAs, cross-tissue signaling is also required to establish expression patterns of the pharyngeal epithelial layers. Shh is strongly expressed in the developing pharyngeal endoderm and the Shh receptor, Ptch, is present in both the pharyngeal ectoderm as well as NCC-derived mesenchyme, highlighting the importance of this signaling molecule in both tissues (Yamagishi et al., 2006). By blocking Shh signaling specifically in NCCs, there is massive apoptosis of the NCC-derived mesenchyme of the arches (Ahlgren and Bronner-Fraser, 1999). This alone shows the importance of Shh signaling in maintenance of the NCC population. However, Shh is also involved in regulating pharyngeal ectoderm expression of other important signaling molecules. Shh expression from the pharyngeal endoderm has been shown to be a major factor in inducing the expression of both Fgf8 and Bmp4 where localized overexpression of Shh within mdPA1 can induce ectopic expression of both factors (Brito et al., 2006 and Haworth et al., 2007). In mutant mice lacking Shh, there is a consequent loss of Fgf8 in the pharyngeal ectoderm of PA1 (Moore-Scott and Manley, 2005). This suggests that Shh itself may not be a direct patterning factor of the NCCs per se, but rather establishes the expression of signaling factors that will ultimately establish the proper pattern and thus is a necessary initiating factor of patterning.
When the NCC fails to be properly patterned or to otherwise develop improperly there can be major morphological defects. In humans, a number of congenital anomalies are associated with improper NCC development. These anomalies of the NCC are collectively known as neurocristopathies (Jones, 1990). Craniofacial defects are some of the more common neurocristopathies and can present as isolated defects in lip or palate development, or more complex anomalies with malformations in multiple NCC derivatives as seen in patients with DiGeorge Syndrome and Treacher Collins Syndrome (Walker and Trainor, 2006). Additionally, neurocristopathies can manifest in cardiac defects (persistent truncus arteriosus and overriding aorta), innervation defects (Hirschsprung Disease), and certain cancers (neuroblastoma, medullary thyroid cancer, and several other neoplasms) (Trainor, 2005 and Farlie et al., 2004). The nature and cause of many of these neurocristopathies is not well understood and it is necessary to continue to elucidate the complex patterning and development of the NCCs in hopes of more completely understanding these diseases.

A number of genes and proteins necessary for NCC development have been intensively studied, yet there are many other factors yet to be fully characterized. For example, there are layers of regulation beyond just establishing gene expression patterns. Major regulatory networks are established both post-transcriptionally and post-translationally to modulate the expression levels and activity of proteins (DeBenedittis and Jiao, 2011 and Shilo and Shejter, 2011). These levels of regulation are not necessarily detectable at the level of gene expression, but rather at the level of protein expression and protein function/activity. Post-translational regulation can include
ubiquitination/degradation, both activating and inhibitory phosphorylation, modification of proteins with a variety of moieties, and activating and inhibitory protein cleavage (Goldberg, 2003, Kuriyan and Eisenberg, 2007 and Casado-Vela et al., 2011). For example, the Shh signaling pathway requires certain cleavages of the Gli proteins for proper functioning of the pathway. This phenomenon will be looked at more in depth later in this work. As for post-transcriptional regulation, many mRNAs are subject to degradation, alternate splicing, and down-regulation of translation due to microRNA (miRNA) binding. The following section will explore the roles that miRNAs play in regulating and tuning protein expression post-transcriptionally.

**miRNA Biogenesis and Function**

miRNAs belong to a class of small (19-21 nucleotides), non-encoding RNAs. miRNAs play crucial roles in a number of different tissue types, ranging from maintenance of stem cell pluripotency to regulation of heart development. As miRNA research has boomed in the past decade, the role that these small RNAs play has been appreciated in nearly every cell and tissue type that they have been studied (Cai et al., 2004, Kanellopoulou et al., 2005 and van Rooij and Olson, 2007). Their main regulatory role comes from binding to complimentary sequences within mRNAs leading to degradation or translational inhibition of the target transcript.

The first miRNA, named lin-4, was discovered in 1993 in a study exploring the roles of non-encoding RNAs in C. elegans (Lee et al., 1993). The authors found that the expression of a very short RNA transcript (lin-4) correlated with down regulation of a
protein known as Lin-14. They discovered that the mRNA transcript encoding Lin-14 had several sequences complimentary to lin-4 and this correlation suggested that Lin-14 protein expression may indeed be mediated through RNA-RNA interaction of the lin-4 miRNA and the Lin-14 mRNA (Lee et al., 1993). This study prompted the study of non-encoding RNAs by a number of groups that went on to identify the pathways responsible for miRNA biogenesis and the machinery that carries out the mRNA targeting. It would be another 10 years until the first mammalian miRNA’s function was described (Lim et al., 2003). As of the writing of this dissertation, there are now over 300 conserved miRNAs, with an additional ~1000 small RNAs structurally resembling miRNAs, identified in mice and humans and bioinformatics have estimated that nearly 1/3 of mammalian mRNAs are subject to miRNA regulation (Mendell and Olson, 2012).

miRNA biogenesis occurs in a step-wise fashion, starting in the nucleus and culminating in the cytoplasm. The first step occurs in the nucleus where RNA Polymerase II transcribes the larger primary miRNA transcripts, known as pri-miRNAs (Cullen, 2004). The pri-miRNAs are then recognized by the RNase III-like enzyme, Drosha. Drosha, along with its binding partner Dger8, will cleave the pri-miRNA into a 60-80 basepair precursor (pre-miRNA). The pre-miRNA transcripts have a characteristic hairpin, comprised of a double-stranded stem region and a single-stranded loop region. The pre-miRNAs also have a characteristic two nucleotide single-stranded overhang on the 3’ end of the transcript (Lee et al., 2003). The 3’ overhang is recognized by the nuclear export protein called Exportin5 (Exp5). Exp5 translocates the pre-miRNA out of the nucleus into the cytoplasm where it is recognized by another RNase III-like enzyme called Dicer. Dicer will cleave the pre-miRNA into a 19-21 basepair double stranded
miRNA duplex, which is deemed as the mature miRNA (Hutvagner et al., 2001). Dicer forms a complex with a member of the Argonaute family (Ago2) where the miRNA duplex is separated into the sense and anti-sense strands. Typically, the anti-sense strand is degraded, leaving a complex of Ago2 with the single-stranded miRNA. The complex, known as the RNA Induced Silencing Complex (RISC), is then guided to specific mRNA targets and functions to modulate protein expression (Liu et al., 2004).

The Ago2-miRNA RISC functions via partially homologous binding of the miRNA sequence with complimentary sequences in the mRNA targets. Although more research is beginning to indicate that important complimentary sequences exists throughout the sequence of the miRNA (Didiano and Hobert, 2006, Grimson et al., 2007, Chi et al., 2009 and Chi et al., 2012), the current dogma suggests that the region known as the seed sequences, from base 2-8 in the miRNA 5’ region, must bind perfectly with the complimentary sequence in the mRNA target (Thomson et al., 2011). Additional complementation along the length of the miRNA is required for tight binding and proper regulation, such that the context of the target site within the mRNA 3’ UTR and the extent of complimentarity along the length of the miRNA helps to determine the downstream effect on protein expression (Zhao et al., 2005). Despite these advances in understanding miRNA-mRNA interactions, the seed sequence still appears to be the major driver of mRNA targeting and is the basis for most of the target prediction algorithms that have been established for the in silico prediction of the miRNA-mRNA targets (Pasquinelli, 2012).
There are four established modes of miRNA function on protein expression, which are all distinct but have the shared effect on downregulating the expression level of the target protein. These modes are mRNA degradation, co-translational repression, repression of translation initiation, and sequestration of the target mRNA to processing-bodies (P-bodies). In mRNA degradation, suggested to be one of the major modes of action, after RISC is loaded on to the mRNA target, the mRNA is cleaved at the site of the miRNA-mRNA complex. Once cleaved, the mRNA is then subjected to degradation due to the loss of the poly-A tail (Yekta et al., 2004). For co-translational repression, evidence suggests that the translation machinery will begin to translate the protein but fail to finish, presumably due to the presence of RISC binding to the target, leading to a truncated form of the protein and subsequent degradation. Studies have shown that upon loading of RISC on to the target mRNA, the translational machinery is present and begins to move along the transcript but no protein product is detected, suggesting that translational initiation occurred but elongation failed (Eulalio et al., 2008). Conversely, the repression of translation initiation involves repressing the translation of the final protein product, however no translational machinery is detected on the mRNA in the presence of miRNA and thus the suggestion is that the translational machinery failed to initiate the translation process (Pillai et al., 2005). Finally, sequestration of mRNA to P-bodies is still somewhat controversial in whether the mRNA is degraded in the P-bodies, which are known to be sites of mRNA degradation, or if the mRNA can simply be preserved in the P-bodies and be available for translation based on certain stimuli (Liu et al., 2005). It is worth noting, that under certain conditions and cellular contexts, that are not completely understood, upon miRNA targeting there is a detectable increase in the resulting protein
product (Vasudevan et al., 2007). Regardless of mode of function, the majority of miRNAs lead to changes, usually inhibitory, in the expression of the protein from the target mRNA. The changes in these protein levels is not nearly dramatic as what is commonly seen when studying transcriptional regulators, and thus the main role of miRNAs in the cell is to fine-tune the level of target proteins.

**Role of miRNAs in Development**

Since their discovery over a decade ago, the study of the role and function of miRNAs has rapidly increased and our knowledge of these protein regulators has grown exponentially. The initial discovery of miRNAs in C. elegans was done in a screen to identify developmental regulators, so it was very exciting early on to hypothesize that miRNAs may contribute significantly to the developmental plan of an organism (Lee et al., 1993). This has lead to a number of knockout models of single miRNAs and studies where miRNA biogenesis was inhibited in specific cell or tissue types. The animal models where miRNA biogenesis is blocked, essentially knocking down all miRNA function, have lead to severe developmental defects (Giraldez et al., 2005, Harfe et al., 2005, Harris et al., 2006 and Zhang et al., 2011). These works have shown that miRNAs as a class of regulators are essential to the development of organisms. However, when one looks at the contribution of individual miRNAs through knockout or knockdown animal models, there are few highly penetrant, developmentally defective phenotypes (Mendell and Olson, 2012). One of the first knockout models in mouse was a knockout
of one of two copies of miR-1, a highly-enriched, muscle-specific miRNA. These mice displayed ventricular septal defects, cardiac electrophysiologic defects, and thickening of the ventricular walls suggesting at least some developmental role for miR-1 (Zhao et al., 2007). This is not the case with many of the other mouse miRNA knockouts that show relatively little developmental phenotype (Park et al., 2010). However, much is yet to be determined about the role of miRNAs in different tissues during development.

Prior to the work being presented here, there was very little known about the role of miRNAs in instructing NCC development. The first indication that miRNAs were involved in NCC development came from Eberhart et al, who utilized a zebrafish model to show that miR-140 negatively regulated PDGF signaling during palatogenesis. Both overexpression of miR-140 and knockdown of PDGFRα lead to similar defects in palatogenesis. They went on to show that miR-140 directly targeted PDGFRα leading to decreased expression of the receptor in vivo (Eberhart et al., 2008). Following this report, Gessert et al used a Xenopus model to show that loss of miR-96, miR-196, and miR-200b resulted in defects in the development of NCC-derived facial cartilages (Gessert et al., 2010). Finally, while our work (presented in Chapter 2) was under editorial review, a report from Zehir et al was the first report in mammals at the role of miRNAs in NCC development. Their group utilized the same Dicerfl/fl, Wnt1-Cre mouse model that our group used to show that loss of miRNA function specifically in the NCCs leads to defects in the development of NCC-derived tissues (Zehir et al., 2010). Our report went on to expand upon their work in showing that a miRNA specifically enriched in NCCs, miR-452, plays an important role in regulating Dlx2 expression, and
loss of miR-452 lead to specific defects in the development of certain craniofacial structures. The culminations of these works have lead to a number of new studies expanding on the role of miRNAs in NCCs. Studies have gone on to show more in depth looks at the role of NCCs in cardiac outflow tract remodeling, NCC-derived neuronal differentiation, the potential role of yet to be described miRNAs in Chromosome 4q deletion syndrome, and the importance of miRNAs in the evolution of avian craniofacial variation (Huang et al., 2010, Huang et al., 2010, Nie et al., 2011, Chapnik et al., 2012, Xu et al., 2012 and Powder et al., 2012). Together, these works have only begun to scratch the surface of the critical role that miRNA-mediated regulation of protein expression has on the proper development of NCC-derived structures. Herein we will begin by reviewing our data first showing that addition of a single miRNA was sufficient to direct the differentiation of multipotent neural crest stem cells in to vascular smooth muscle cells. Then, we will further elucidate the full role that miRNAs play in NCC development and help elucidate some novel tissue-tissue interactions involving miR-452, Wnt5a, and Shh.

In Chapter 1, we sought to understand the role of miR-143 and miR-145 in cardiac development. Through these studies we discovered the miR-145 was a potent regulator of vascular smooth muscle cell (VSMC) differentiation. Interestingly, miR-145 was also capable of efficiently directing the differentiation of multipotent NCSCs in to VSMCs, being the first indications that miRNAs could play an important role in directing NCC cell fate.
In Chapter 2, we pushed forward with our understanding of miRNA roles in the neural crest by knocking out Dicer within the NCCs and thus inhibiting nearly all miRNA functions. These mutant mice lacked most NCC-derived tissue and massive apoptosis was observed in the PAs prior to NCC differentiation. In addition to phenotypic analysis, we also determined that Dlx2 and Fgf8 expression patterns were disrupted in the mutant mice, with much reduced expression in PA1. We also sought to determine which miRNAs were highly enriched in the NCC population as compared to non-NCC populations. Our study identified 9 miRNAs that were highly enriched and assumed to play some important role in NCC development. One of these miRNAs in particular, miR-452, was required for proper expression of the NCC patterning gene Dlx2. Loss of miR-452 in vivo led to decreased expression of Dlx2 in mdPA, resulting in specific defects during craniofacial development. Furthermore, we found that miR-452 directly targeted Wnt5a mRNA. Finally, we went on to show a novel interaction between Wnt5a and Shh signaling in PA1.

In Chapter 3, we sought to better understand the mechanism by which Wnt5a signaling inhibits Shh signaling seen in Chapter 2. We found that in multiple cell types, overexpression of Wnt5a could lead to the inhibition of Shh signaling, suggesting a conserved role for this interaction. We determined that Wnt5a was acting through G-protein signaling that culminated in the upregulation of cyclic AMP (cAMP). Furthermore, the influx of cAMP by Wnt5a caused a concordant increase in PKA activity. Inhibition of PKA activity in the presence of Wnt5a, could nullify the inhibitory
action of Wnt5a on Shh signaling, suggesting that overexpression of Wnt5a leads to activation of PKA and subsequent inhibition of Shh signaling.

Overall, the research findings described in this thesis make an invaluable contribution to our understanding of the cellular and molecular interactions governing the development of the neural crest – encompassing both miRNA mediated regulation of neural crest patterning and the exploration of an interaction between two important developmental signaling pathways, namely Wnt5a and Shh. Additionally, identification of miR-452 as a regulator of Dlx2 expression lends significant insight into the general regulatory network governing the proper expression of this important neural crest transcription factor during craniofacial development.

FIGURE LEGENDS

Figure 1: Migration patterns of the neural crest
The NCCs migrate down stereotypical routes as they populate specific embryonic structures. The anterioposterior positioning of the NCCs as they arise from the neural tube determine the migratory pathways that they will follow. The colors denote the NCC populations as they arise from the neural tube and their final migratory destinations. The pharyngeal arches are denoted by roman numerals. The neural crest cells arising from r3 and r5 (yellow, asterisk) provide minimal contributions to the pharyngeal arches due to apoptosis of the NCCs arising from those regions. Mdb, midbrain; ov, otic vesical; r, rhombomere. (Chapman, 2011)
**Figure 2: Hox gene expression pattern establishes neural crest identity**

The Hox genes are expressed in and overlapping pattern along the anterioposterior axis of the developing embryo. The color coded bars represent the expression patterns of the different Hox genes in the various NCC populations as the migrate in to the pharyngeal arches. Each pharyngeal arch is color coded to represent the combinations of Hox genes that each express, except for PA1 (yellow) because it is Hox-negative. PA, pharyngeal arch; r, rhombomere. Adapted from (Minoux et al., 2009).

**Figure 3: The nested expression pattern of the Dlx genes pattern the pharyngeal arches**

The Dlx genes are expressed in a nested pattern within PA1 and PA2 of the developing embryo, shown both in schematic form and with in-situ hybridization data at E10.5 of mouse embryos. The nested expression patterns of the six Dlx genes are color coded to show their representative expression throughout the PAs, with Dlx1/2 being expressed throughout and Dlx3/4 being restricted to the most distal tips of the PAs. mxBA1, maxillary component of branchial arch 1; mdBA2, mandibular component of branchial arch 2; BA2, branchial arch 2; FNP, frontonasal process. Adapted from (Depew et al., 2005).
Figure 1: Migration patterns of the neural crest

(Chapman, 2011)
Figure 2: Hox gene expression pattern establishes neural crest identity

(Minoux et al, 2009)
Figure 3: The nested expression pattern of the Dlx genes pattern the pharyngeal arches

(Depew et al, 2005)
CHAPTER 1

miR145 directs neural crest stem cell differentiation into smooth muscle

BACKGROUND

Proper specification of the cardiac NCC is critical for the development of the heart. The heart is derived from essentially three populations of progenitor cells. Two of these populations, the first and second heart fields, will give rise to the working myocardium of the heart and provide the structural background for the organ. However, it is the cardiac NCCs which are responsible for the patterning and positioning of the outflow tract (Cordes and Srivastava, 2009). The cardiac NCC progenitors will migrate into the outflow tract and undergo a differentiation into vascular smooth muscle cells (VSMCs). The VSMCs make up the interior lining of the blood vessels and are a critical barrier between the vascular system and the rest of the body (Vincent and Buckingham, 2010). The NCC-derived VSMCs provide important signals not only instructing the proper organization of the outflow tract, but also to the underlying myocardium, where ablation of the cardiac NCC leads to mispatterned outflow tract and a loss of myocardium suggesting critical interactions between these cellular populations (Kirby et al., 1985 and Porras and Brown, 2008). Thus, the regulation of these cardiac NCCs must be precisely maintained. Disruptions in these cell types are known to cause human diseases, manifested in persistent truncus arteriosus and aortic arch malformations.

The role of miRNAs in regulating the development of the heart has been extensively studied, but still remains insufficient. Importantly, the contribution of miRNAs in the
development of the first and second heart fields have been studied using multiple models (Cordes and Srivastava, 2009). However, very little is known about how miRNAs can instruct cardiac NCC development. At the time that we performed the experiments reported in this chapter, there were no descriptions of miRNAs regulating any portion of the cardiac NCC. Subsequently, our group and others have described mandatory roles of miRNAs in the proper development of the cardiac NCCs (Sheehy et al., 2010, Huang et al., 2010 and Nie et al., 2011). To that end, complete loss of miRNA biogenesis in NCCs leads to defective organization of the outflow tract (Chapnik et al., 2012). However, the work described in this chapter will focus exclusively on a pair of bicistronic miRNAs, miR-143 and -145, and how miR-145 plays an important role in instructing the differentiation of progenitor NCCs into VSMCs.

The initial studies regarding miR-143 and miR-145 were mainly focused on their regulation and tumor suppressor roles in a number of different cancers (Wang and Lee, 2009). Our group initially reported that miR-143 is the most enriched miRNA during differentiation of mouse embryonic stem (mES) cells into multipotent cardiac progenitors (Ivey et al., 2008). This prompted our group to focus our efforts in understanding the role that miR-143 and miR-145 play during cardiac development.

Both miR-143 and miR-145 are highly conserved miRNAs and lie within 1.7 kb of one another. This bicistronic miRNA pair is expressed as early as E7.5 in the cardiac progenitor cells of the cardiac crescent region of the developing mouse (Figure 4A). As the heart continues to develop through E8.5 and E9.5, miR-143/145 is widely expressed
throughout the heart and cardiac outflow tract region of the embryo (Figure 4B-D). Furthermore, after the outflow tract region has undergone proper patterning and remodeling by E15.5, expression of miR-143/145 is lost in the outflow tract region and is mainly restricted to the ventricles and atria of the heart (Figure 4E). Interestingly, in the post-natal heart, there is a dramatic switch in miR-143/145 expression where the miRNAs become once again upregulated in the outflow tract and ventricular expression is lost (Figure 4F). Looking more closely at the expression of miR-143/145 in the post-natal heart, it was found that these miRNAs are highly expressed in the VSMCs of the aorta and coronary vessels, with miR-145 expression being greater than 50-fold higher in the aorta than that of the myocardium (Figure 5A-E).

During VSMC induction, the cells mature from an actively proliferating, undifferentiated progenitor in to a quiescent, fully-differentiated cell type. Interestingly, under cardiac stress conditions the VSMCs can revert back to their proliferative state and is known to contribute to a number of human vascular diseases (Ross, 1993). This proliferative state is similar to the progenitor cell types seen in the developing embryo. This switching of VSMC fate requires the coordinated expression of a number of pro-proliferative factors, such as Elk-1, and the inhibition of pro-differentiation genes, such as smooth muscle actin (Sm-actin) and calponin (Owens et al., 2004 and Yoshida and Owens, 2005). Our group found that miR-145 could play a role in this process by reinforcing the pro-differentiation genetic program. In in vitro studies, it is possible to force the differentiation of 10T1/2 fibroblast cells in to VSMCs through the addition of high levels of recombinant Myocd protein (Hirschi et al., 1998 and Wang et al., 2003).
This leads to differentiation of the fibroblasts, as evidenced by an increase of smooth muscle cell markers (Figure 6A-C). If one uses an antagonir directed towards miR-145, in order to block miR-145 activity, the cells can no longer efficiently differentiate in to VSMCs (Figure 6A-C). This suggests that there is a requirement of miR-145 for aiding in this process. Importantly, low levels of Myocd will not efficiently lead to the differentiation of these fibroblasts in to VSMCs (Figure 6D). Interestingly, if miR-145 is added to the cells along with the low levels of Myocd the cells will once again undergo the differentiation process (Figure 6D).

Combining the data showing miR-145 expression in the NCC-derived VSMCs of the aorta and the ability of miR-145 to aid in the differentiation of VSMCs, we sought to determine whether or not miR-145 could direct the differentiation of undifferentiated NCSCs in to a VSMC cellular fate.

RESULTS

During mammalian development, the cardiac NCCs will populate the pharyngeal arteries and differentiate in to the VSMCs that line the interior of the vessels (Bergwerff et al., 1998). The lack of a reliable genetic marker of these cardiac NCCs and the proclivity of primary progenitor NCCs isolated from the neural tube to sporadically differentiate in vitro has made studying the factors that direct cardiac NCC cell fate challenging. Recently, a cell line has been established where undifferentiated neural crest stem cells (NCSCs) can be maintained indefinitely in culture. This cell line, called Joma1.3, was derived from a transgenic mouse expressing a tamoxifen-inducible e-Myc
gene that, when activated, will force the cells to maintain a highly proliferating, undifferentiated fate (Maurer et al., 2007). By supplementing the Joma1.3 cell media with 4-hydroxytamoxifen (4OHT), the cells will maintain their pluripotency. Upon removal of 4OHT, the cells will undergo random differentiation into the various NCC-derived cell types, such as smooth muscle, neuronal, cartilage, and melanocyte. Importantly, in the absence of 4OHT, addition of specific factors allows for the directed differentiation of the NCSCs into specific cell types. For example, addition of VEGFβ to Joma1.3 cells for 6-days leads to nearly 90% of the cells to upregulate the VSMC marker Sm-actin (Maurer et al., 2007).

We utilized the Joma1.3 cells to test the ability of miR-145 to direct the differentiation of NCSCs into VSMCs. As mentioned, if the cells are maintained under culture conditions containing 4OHT they will remain in a proliferative, undifferentiated state. If 4OHT is removed, the cells will undergo random differentiation and we could only detect a small upregulation of VSMC-markers by qPCR (Figure 7A), suggesting a low-level of differentiation down a VSMC fate. However, when miR-145 is added to the cells in the absence of 4OHT (-4OHT), we detected a dramatic increase in the expression level of a number of VSMC-markers (Figure 7A). We also looked at the upregulation of Sm-actin and calponin protein levels via Western Blot upon addition of either miR-143 or miR-145 and found that only miR-145 could generate a robust increase in either protein, with Sm-actin being induced nearly 60-fold over cells where miR-145 was not added (Figure 7B). Interestingly, these data were generated after only 24 hours of miR-145 exposure, which is nearly 5 days less than what is needed to see efficient VSMC differentiation using the
conventional TGFβ method. This suggests that miR-145 is both an efficient and rapid inducer of VSMC differentiation in Joma1.3 cells.

We next sought to determine if the robust increase of VSMC-marker expression upon miR-145 addition corresponded to a large percentage of the cells undergoing differentiation down a VSMC fate. To do this, we stained cells by immunocytochemistry for the VSMC-markers Sm-actin, calponin, and caldesmon upon addition of miR-145. We found that the nearly 75% of the cells treated with miR-145 stained positive for Sm-actin. Importantly, the majority of the cells that stained positive for the VSMC-markers underwent a morphological change where the cells flattened out and possessed the characteristics of a fully-differentiated VSMC (Figure 8A,B).

DISCUSSION

At the time the work in this chapter was initially published, the ability of miR-145 to efficiently direct VSMC differentiation from multipotent stem cells was the first evidence, to our knowledge, of a miRNA capable of directing VSMC fate. Since that time, a number of works have followed and elaborated on the functions of miR-145. It is now appreciated that miR-143 and miR-145 are integral parts of the VSMCs’ ability to respond to vascular injury where mice lacking both miR-143 and miR-145 are profoundly impeded in the VSMCs’ ability to undergo necessary cytoskeletal rearrangement and migration after injury (Xin et al., 2009 and Elia et al., 2009). In addition to its role in VSMC functions, miR-145 has recently been reported to be downregulated in the cardiac myocardium after acute myocardial infaction (AMI) in mice and is associated with an
increase in Dab2 expression in the infarct border zone. The resulting increase in Dab2 results in a deleterious decrease in β-catenin activity, suggesting that in AMI models downregulation of miR-145 may be deleterious to the heart post-infarct, further supporting the importance of this miRNA in the cardiovascular system (Mayorga and Penn, 2012).

However, the most highly studied role of miR-145 has been in its role as a tumor suppressor for a number of different cancers (Wang and Lee, 2009, Tazawa et al., 2011 and Luo et al., 2011). Interestingly, miR-145 overexpression has been studied as a potential therapy in the treatment of breast cancer (Kim et al., 2011). These works have highlighted the important role that miR-145 plays in maintaining proper cell differentiation and proliferation in a variety of cell types. It will be intriguing to continue following the literature as further research elucidates new mechanisms, and excitingly, possible therapeutic application of this miRNA.

Despite all the recent advances in miR-145 research, I was particular interested in its ability to control the differentiation of NCSCs. Especially, since it was one of the first miRNAs to show a role in directing specific NCC fates. To follow on this work, we further studied the role of other miRNAs that participate in NCC development and sought to understand how loss of miRNA function affects the developing neural crest. These topics will be the focus of Chapter 2.
FIGURE LEGENDS

Figure 4: miR-143/145 are cardiac and smooth muscle-specific miRNAs

(A-C) Whole mount embryos at the indicated time points showing B-gal activity in transgenic animals with a miR-143/145 enhancer-lacZ construct showing cardiac and pharyngeal mesodermal expression. (D) Transverse section of (C). (E,F) B-gal expression at E15.5 (E) and P21 (F) showing the bimodal switch in miR-143/145 expression as it goes from strong myocardial expression at E15.5 to strong VSMC expression post-natally. Pcm, precardiac mesoderm; ht, heart; ot, outflow tract; rv, right ventricle; lv, left ventricle; pm, pharyngeal mesoderm; cv, cardinal vein; nt, neural tube; da, dorsal aorta; pe, pharyngeal endoderm; ec, endocardium; mc, myocardium; ra, right atrium; la, left atrium; co, coronary arteries.

Figure 5: Strong expression of miR-145 in VSMCs

(A-D) B-gal expression from transgenic miR-143/145 enhancer-LacZ mice and section in-situ hybridization of miR-145 in P21 sections of the heart showing specific expression within the smooth muscle cells of the aorta and coronary arteries. (E) qRT-PCR data comparing miR-143 and miR-145 expression from tissue taken from the aorta and the rest of the heart showing enrichment for both miRNAs in the aorta. Ao, aorta; ra, right atrium; rv, right ventricle; lv, left ventricle; vs, ventricular septum; sm, smooth muscle; co, coronary artery.
Figure 6: miR-145 directs VSMC fate in 10T1/2 fibroblasts

(A,B) Immunocytochemistry of 10T1/2 fibroblasts showing smooth muscle (Sm)–actin expression upon addition of high-levels of myocardin (Myocd) protein. The increase in Sm-actin expression can be blocked by addition of anti-miR-145 but not anti-miR-143. Also shows that addition of miR-145 can induce VSMC fate in the presence of low-levels of Myocd. Sm-actin-positive cells are quantified in (B). (C,D) qRT-PCR quantification of specific VSMC-markers in conditions similar to (A,B) showing that multiple marker genes besides Sm-actin are induced by miR-145.

Figure 7: miR-145 addition upregulates VSMC-marker genes in neural crest progenitor stem cells

(A) Joma1.3 NCSCs were removed from 4OHT-containing media (-4OHT, blue bars) and subsequently treated with miR-145 mimic (+miR-145, red bars) for 24-hours. Relative levels of the various VSMC-markers were quantified via qRT-PCR. (B) Western blots for Sm-actin and Calponin protein expression after treatment with either miR-145 or miR-143 mimics. Loading levels were normalized to Gapdh. Quantification of protein levels from western blot analysis using LI-COR software is shown in the graph on the right. After loading normalization, relative protein levels were normalized to +4OHT levels. *P<0.05; error bars represent 95% confidence intervals.
Figure 8: miR-145 alone differentiates NCSCs down a smooth muscle cell fate

(A) Representative images of immunocytochemistry from Joma1.3 cells for various VSMC-markers with or without the addition of miR-145 mimic. (B) Quantification of Sm-actin positive cells represented in (A). Data normalized to +4OHT cell culture condition. *P<0.05; error bars represent 95% confidence intervals.
Figure 4: miR-143/145 are cardiac and smooth muscle-specific miRNAs

(Cordes et al, 2009)
Figure 5: Strong expression of miR-145 in VSMCs

(Cordes et al, 2009)
Figure 6: miR-145 directs VSMC fate in 10T1/2 fibroblasts

(Cordes et al, 2009)
Figure 7: miR-145 addition upregulates VSMC-marker genes in neural crest progenitor stem cells
Figure 8: miR-145 alone differentiates NCSCs down a smooth muscle cell fate
CHAPTER 2

The neural crest-enriched miRNA, miR-452, regulates epithelial-mesenchymal signaling in the first pharyngeal arch

BACKGROUND

The proper migration and differentiation of neural crest cells (NCCs) is essential for craniofacial, cardiac, peripheral and enteric nervous system, melanocyte, and thymic development (Jiang et al., 2000, Helms and Schneider, 2003, Le Douarin et al., 2004 and Lee et al., 2004). After delaminating from the dorsal portion of the neural tube, NCCs migrate ventrolaterally along stereotypical routes and are induced to differentiate through reciprocal signaling with neighboring cells (Sauka-Spengler and Bronner-Fraser, 2008). Cranial NCCs populate the pharyngeal arches (PAs), where the neural crest–derived mesenchyme encounters a number of instructive signals from the pharyngeal epithelia (i.e., endoderm and ectoderm), resulting in differentiation into the proper cell lineages (Kameda, 2009 and Le Douarin et al., 2004). Similarly, NCCs that populate the outflow tract of the heart and the developing aortic arch arteries rely on reciprocal signaling with neighboring cardiac progenitor cells derived from the second heart field (Waldo et al., 2005). Disruption of NCC development, cell- or non-cell-autonomously, results in numerous forms of human birth defects, including DiGeorge and Treacher-Collins syndromes (Epstein and Parmacek, 2005). Although many signaling pathways and transcription factors involved in NCC development are known (Meulemans et al., 2004
and Trainor et al., 2002), the mechanism by which post-transcriptional regulation affects NCC development has not been established.

MicroRNAs (miRNAs) are an important class of post-transcriptional regulatory molecules. They typically bind to sequence-specific binding sites within the 3’-untranslated region (3’-UTR) of target mRNAs to repress translation, degrade the target message, or both (Bartel, 2009). The RNase III enzyme, Dicer, is required for the cleavage of precursor-miRNAs into fully functional, mature miRNAs (Lee et al., 2004). Studies with specific miRNA and conditional Dicer deletions revealed that miRNAs are required for proper development of a number of different tissues, including lungs, cardiac muscle, chondrocytes, skin, and limbs (Harfe et al., 2005, Harris et al., 2006, van Rooij et al., 2007, Zhao et al., 2007, Kobayashi et al., 2008 and Yi et al., 2009). Deletion of Dicer in neural crest cells disrupts proper cranial neural crest cell development (Zehir et al., 2010); however, the individual miRNAs that contribute to neural crest development and the mechanism by which they do so remain unknown.

Here, we show that disruption of miRNA biogenesis in NCCs not only affects cranial and cardiac neural crest development, but also specifically affects the expression of Dlx2 in the mandibular component of the first pharyngeal arch (PA1). We profiled miRNAs enriched in NCCs and found that one NCC-enriched miRNA, miR-452, was sufficient to rescue proper expression of Dlx2, a known PA patterning gene, in the mandibular-component of PA1. Additionally, we found that miR-452 regulated reciprocal epithelial-mesenchymal signaling in PA1, involving Wnt5a, Shh, and Fgf8, converging on Dlx2.
expression. Thus, our study reveals a novel miRNA-regulated signaling cascade within
NCCs and the pharyngeal apparatus.

RESULTS

\textit{Dicer}^{\text{flax/flox}} \textit{Wnt1}^{\text{Cre}} \text{ mutant mouse embryos have defective craniofacial,}
cardiovascular, and thymic development

To determine if miRNA biogenesis is required for the proper development of NCCs,
we disrupted Dicer, a miRNA-processing enzyme, with a \textit{Dicer} allele in which the exon
encoding the second RNase III domains was flanked by loxP sites (Harfe et al., 2005).
We crossed \textit{Wnt1}^{\text{Cre}} transgenic mice, in which Cre recombinase is expressed in pre-
migratory NCC progenitors and progeny at embryonic day 8.5 (E8.5) (Danielian et al.,
1998), with \textit{Dicer}^{\text{flax/flox}} mice. Embryos lacking \textit{Dicer} in NCCs had severe defects in the
development of NCC-derived tissues and died soon after E16.5. In mutants, severe
craniofacial defects were apparent by E14.5 (Figure 9A,B). The NCC-derived maxillary
and mandibular regions of the face and the frontonasal process lacked cartilaginous tissue
(Figure 9C,D). However, the presence of mesodermally-derived cartilage near the base of
the skull and non-NCC-derived tissue in the head (Figure 9D, dashed lines) suggested the
defects were mainly restricted to NCC-derived cartilages at E14.5. We also observed loss
of NCC-derived neuronal tissue from the dorsal root ganglia (DRG) and thoracic
sympathetic ganglia (TSG) in \textit{Dicer}^{\text{flax/flox}} \textit{Wnt1}^{\text{Cre}} mutants by staining with H&E or the
pan-neuronal marker neurofilament-M (Figure 10A,B and 10C,D, respectively).
In addition to defective craniofacial and neuronal development, we also observed defects in the development of other NCC-derived tissues. In some cases, the outflow tract did not fully septate into a pulmonary artery and aorta, resulting in persistence of a common outflow vessel (truncus arteriosus) (Figure 11A,D). In humans, persistent truncus arteriosus is a consequence of NCC defects and often accompanied by a ventricular septal defect and patterning defects of the aortic arch (Srivastava, 2006). 

$Dicer^{floxflox} Wnt1^{Cre}$ mice also had a ventricular septal defect (Figure 11B,E and 11C,F). Furthermore, thymus development, also dependent on NCCs, was absent in $Dicer^{floxflox} Wnt1^{Cre}$ mutants (Figure 12A,C) and delayed in $Dicer$ heterozygous embryos ($Dicer^{floxc+} Wnt1^{Cre}$) at E14.5 (Figure 12B). The haploinsufficiency of $Dicer$ was transient, as the thymus was morphologically indistinguishable in wildtype and heterozygous embryos by E16.5. These diverse anomalies suggest that proper miRNA-mediated post-transcriptional regulation of gene expression is required for development of NCC-derived tissues.

The severe defects in $Dicer$ mutants might be caused by failure of NCCs to delaminate from the neural tube and/or migrate properly. To address this issue, we crossed a Cre-dependent reporter mouse line, R26R-YFP (Soriano, 1999), which marks all progeny of Cre-expressing cells with yellow fluorescent protein (YFP), into the $Dicer^{floxflox} Wnt1^{Cre}$ background. We first investigated the early migratory behavior of NCCs at E8.5 and found no gross defect in the ability of mutant NCCs to delaminate and migrate (Figure 13 A,D). Additionally, NCC migration was grossly unaffected both at
E9.5 (Figure 13B,E and 13C,F) and at E10.5 (Figure 13G,I) in mutants, with observable streams of YFP⁺-NCCs migrating towards the PAs (arrowheads) and normal occupancy of YFP⁺-NCCs in the PAs. YFP was expressed in the craniofacial region of E14.5 mutants, albeit at lower intensity than in wildtype littermates, suggesting that NCC progeny condensed in the proper locations (Figure 13H,J).

In addition to relatively normal migratory behavior YFP⁺-NCCs in the craniofacial region, cells also lined the aorta and common carotid arteries of mutants (Figure 14A,C), although the transverse aorta was absent, consistent with loss of the left fourth aortic arch artery and a discontinuance of the ascending aortic arch with the descending aorta, reflecting improper patterning of the aortic arch due to the left fourth aortic arch defect (Fig. 14C, asterisk, also observable in Figure 12C, asterisk). Although we detected YFP⁺ cells in the outflow tract, these cells did not differentiate into smooth muscle in the wall of the ductus arteriosus (arrows), which provides a necessary vascular connection between the aorta and pulmonary artery in the fetus (Figure 14B,D). These results suggest that abnormal NCC patterning, differentiation or maintenance, rather than delamination or migration, causes the defects seen in Dicer mutant NCC-derived tissues.

Dlx2 expression is decreased in PA1 of Dicer<sup>flax/flox</sup> Wnt1<sup>Cre</sup> mutant mouse embryos

Next, we investigated whether patterning or maintenance of the bilaterally symmetric PAs was defective in Dicer<sup>flax/flox</sup> Wnt1<sup>Cre</sup> mutants. Morphologically, the emergence, size, and shape of the PAs, patterned along the anterioposterior axis, were indistinguishable in mutant and wildtype embryos at E10.5. However, expression of the distal-less homeobox
gene (Dlx2), a regulator of NCC patterning in the first pharyngeal arch (PA1) (Qiu et al., 1995), was downregulated in the mandibular and, less severely, in the maxillary portions of PA1 in mutants (Figure 15A,B). The mandibular component of PA1 (md) contributes to the lower jaw, while the maxillary region of PA1 (mx) populates the palatal region and other craniofacial bones. Other markers of NCC-derived PA mesenchyme, such as Msx1 (Satokata and Maas, 1994), were not significantly diminished (Figure 15C,D). In contrast, expression of fibroblast growth factor 8 (Fgf8), which is required for survival of NCC-derived PA mesenchyme (Trumpp et al., 1999 and Macatee et al., 2003), was also greatly down-regulated in the adjacent mandibular ectoderm of PA1 in mutants (Figure 15E,F).

To determine if loss of Dlx2 and Fgf8 gene expression in PA1 affected NCC cell maintenance, we assessed cell proliferation with an anti-phospho-histone H3 antibody and apoptosis using TUNEL assay. At E10.5, no statistically significant difference between mutant and wildtype embryos was detected by either assay (Figure 16A,E and 16B,F; quantified in Figure 16I,J). Concordantly, at E11.5 proliferation remained unchanged in wildtype and mutant embryos (Figure 16C,G; quantified in Figure 16K); however, apoptosis increased dramatically in the pharyngeal arch region of the mutants (Figure 16D,H; quantified in Figure 16L). Thus, loss of Dicer activity in NCCs disrupted normal PA1 gene expression and survival of NCC-derived mesenchyme.
miR-452 is enriched in neural crest cells and sensitive to Dicer dosage

We sought to identify specific miRNAs whose disruption could contribute to the gene expression changes in \(\text{Dicer}^{\text{floox/floox}}\ Wnt1^{\text{Cre}}\) mutants. We searched for miRNAs enriched in normal NCC-derived PA mesenchyme. Cells from the frontonasal process and PAs of \(\text{Wnt1}^{\text{Cre}}\text{R}26\text{R}^{\text{YFP}}\) embryos were sorted into YFP\(^+\) (NCCs) and YFP\(^-\) (non-NCCs) populations at E10.5 and E11.5 and analyzed by miRNA microarrays. The NCC population at E10.5 was enriched for a single miRNA, miR-452 and for eight additional miRNAs at E11.5 (Figure 17A). Interestingly, a few miRNAs were downregulated in E10.5 NCCs heterozygous for Dicer (\(\text{Dicer}^{\text{floox/+}}\ Wnt1^{\text{Cre}}\text{R}26\text{R}^{\text{YFP}}\)) and among those, miR-452 was the most downregulated of all (Figure 17B). Thus, miR-452 was abundant in the NCC population and was most sensitive to the protein dosage of Dicer.

To determine the relative levels of miR-452 within the NCCs, qRT-PCR revealed a seven-fold enrichment of miR-452 in E10.5 YFP\(^+\) (NCCs) compared to YFP\(^-\) (non-NCCs), thus confirming our microarray data (Figure 17C). Next, we sought to determine the relative levels of PA1 compared to other regions of the embryo, we harvested E10.5 wildtype or \(\text{Dicer}^{\text{floox/floox}}\ Wnt1^{\text{Cre}}\) mutant embryos and dissected them into PA1, midbrain region of the head, heart, limb buds, and tail to measure miR-452 levels via qRT-PCR. The qRT-PCR data revealed that miR-452 was most highly expressed in PA1 and the tail, with lower levels within head and the limb buds, and was nearly undetectable in the heart (Figure 17D). We observed a 2.5-fold decrease in the levels of miR-452 in PA1 of \(\text{Dicer}^{\text{floox/floox}}\ Wnt1^{\text{Cre}}\) embryos, but not other regions of the embryo, suggesting significant expression within the NCC-derived mesenchyme of PA1 (Figure 18B).
miR-452 regulates Dlx2 expression in PA1

The downregulation of Dlx2 in PA1 of Dicer\textsuperscript{flax/flax}Wnt1\textsuperscript{Cre} mutant embryos provided an important assay to determine the contribution of specific miRNAs to Dlx2 expression in the PA. We developed a lipofectamine-based transfection approach to efficiently introduce individual mature miRNA locked-nucleic-acid (LNA) mimics into cultured PAs of E9.5 Dicer\textsuperscript{flax/flax}Wnt1\textsuperscript{Cre} mutant embryos to test for rescue of Dlx2 expression (Figure 18A-C). In situ hybridization on cultured PA1 from wildtype and Dicer\textsuperscript{flax/flax}Wnt1\textsuperscript{Cre} mutants revealed that Dicer\textsuperscript{flax/flax}Wnt1\textsuperscript{Cre} mutant PA1 had Dlx2 downregulation in culture similar to that observed in vivo (Figure 19A,D). We introduced mimics for five of the nine NCC-enriched miRNAs. Only miR-452 consistently rescued Dlx2 expression in Dicer\textsuperscript{flax/flax}Wnt1\textsuperscript{Cre} mutant PAs (Figure 19G and data not shown; representative PA1s shown in Figure 19A-F).

We used antagomirs (Krutzfeldt et al., 2005) targeted against miR-452 to determine if loss of miR-452 activity affects Dlx2 expression in vivo. Antagomirs are cholesterol-modified, antisense oligonucleotides that bind to and inhibit the function of endogenous miRNAs by blocking their incorporation into the RNA-induced silencing complex. Because antagomirs do not cross the placental barrier, we injected miR-452 antagomirs directly into the embryonic sac of wildtype embryos in utero. To validate this delivery method, we injected a fluorophore-modified antagomir into the embryonic sac of E8.5 embryos and observed near ubiquitous uptake of the antagomir throughout the embryos at E10.5 (Figure 20A,B). After confirming the delivery method, we next injected miR-452 antagomir in utero at E8.5. At E11.5, miR-452 knockdown was variable and bimodal as
observed by qRT-PCR (Figure 20C). We found that this method of in vivo miR-452 knockdown resulted in ~35% of embryos with efficient miR-452 knockdown of >70% (n= 9; mean = 86%; median = 94%) and these embryos were used for subsequent experiments; the remainder were considered failed knockdowns. In embryos with efficient miR-452 knockdown, Dlx2 expression in the distal mandibular portion of PA1 in E11.5 embryos was significantly reduced, similar to that in Dicer$^{flox/floxWnt1^{Cre}}$ mutants (Figure 20D,E). Maxillary expression of Dlx2 was only slightly affected, consistent with what was observed in Dicer$^{flox/floxWnt1^{Cre}}$ mutants.

At E16.5, some craniofacial structures of miR-452 antagonir-injected embryos were hypoplastic (Figure 21A-D), particularly the ala orbitalis, palatal process of palatine, and anterolateral process of ala temporalis (Figure 21B,D). Interestingly, some mesoderm-derived tissues, such as the parietal bone, were also affected in the antagonir-injected embryos. This finding implies that a non-NCC function of miR-452 or a non-cell-autonomous affect in the NCCs results in disruption of mesodermal bone development. Not surprisingly, the defects upon miR-452 knockdown were less severe than upon complete loss of miRNA biogenesis, reflecting the function of other miRNAs in craniofacial development or the incomplete loss of miR-452. Nevertheless, these results indicate that miR-452 is involved in proper Dlx2 gene expression in NCC-derived mesenchyme and also for a subset of subsequent craniofacial development.
miR-452 directly targets Wnt5a and loss of miR-452 leads to decreases in Shh and Fgf8 signaling in the mandibular component of PA1

To understand the mechanism by which miR-452 regulated Dlx2 expression, we sought to identify the direct target(s) of miR-452 in NCCs and their derivatives. Putative targets were identified with an in-house miRNA:mRNA algorithm that considers sequence specificity, binding site accessibility, and evolutionary conservation of the binding site, and potential sites were then tested experimentally (Cordes et al., 2009). The neural crest stem cell (NCSC) line Joma1.3 (Maurer et al., 2007) was transfected with miR-452 mimic along with constitutively active luciferase reporter genes containing the 3´-UTR of putative targets. Repression of luciferase activity was only observed in the presence of the Wnt5a 3´-UTR (Figure 22A). Wnt5a, a non-canonical member of the Wnt family of signaling molecules, is expressed in NCC-derived mesenchyme of the PAs (Gavin et al., 1990), and its 3´-UTR contains a miR-452 binding site (Figure 22B). Mutating the seed region of the miR-452 binding site within the Wnt5a 3´-UTR led to loss of the miR-452-dependent decrease in luciferase activity, suggesting that the activity of the predicted binding site depends on miR-452 binding (Figure 22B). Furthermore, co-transfection of miR-452 inhibitors into NCSCs containing a luciferase vector with the Wnt5a 3´-UTR resulted in a dose-dependent increase in luciferase activity (Figure 22D). In addition to changes in luciferase activity, western blot revealed reduced Wnt5a protein levels, but not mRNA, in NCSCs transfected with miR-452 mimics (Figure 23, top lanes). We also observed a decrease in Wnt5a protein levels in our PA culture system upon transfection of miR-452 mimic (Figure 23, bottom lanes and graph). These results
suggest that endogenous Wnt5a protein levels are sensitive to miR-452 overexpression in vitro and in vivo.

In various developmental roles, Wnt5a acts as a signaling mediator of epithelial-mesenchymal interactions. For example, Wnt5a expression in lung epithelium negatively regulates Shh responsiveness of the underlying mesenchyme (Li et al., 2005). In PAs, Wnt5a is primarily expressed in NCC-derived mesenchyme (Gavin et al., 1990), and whole-mount in situ hybridization revealed higher levels in the mandibular component of PA1 (data not shown). In contrast, Shh is produced in the pharyngeal endoderm where it diffuses into the NCC-derived mesenchyme and activates downstream signaling cascades (Yamaguchi et al., 1999 and Washington Smoak et al., 2005). Shh is required for proper development of PAs and, by positively regulating Fgf8 expression in the pharyngeal ectoderm, supports Dlx2 expression in the NCC-derived mesenchyme (Thomas et al., 1998, Haworth et al., 2007 and Yamagishi et al., 2006).

To determine if Wnt5a inhibits the responsiveness of NCSCs to Shh stimulus, we transfected NCSCs with a human Wnt5a expression plasmid, then 24 hours later, treated cells with recombinant Shh protein and measured the expression levels of two Shh-responsive genes, Gli1 and Ptch1. Overexpression of Wnt5a blunted the increase in Gli1 and Ptch1 mRNA expression in response to Shh treatment (Figure 24A). To test this in vivo, we implanted beads soaked in recombinant Wnt5a or bovine serum albumin (control) into PA1 of mice harboring a lacZ gene in the Ptch1 locus (Goodrich et al., 1997) as a reporter of Shh signaling. β-Gal expression was decreased circumferentially
around the Wnt5a bead, but not around the control bead placed in the contralateral PA (Figure 24B). Next, we asked if increased Wnt5a protein levels was sufficient to result in decreased \( \text{Dlx2} \) expression, as observed in the \( \text{Dicer} \) mutants and miR-452 knockdown experiments. Again, Wnt5a- and BSA-soaked beads were implanted into the mandibular component of cultured PA1 of wildtype E10.5 embryos. In-situ hybridization to assay \( \text{Dlx2} \) expression revealed a marked decrease in \( \text{Dlx2} \) expression specifically surrounding the Wnt5a soaked beads, while \( \text{Dlx2} \) expression was unaffected surrounding the contralateral BSA soaked beads (Figure 24C). Thus, increased Wnt5a protein levels in the mandibular component of PA1 led to a decrease in Shh signaling and \( \text{Dlx2} \) expression.

We investigated whether miR-452 promotes \( \text{Dlx2} \) expression in part by repressing the translation of Wnt5a, allowing efficient Shh signaling to NCCs. Shh positively regulates expression of \( \text{Fgf8} \) in the pharyngeal ectoderm, and \( \text{Fgf8} \) expression is required for \( \text{Dlx2} \) expression in the NCC-derived mesenchyme (Thomas et al., 2000 and Haworth et al., 2007). Thus, we hypothesized that loss of miR-452 would decrease endogenous Shh signaling and \( \text{Fgf8} \) expression within the endoderm and ectoderm, respectively, of PA1. Indeed, expression of \( \text{Gli1} \) was decreased in the mandibular component of PA1 in miR-452 antagonir-injected embryos at E11.5 and expression of \( \text{Fgf8} \) was markedly lower in the mandibular ectoderm (Figure 25A-D). The expression of \( \text{Gli1} \) and \( \text{Fgf8} \) were unchanged in the maxillary component of PA1, providing an internal control for expression. The mandibular-specific effects of miR-452 knockdown were consistent with this miRNA participating in the greater mandibular loss of \( \text{Fgf8} \) and \( \text{Dlx2} \) expression in
the Dicer\textsuperscript{flx/flx} Wnt\textsuperscript{Cre} mouse and the mandibular enrichment of Wnt5a expression. These data suggest that loss of miR-452, resulting in an increase in Wnt5a protein, leads to decreases in Shh signaling and Fgf8 expression, which in turn results in the decrease in Dlx2 expression seen in both Dicer mutant and miR-452 antagonir-injected embryos.

**DISCUSSION**

Here we show that miRNA biogenesis is essential for the proper development of NCC-derived tissues, including those that contribute to craniofacial, cardiovascular, thymic, and nervous system structures. More importantly, we found that miR-452 was the most enriched miRNA in early NCCs and regulated epithelial-mesenchymal interactions in the mandibular region of PA1 by directly targeting Wnt5a in the NCC-derived mesenchyme. Our findings suggest that miR-452 negatively regulates secretion of Wnt5a from the NCC-derived mesenchyme of PA1, and that Wnt5a normally negatively regulates Shh signaling to the ectoderm (see model, Figure 26). Since Shh produced in the endoderm activates ectodermal expression of Fgf8 (Haworth et al., 2007) and secreted Fgf8 functions to promote Dlx2 expression in the neighboring NCC-derived mesenchyme (Thomas et al., 2000), miR-452 may regulate Dlx2 positively in part through this complex epithelial-mesenchymal interaction. Of course, miR-452 and other NCC-enriched miRNAs likely target other mRNAs in the NCC-derived mesenchyme of PA1 that further regulate epithelial-mesenchymal interactions required for proper PA development.
In the present work, we did not address which specific NCC-enriched miRNAs function in the other arches that give rise to the thymus (PA3) or the cardiac outflow tract and aortic arch arteries (PA3, -4, and -6), all of which were affected in the \textit{Dicer} mutants. One or more of the other eight NCC-enriched miRNAs likely contribute to development of these structures in a stage-specific manner. Notably, the thymus and cardiac outflow tract also require cross-tissue interactions for their development and maturation (Olson, 2006 and Gordon et al., 2010). For example, reciprocal signaling between the second heart field and cardiac NCCs is required for proper patterning of the aortic arch arteries (Waldo et al., 2005). Deletion of Notch signaling specifically in the second heart field leads to altered NCC behavior, resulting in a decrease of NCC-derived tissue in the outflow tract and improper patterning of the arch arteries (High et al., 2009). Other NCC-enriched miRNAs probably regulate similar pathways to coordinate the complex tissue-tissue interactions required to pattern the outflow tract and aortic arch arteries.

Interestingly, the phenotype of the \textit{Dicer}^{\text{flax/flax}}\textit{Wnt1}^{\text{Cre}} mutant mice has overlapping features with the human disease, DiGeorge syndrome (DGS). DGS patients typically present with craniofacial, cardiovascular and thymic anomalies arising from a 3-megabase deletion of chromosome 22q11 (Lindsay, 2001). In that region, \textit{Tbx1} has been strongly implicated in the etiology of DGS. However, \textit{Tbx1} is not expressed in the NCCs, rather, it is expressed in the pharyngeal ectoderm, endoderm and early second heart field progenitors (Merscher et al., 2001 and Yamagishi et al., 2003). Tbx1 is downstream of Shh in the pharyngeal endoderm and regulates the expression of \textit{Fgf8}, resulting in a secondary effect on the adjacent neural crest-derived mesenchyme that expresses Fgf
receptors (Anselmo et al., 1992, Garg et al., 2001, Hu et al., 2004 and Zhang et al., 2005). Another intriguing gene in the DGS-deleted region is DGCR8, which is required for miRNA biogenesis (Gregory et al., 2004), and thus suggests a possible contribution of miRNA dysregulation in DGS. Our finding that $Dicer^{\text{flox/+}} Wnt1^{\text{Cre}}$ heterozygous embryos have hypoplastic thymus primordia indicates that a hemizygous deletion of members of the miRNA biogenesis pathway in NCCs may pre-dispose to some DGS-like phenotypes. Disruption of cross-tissue signaling cascades has been found in a number of human neural crest disorders (Lindsay et al., 2001 and Ornitz and Marie, 2002). The results presented here suggest a critical role for miRNAs in titrating such reciprocal signaling during NCC development and may have implications for the potential contribution of miRNA dysregulation in human neural crest disorders.

Finally, the inhibition of Shh signaling imposed by Wnt5a overexpression represents a novel interaction between the two signaling pathways in PA1. A similar tissue-tissue interaction involving Wnt5a and Shh signaling has been observed in the epithelial and mesenchymal tissues of the developing lung (Li et al., 2002 and Li et al., 2005). This work shows that the ability of Wnt5a to inhibit Shh signaling in a paracrine fashion is conserved in the PAs. However, the mechanism by which these two signaling pathways interact is still unknown. In Chapter 3, we will examine the nature of this interaction and show that Wnt5a can inhibit Shh signaling mainly through activation of PKA and the likely increased processing of the Gli repressor proteins.
FIGURE LEGENDS

Figure 9: miRNAs are required for proper craniofacial development

(A,B) Lateral view of E14.5 wildtype (A) and Dicer\textsuperscript{flax/flax} Wnt\textsuperscript{Cre} mutant (B) mouse embryos. (C,D) Cartilage staining with Alcian blue shows loss of craniofacial cartilaginous tissue (dashed line, arrow) in Dicer mutants (D) but not wildtype embryos (C). SP, styloid process; MC, Meckel’s cartilage; FN, frontonasal cartilage. Scale bars are represented as 2 mm.

Figure 10: NCC-derived neuronal tissue is lost upon Dicer ablation

(A,B) H&E staining of transverse sections from E13.5 wildtype (A) and Dicer mutant (B) embryos showing loss of dorsal root ganglia (DRG, arrowheads) and thoracic sympathetic ganglia (TSG, arrows). (C,D) Transverse sections through the neural tube of E13.5 wildtype (C) and Dicer mutant (D) embryos focused on the dorsal root ganglia (DRG, arrowheads) immunostained for the pan-neuronal marker neurofilament-M (NF-M). DRG, dorsal root ganglia; TSG, thoracic sympathetic ganglia; NT, neural tube; NF-M, neurofilament-M. Scale bars are represented as 100 µm.

Figure 11: Loss of NCC-miRNAs leads to persistent truncus arteriosus and ventricular septal defects

(A,D) Transverse sections of the outflow tract region at E14.5 showing normal septation of the pulmonary artery (PA) and aorta (Ao) in wildtype embryos (A), but communication between the two vessels remains, reflecting a truncus arteriosus (TA) in Dicer mutants (D). (B,E) Transverse sections of the right and left ventricles of E14.5
wildtype (B) and Dicer mutant (E); arrowhead indicates ventricular septal defect. (C,F) Latex injections into the left ventricle of E14.5 embryonic hearts show a ventricular septal defect (arrowhead) in Dicer mutants (F) with latex flow from the left to right ventricle. Tr, trachea; Ao, aorta; PA, pulmonary artery; LA, left atrium; TA, truncus arteriosus; RV, right ventricle; LV, left ventricle. Scale bars are represented as 100 µm.

**Figure 12: Thymus development is sensitive to Dicer-dosage in NCCs**

(A–C) Brightfield images of thymus (dashed lines; image contrast-enhanced to visualize thymus) from E14.5 wildtype (A), Dicer\(^{+/+}\) Wnt1\(^{Cre}\) heterozygous (B), and Dicer\(^{\beta/\beta}\) Wnt1\(^{Cre}\) mutant (C) embryos; arrow indicates absence of thymus in mutant. Asterisk in (C) indicates the interrupted communication between the ascending aorta and descending aorta. Scale bars are represented as 2 mm.

**Figure 13: NCC delamination and migration is unaffected in Dicer-mutant embryos**

(A,D) Transverse sections of E8.5 Dicer\(^{+/+}\) Wnt1\(^{Cre}\) R26R\(^{YFP}\) (A) and Dicer\(^{\beta/\beta}\) Wnt1\(^{Cre}\) R26R\(^{YFP}\) (D) embryos through the neural tube stained for YFP (NCCs) showing YFP\(^+\)-NCCs migrating from the neural tube towards the pharyngeal arches (arrowheads). (B,E) Lateral view of E9.5 pharyngeal arch region of Dicer\(^{+/+}\) Wnt1\(^{Cre}\) R26R\(^{YFP}\) (B) and Dicer\(^{\beta/\beta}\) Wnt1\(^{Cre}\) R26R\(^{YFP}\) (E) embryos showing lineage tracing of NCCs; arrowheads indicate migrating streams of NCCs. (C,F) Transverse sections of E9.5 Dicer\(^{+/+}\) Wnt1\(^{Cre}\) R26R\(^{YFP}\) (C) and Dicer\(^{\beta/\beta}\) Wnt1\(^{Cre}\) R26R\(^{YFP}\) (F) embryos showing YFP\(^+\)-NCCs migrating from the neural tube towards the pharyngeal arches (arrowheads). (G,I) Lateral views of E10.5 pharyngeal arch region of Wnt1\(^{Cre}\) R26R\(^{YFP}\)
(G) and \( \text{Dicer}^{\beta/\beta} \text{Wnt1}^{\text{Cre}r26R^{YFP}} \) (I) embryos showing lineage tracing of NCCs; arrowheads indicate migrating streams of NCCs. (H,J) Lateral view of the craniofacial region of E14.5 \( \text{Dicer}^{+/+} \text{Wnt1}^{\text{Cre}r26R^{YFP}} \) (H) and \( \text{Dicer}^{\beta/\beta} \text{Wnt1}^{\text{Cre}r26R^{YFP}} \) (J) embryos. Scale bars are represented as: 2 mm, whole embryos; 100 µm, sections.

Figure 14: miRNAs are required for proper differentiation of NCC-derived smooth muscle cells of the cardiac outflow tract

(A,C) Images of the outflow tracts and aortic arch of E14.5 \( \text{Wnt1}^{\text{Cre}r26R^{YFP}} \) (A) and \( \text{Dicer}^{\beta/\beta} \text{Wnt1}^{\text{Cre}r26R^{YFP}} \) (C) embryos; asterisk denotes the transverse aorta, absent in mutant (C), with the vascular connection to the DAo being the ductus arteriosus (arrow). (B,D) Immunofluorescence of transverse sections through the aortic arch of (A) and (C) stained for YFP (green) and smooth-muscle actin (SMA, red); arrow in (D) indicates the absence of NCC-derived smooth muscle cells in the wall of the ductus arteriosus (DA) as is present in (C), connecting the Ao and PA. RCC, right common carotid artery; LCC, left common carotid artery; RSCA, right subclavian artery; DA, ductus arteriosus; AAo, ascending aorta; DAo, descending aorta; Ao, aorta; PA, pulmonary artery. Scale bars are represented as: 2 mm, whole embryos; 100 µm, sections.
Figure 15: Dicer^{fl/fl} Wnt1^{Cre} mutant mouse embryos have decreased Dlx2 and Fgf8 expression in the mandibular component of the first pharyngeal arch

In situ hybridization staining of E10.5 wildtype (A,C,E) and Dicer mutant (B,D,F) embryos for expression of Dlx2 (A,B), Msx1 (C,D), and Fgf8 (E,F); arrows indicate the mandibular component of PA1 and insets are higher magnification views of this region. H, head; mx, maxillary component of PA1; md, mandibular component of PA1; LB, limb bud. Scale bars are represented as 500 µm.

Figure 16: Dramatic increase in cell death of NCCs in Dicer mutant embryos

(A,E,I) Phospho-histone H3 (pH3) immunohistochemistry to assay proliferating cells of representative frontal sections from E10.5 wildtype (A) and Dicer mutant (E) mouse embryos, quantified from multiple sections in (I). (B,F,J) TUNEL assay to quantify apoptotic cells of representative frontal sections from E10.5 wildtype (B) and Dicer mutant (F) mouse embryos, quantified from multiple sections in (J). (C,G,K) pH3 immunohistochemistry to assay proliferating cells of representative frontal sections from E11.5 wildtype (C) and Dicer mutant (G) mouse embryos, quantified from multiple sections in (K). (D,H,L) TUNEL assay in frontal sections of E11.5 wildtype (D) and Dicer mutant (H) embryos, quantified from multiple sections in (L); *P<0.05; error bars indicate 95% confidence intervals. mx, maxillary component of PA1; md, mandibular component of PA1; LB, PA2, pharyngeal arch 2; FN, frontonasal process; n.s., not statistically significant. Scale bars are represented as 200 µm.
Figure 17: miR-452 is highly enriched in NCCs and is sensitive to Dicer dosage

(A) NCCs (YFP⁺) and non-NCCs (YFP⁻) were sorted by FACS from the PA and frontonasal process regions of Wnt1CreR26RYFP E10.5 and E11.5 embryos. miRNA levels were measured by microarray analysis of YFP⁺ vs. YFP⁻ cells. (B) NCCs (YFP⁺) were sorted by FACS from wildtype (Wnt1Cre R26RYFP) and Dicer heterozygous (Dicerfl/+ Wnt1Cre R26RYFP) E10.5 embryos. miRNA levels were measured by microarray analysis of heterozygous and wildtype NCCs. (C) qRT-PCR results measuring relative miR-452 expression levels from the E10.5 FACS sorted cells in (A). (D) qRT-PCR results measuring relative miR-452 expression levels in the tissues indicated from E10.5 wildtype (blue bars) or Dicer mutant (red bars) embryos; data normalized to miR-452 levels in the heart. *P<0.05; error bars indicate 95% confidence intervals.

Figure 18: Efficient transfection of PA explants with miRNA mimics

(A) qRT-PCR quantification of miR-452 levels from cultured first pharyngeal arch (PA1) explants transfected with increasing concentration of locked-nucleic acid miR-452 mimic (red bars) or with control mimic (blue bars). (B,C) Cultured PA1 explants transfected with AlexaFluor Block-IT reagent to determine small-oligo transfection efficiency via brightfield (B) and fluorescence imaging (C) to show efficiency of transfection by this technique. Data normalized to miR-452 expression levels transfected with control mimic. Error bars indicate 95% confidence intervals. Md, mandibular component of PA1; BF, brightfield; Fluor, fluorescence. Scale bars are represented as 200 µm.
Figure 19: miR-452 rescues Dlx2 expression in Dicer mutants

(A-F) Representative images of Dlx2 expression in wildtype or Dicer mutant E9.5 PA1 cultures with or without transfection of control, miR-452, or miR-513 mimics. (G) The PA1 area that stained positive for Dlx2 relative to total area of the PA in Dicer mutants and wildtype embryos is quantified (area defined by dashed lines). The PAs were transfected with control mimic (n = 17) or with a miR-452 mimic (n = 21) or a miR-513 mimic (n = 9). *P<0.05; error bars indicate 95% confidence intervals. Scale bars are represented as 200 µm.

Figure 20: In utero delivery of anti-miR-452 leads to loss of Dlx2 expression similar to Dicer mutants

(A,B) E8.5 embryos were transfected in utero with a fluorophore-modified antagonomir and harvested at E10.5 to determine the extent of antagonomir uptake in vivo. (A) Brightfield images and (B) fluorescence images of injected embryos and embryonic sac showing nearly ubiquitous embryonic uptake of the fluorophore-modified antagonomir. (C) Box plots representing qRT-PCR quantification of miR-452 levels from the PA region of littermate embryos injected in utero with PBS as a control (black box, n=25) or miR-452 antagonomir (blue and red boxes, n = 28); boxes represent interquartile range, thick bar represents the median for the sample set, and error bars indicate one standard deviation from the median. The miR-452 antagonomir injected embryos were separated into two cohorts; those with greater than 70% knockdown of miR-452 (red box; n = 9) and those with less than 70% knockdown (blue box; n = 19). Only those embryos with greater than
70% knockdown were considered for further study. (D,E) Dlx2 in situ hybridization of littermate E11.5 embryos injected in utero at E8.5 with PBS (D) or miR-452 antagonir (E). Arrowhead indicates the decreased Dlx2 expression specifically in the mandibular component of PA1. Mx, maxillary component of PA1; md, mandibular component of PA1; LB, limb bud. Scale bars are represented as 500 µm.

Figure 21: Loss of miR-452 leads to specific craniofacial skeletal defects

(A–D) Alcian blue (cartilage) and alizarin red (bone) skeletal staining of littermate E16.5 embryos injected in utero at E8.5 with PBS (A,C) or miR-452 antagonir (B,D) imaged both in oblique lateral (A,B) and ventral (C,D) views; asterisk in (B) marks the missing cartilaginous structure, the ala orbitalis (alo). Dashed lines in (B) indicate the absence of the parietal bone (pb). Dnt, dentary; alo, ala orbitalis; pb, parietal bone; ppmx, palatal process of maxilla; iov, incisura ovale; pppl, palatal process of palatine; alat, anterolateral process of ala temporalis. Scale bars are represented as 1 mm.

Figure 22: The Wnt5a 3’-UTR is a direct target of miR-452

(A) Fold change in luciferase reporter activity relative to luciferase levels in the presence of the putative target 3’-UTRs in the sense orientation (S-target) or, as a control, in the anti-sense direction (AS-target), in JoMa1.3 NCSCs with or without co-transfection with a miR-452 mimic. *P<0.05 vs. S-target. (B) Putative miR-452 binding site in the mouse Wnt5a 3’-UTR. Vertical lines indicate sequence matching and residues mutated are
underlined. Relative luciferase activity in the presence of wildtype or mutant miR-452 binding site with or without miR-452 mimic in Joma1.3 NCSCs. *P<0.05. (C)

Quantification of Wnt5a mRNA levels via qRT-PCR from NCSCs transfected with control mimic (black bar) or miR-452 mimic (blue bar). (D) Luciferase values from NCSCs co-transfected with a luciferase reporter vector containing the Wnt5a 3’-UTR and an increasing concentration of a miR-452 inhibitor (colored bars). Values were normalized to NCSCs transfected with no inhibitor (black bar); n.s., not statistically significant. All experiments were done at least in triplicate. Error bars indicate 95% confidence intervals.

Figure 23: Wnt5a protein levels are diminished upon miR-452 expression

Wnt5a protein from JoMa1.3 NCSCs (top lanes) or cultured wildtype E10.5 PAs (bottom lanes) transfected with or without miR-452 mimic assessed by western blot. Quantification of Wnt5a protein levels from western blot analysis of PAs using LI-COR software is shown (n=5). *P<0.05; error bars indicate 95% confidence intervals.

Figure 24: Wnt5a inhibits Shh signaling in NCSCs and PA culture

(A) Expression of indicated genes by qRT-PCR in NCSCs transfected with or without an expression plasmid harboring human Wnt5a cDNA (+Wnt5a) treated with or without 0.5 µg of recombinant Shh protein. Graph shows change in gene expression relative to control (0 µg Shh); error bars indicate 95% confidence intervals. *P<0.05, **P<0.05 vs.
control. (B) Ptch1 expression, as visualized by β-Gal staining from Ptch1-LacZ E10.5 mouse embryo PA explants cultured with a bead soaked in bovine serum albumin (BSA) or recombinant Wnt5a protein (arrowheads). (C) Dlx2 expression, as visualized by in situ hybridization, from wildtype E10.5 mouse embryo PA explants with beads soaked in BSA or recombinant Wnt5a protein (arrowheads). Frontal view of pharyngeal arch is shown in (B and C). Scale bars are represented as 100 μm.

**Figure 25: Loss of miR-452 leads to decreased Shh signaling in PA1**

(A-D) Whole-mount in situ hybridization for Gli1 (A,C) and Fgf8 (B,D) in E11.5 embryos injected at E8.5 in utero with PBS (A,B) or miR-452 antagonir (C,D) reveal downregulation of gene expression in the mandibular component of PA1 (arrows). Insets represent higher magnification of the mandibular components of PA1 indicated by red boxes. md, mandibular component of PA1; mx, maxillary component of PA1. Scale bars are represented as 250 μm.

**Figure 26: Model depicting the mechanism by which miR-452 regulates an epithelial-mesenchymal signaling cascade in PA1 converging on Dlx2 expression.**

Our data show that Wnt5a is a direct target of miR-452, leading to decreased levels of the Wnt5a protein in NCCs. Upon loss of miR-452, Wnt5a protein levels increase and lead to inhibition of Shh signaling in the PAs, by a mechanism that will be explored in Chapter 3. The inhibition of Shh signaling leads to an expected decrease in Fgf8
expression, resulting in disrupted expression of Dlx2. Our data suggests that miR-452 is positively regulates PA expression of Dlx2 through an epithelial-mesenchymal signaling cascade controlled by Wnt5a. Ect, pharyngeal ectoderm; NCC, NCC-derived mesenchyme; End, pharyngeal endoderm.
Figure 9: miRNAs are required for proper craniofacial development
Figure 10: NCC-derived neuronal tissue is lost upon Dicer ablation
Figure 11: Loss of NCC-miRNAs leads to persistent truncus arteriosus and ventricular septal defects
Figure 12: Thymus development is sensitive to Dicer-dosage in NCCs
Figure 13: NCC delamination and migration is unaffected in Dicer-mutant embryos
Figure 14: miRNAs are required for proper differentiation of NCC-derived smooth muscle cell of the cardiac outflow tract
Figure 15: Dicer^{fl/fl} Wnt1^{Cre} mutant mouse embryos have decreased Dlx2 and Fgf8 expression in the mandibular component of the first pharyngeal arch.
Figure 16: Dramatic increase in cell death of NCCs in Dicer mutant embryos
Figure 17: miR-452 is highly enriched in NCCs and is sensitive to Dicer dosage
Figure 18: Efficient transfection of PA explants with miRNA mimics
Figure 19: miR-452 rescues Dlx2 expression in Dicer mutants
Figure 20: In utero delivery of anti-miR-452 leads to loss of Dlx2 expression similar to Dicer mutants.
Figure 21: Loss of miR-452 leads to specific craniofacial skeletal defects
Figure 22: The Wnt5a 3’-UTR is a direct target of miR-452
Figure 23: Wnt5a protein levels are diminished upon miR-452 expression
Figure 24: Wnt5a inhibits Shh signaling in NCSCs and PA culture
Figure 25: Loss of miR-452 leads to decreased Shh signaling in PA1
Figure 26: Model depicting the mechanism by which miR-452 regulates an epithelial-mesenchymal signaling cascade in PA1 converging on Dlx2 expression.
CHAPTER 3

The cross-tissue interaction between Wnt5a and Shh signaling is a result of increased PKA signaling, and likely, disrupted Gli processing

BACKGROUND

Wnt-family proteins are secreted signaling molecules that represent a crucial family of developmental regulators that influence a number of processes; including differentiation, proliferation, apoptosis, cell polarity, and migration (Wodarz and Nusse, 1998). In mouse and human there are 19 highly conserved Wnt ligands that bind to a number of different receptors. The Wnt receptors include 10 different Frizzled members (Fzd), Lrp5, Lrp6, Ror2, and Ryk (Li et al., 2008 and Angers and Moon, 2009). Throughout development, the expression of the various Wnt ligands and receptors is temporally and spatially regulated, allowing for the regulation of a vast array of developmental processes. The most studied intracellular signaling processes regulated by the Wnt ligands is the β-catenin-dependent pathway (known as the “canonical” Wnt pathway) (He et al., 2004). Although the canonical pathway is the best studied Wnt pathway, there are a number of Wnts that activate the non-canonical, β-catenin-independent pathway and classically act in regulating cell polarity and migration (Veeman et al., 2003). The non-canonical Wnt signaling pathway has been shown to activate a variety of different pathways. The various pathways activated by non-canonical Wnt ligands has made studies difficult, but a number of these pathways have been identified and characterized, such as PKC, NFAT, JNK, and RHOA among others (Katoh and Katoh, 2007). Wnt5a is
one of the main representative ligands for the β-catenin-independent pathways. Mice homozygous for a Wnt5a- null allele are truncated caudally, displaying an inability to extend the embryonic anterioposterior axis. In addition, affected structures, including fore- and hindlimbs, snout, mandible, tongue, genital tubercle, and tail are shortened along the outgrowing axis. Finally, these Wnt5a-null mice suffer perinatal lethality due to asphyxia (Yamaguchi et al. 1999).

Mutant animals that have a truncated anterioposterior body axis typically have mutations in genes involved in planar cell polarity (PCP) of which the non-canonical Wnt signaling pathway is an important regulator (Seifert and Mlodzik, 2007). In addition to establishing the anterioposterior axis, the PCP pathway is also responsible for establishing proper orientation of the centrosomes and the cilia that eminate from them. Importantly, both Wnt5a- and Ror2-null embryos show characteristics of misaligned cilia (Li et al., 2002 and Minami et al., 2010). The cilia are microtubule-based projections that stick out into the extra-cellular space from nearly all cells. In addition to acting as cellular projections that participate in mechanoreception, the cilia have more recently been shown to be major signaling centers where a number of different signaling pathways become concentrated (Lin et al., 2003 and Singla and Reiter, 2006). One of the best studied signaling pathways that require cilia for proper activity is the hedgehog signaling pathway.

Sonic hedgehog (Shh) is the most well studied member of the hedgehog family of signaling ligands. Shh requires two transmembrane proteins to transmit the proper
signal: Patched (Ptc) and Smoothened (Smo) (Alcedo et al., 1996). In the absence of the Shh ligand, Ptc binds to Smo and inhibits it from transducing the signal to subsequent downstream effectors. Upon Shh binding to Ptc, the inhibition on Smo is released and the protein is able to actively relay the signal (Taipale et al., 2002). Downstream of Smo, the Shh signaling pathway culminates on the regulation of the Gli-family of transcription factors which can act as repressors or activators of gene expression dependent on the presence or absence of Shh ligand binding, respectively (Huangfu and Anderson, 2006). In vertebrates, the Gli-family consists of three members; Gli1, which acts solely as an activator of gene expression, and Gli2 and Gli3 which show both inhibitory and activating activity on gene expression. However it should be noted that only a small fraction of Gli2 protein is processed in to an inhibitory form and that Gli3 acts as the major inhibitory factor in Shh signaling (Pan et al., 2006 and Pan et al., 2009). These different functions of the Gli proteins is dependent on the activity of Smo (Jacob and Briscoe, 2003). Importantly, in the absence of Shh ligand, and thus the inhibition of Smo activity, Gli3 is converted into an inhibitory transcription factor (Gli3R) by the proteolytic cleavage of the C-terminal protein domain. After cleavage of this domain, Gli3R enters the nucleus and inhibits the expression of Shh-regulated genes (Dai et al., 1999, Ruiz i Altaba, 1999, Sasaki et al., 1999 and Wang et al., 2000). Recently, it was shown that the cleavage of Gli3 requires a phosphorylation that is mediated by protein kinase A (PKA) and that binding of the Shh ligand leads to repression of PKA activity, thus inhibiting the formation of Gli3R (Wen et al., 2010).
In the previous chapter we discovered that, in addition to the reported interaction of Wnt5a and Shh signaling seen in lung tissues (Li et al., 2002 and Li et al., 2005), Shh signaling is diminished upon upregulation of Wnt5a in both NCSCs and ex vivo PA explant cultures (Sheehy et al., 2010). In this chapter we will further investigate the mechanism by which Wnt5a signaling leads to the inhibition of the Shh signaling pathway. We found that besides lung and PA tissue, this interaction between Wnt5a and Shh signaling persists in a number of cell types. We also show that despite the intriguing link between Wnt5a-dependent PCP signaling and its role in cilia function, it does not appear to be the major mechanism by which Wnt5a inhibits Shh signaling. Rather, the overexpression of Wnt5a leads to the activation of PKA, through increased cyclic AMP (cAMP) production, and likely leads to increased production of Gli3R.

**RESULTS**

**Wnt5a inhibits Shh responsiveness in multiple cell types.**

Based on the complimentary expression patterns of Wnt5a and Shh throughout development, we hypothesized that the Wnt5a-Shh signaling interaction is conserved in multiple tissue types. To test this, we overexpressed Wnt5a in three different cell types; multipotent NCSCs (Joma1.3), mouse embryonic fibroblasts (NIH3T3) and human embryonic kidney cells (HEK293). The cells were treated in the presence or absence of exogenous Shh recombinant protein to activate the pathway, following either control plasmid or an expression plasmid that forced the overexpression of Wnt5a. We
determined Shh signaling activity via two different methods; qRT-PCR for Gli1 expression, a well established readout for active Shh-mediated signaling, or expression of a Gli-responsive luciferase vector, where increases in Shh signaling leads to upregulation of luciferase expression. In all cell lines and both test types, overexpression of Wnt5a leads to inhibition of Shh signaling based on Gli expression and activity (Figure 27A,B). Interestingly, we found that in HEK293 cells there was a high level of autocrine Shh signaling by which addition of exogenous Shh protein did not lead to further increases in Gli1 expression. Despite this high level of autocrine signaling, Wnt5a was still seen to be a potent inhibitor of Gli1 expression (Figure 27A). These results show that Wnt5a can inhibit Shh signaling in multiple cell types, suggesting that it may be a conserved regulator of Shh signaling pathway during development.

**Wnt5a activity does not affect ciliogenesis or overall cilia length**

It has been recently appreciated that the majority of Shh signaling is coordinated through the cilia, with a number of members of the Shh signaling pathway being localized on or near the cilia (Corbit et al., 2005, Rohatgi et al., 2007 and Goetz and Anderson, 2010). Additionally, ablation of ciliogenesis has been shown to disrupt Shh signaling (Huangfu et al., 2003). Combining these data with the fact that loss of either Wnt5a or Ror2 leads to defects in ciliogenesis and cilia positioning (Li et al., 2002 and Minami et al., 2010) lead us to hypothesize that overexpression of Wnt5a in our system may be leading defects in ciliogenesis.
To test this hypothesis, we cultured Joma1.3 NCSCs under conditions where Wnt5a was being overexpressed. In both control (wildtype) and Wnt5a overexpressing (Wnt5a OE) we stained the cells via immunofluorescence for acetylated-tubulin (AcTub), the stabilized form of tubulin that is present in cilia, and Ninein, a marker of the centrosomes. Under both conditions, cilia formation appeared to be unaffected and the cilia appeared to be localized properly (Figure 28A,B). We counted nearly 2000 cells from multiple experiments for both conditions and found that ~75% of the cells were ciliated properly in each condition with no significant difference between the two conditions (Figure 28C). We also measured the length of the cilia on those ciliated cells and found no significant difference in cilia length under either condition (Figure 28D). These results suggested that the formation of cilia was unaffected by overexpression of Wnt5a.

**Wnt5a activity relies on G-protein signaling to inhibit Shh signaling**

Based on our findings that Wnt5a overexpression did not appear to affect ciliogenesis, we next sought to determine the pathway(s) downstream of Wnt5a binding that may be activated and lead to the inhibition of Shh signaling. The β-catenin-independent pathway has multiple downstream routes and it would be necessary to determine which pathway is responsible for the interaction with Gli activity (Katoh and Katoh, 2007). In order to narrow down the pathways responsible for inhibiting Gli activity, we took a candidate screen approach to knockdown pathways which have been shown to be activated upon Wnt5a stimulation. We utilized the following inhibitors to knockdown the activity of various pathways: cyclosporine A (CsA, calcineurin inhibitor), KN93 (CaMKII inhibitor), pertussis toxin (PTX, G-protein inhibitor), JNK inhibitor II (JNK), 2’Z,3’E-6-
bromoindirubin-3’-oxime (BIO, GSK-3β inhibitor), and bisindolylmaleimide I (BIMI, PKC inhibitor). Although we determined that β-catenin activity was unlikely to be changed via Wnt5a overexpression based on expression of Axin2 (data not shown), we decided to also test the possibility of canonical-Wnt signaling by using the GSK-3β inhibitor BIO to artificially activate β-catenin stability and thus activate β-catenin-dependent signaling.

These inhibitors were added to either NCSCs or HEK293 cells with or without Wnt5a overexpression. NCSCs do not express the Shh ligand in vitro, so in order to test the activity of the Shh signaling pathway exogenous Shh protein was added to the media (Figure 29A). As mentioned, HEK293 cells already have a highly active Shh signaling pathway, so exogenous Shh protein addition was unnecessary (Figure 29B). The resulting changes in Gli activity was tested by both qRT-PCR expression levels of Gli1 and through a Gli-responsive luciferase assay. In both cell types, the only inhibitor that could restore Gli1 expression and Shh activity in the presence of Wnt5a overexpression, was the G-protein inhibitor PTX (Figure 29A,B). It should be noted that both BIO and BIMI addition lead to inhibition of Shh signaling even in the absence of Wnt5a, however this was mostly due to high levels of cell death in these assays (data not shown). Even after performing dose response curves for BIO and BIMI, getting high enough levels of inhibition lead to cell death, suggesting that β-catenin-dependent and PKC signaling are required for survival of these cells and overexpression of Wnt5a is unlikely to lead to the inhibition of these pathways. Together this data suggests that Wnt5a signaling is leading
to the activation of certain G-proteins that lead to the inhibition of Gli1 expression and Shh activity.

**PKA is downstream of G-proteins and is activated by Wnt5a overexpression**

G-proteins compose a large family of effector proteins that catalyze the conversion of GTP to GDP, activating a number of different signaling pathways (Neves et al., 2002, Goldsmith and Dhanasekaran, 2007 and Teicher and Fricker, 2010). One of the classical downstream mediators of G-protein signaling is production of the secondary messenger cyclic AMP (cAMP). Adenylyl cyclases are activated via G-proteins and catalyze the conversion of ATP into cAMP (Beavo and Brunton, 2002 and New and Wong, 2007). Although cAMP can lead to a number of different changes in the cell, one of the most prominent secondary-signaling functions of cAMP is to activate protein kinase A (PKA). PKA is one of the most well studied transducers of the cAMP signal (Taylor et al., 1990). PKA resides in the cytoplasm as an inactive tetrameric holoenzyme, however in the presence of cAMP, the holoenzyme dissociates into two free regulatory units bound by cAMP, and two free and active catalytic units. The activated catalytic units are then free to catalyze the phosphorylation of a large number of possible protein targets (Taylor et al., 1990).

In order to determine whether overexpression of Wnt5a causes an increase in PKA activity and a concordant increase in cAMP production, we first utilized a PKA-responsive luciferase assay. In this assay, luciferase expression is under the control of a CREB-responsive promoter. CREB binding to the promoter is dependent on
phosphorylation by PKA, and thus increased luciferase expression is a reliable indication of increased PKA activity. Indeed upon addition of Wnt5a, PKA activity increased by nearly 2-fold. And as expected, addition of the G-protein inhibitor PTX lead to a blockage of the Wnt5a-induced PKA activity (Figure 30A). We next sought to determine whether the increase of PKA activity due to Wnt5a overexpression was caused by an increase in cellular cAMP levels. We found that overexpression of Wnt5a in cells expressing a cAMP-reporter construct showed an increase in cAMP levels compared to cells with normal levels of Wnt5a expression. Additionally, this increased cAMP levels could be blocked with PTX treatment (Figure 30B). Together, this suggests that Wnt5a signaling in these cells leads to activated G-protein signaling, resulting in increased cAMP levels and an increase in PKA activity.

Based on these results we further sought to determine whether changes in PKA activity had an effect on Shh signaling. It has been established that in the absence of Shh signaling, Gli2 and Gli3 are phosphorylated by PKA, which primes these proteins for cleavage of their C-terminal domain via the ubiquitin-proteasome pathway (Pan et al., 2006 and Tempe et al., 2006). The remaining N-terminal portion of Gli2 and Gli3 are then able to enter the nucleus and act as transcriptional repressors (Gli2R and Gli3R) of the Shh signaling pathway (Pan and Wang, 2007). These findings lead us to hypothesize that the increase in Wnt5a-induced PKA activity could be responsible for the Wnt5a-dependent inhibition of Shh signaling. To test this hypothesis, we utilized protein kinase A inhibitor fragment 14-22 (PKAi), an inhibitor of PKA activity, and Sp-adenosine 3’,5’-cyclic-AMP (PKAa), a stabilized analogue of cAMP and potent activator of PKA. We then inhibited or activated PKA in the presence or absence of Wnt5a overexpression and
measured Shh signaling (Figure 30C). Our results show that upon addition of PKAi in the presence of Wnt5a overexpression, Shh signaling was restored to baseline, suggesting that PKA activity is required for Wnt5a-dependent inhibition of Shh signaling. Conversely, activation of PKA in the absence of Wnt5a overexpression lead to a dramatic decrease in Shh signaling.

In summary, our data show that Wnt5a is a potent activator of cAMP production and a subsequent increase in PKA activity. The activation of PKA activity is necessary for Wnt5a to impose inhibition upon the Shh signaling, since inhibition of PKA activity in the presence of Wnt5a overexpression restores normal Shh signaling. And finally, PKA activation alone, in the absence of Wnt5a, is capable of inhibiting Shh signaling to the same extent as Wnt5a overexpression, presumably through increased production of the repressor forms of Gli2 and Gli3 (see model in Figure 31).

DISCUSSION

Our initial findings that the non-canonical Wnt pathway, specifically through the ligand Wnt5a, had an inhibitory interaction with the Shh signaling pathway represented an understudied and potentially important regulatory interaction during development. In this chapter, we sought to more clearly understand the mechanism by which these two pathways interacted. First, we established that the Wnt5a-Shh signaling interaction could be observed in multiple cell types, including NCSCs, NIH3T3, and HEK293. Indeed, in all three cell types we were able to observe decreased Gli1 expression and activity, and thus Shh signaling, upon overexpression of Wnt5a. Interestingly, all three cell types
express Wnt5a endogenously, but only HEK293 cells express high enough levels of Shh ligand to register significant levels of Gli1 expression and activity in an autocrine fashion. Thus, NCSCs and NIH3T3 cells required exogenous activation of the Shh signaling pathway via recombinant Shh or pharmacological agonists. Importantly, all three cell types behaved similarly when challenged with overexpression of Wnt5a. This suggests, that the source of Shh, either autocrine or exogenous, does not change the interaction between the two signaling pathways. This strengthens the hypothesis that the interaction between these two pathways may regulate tissue-tissue interaction where Wnt5a signaling in one tissue type can blunt Shh signaling, regardless if the Shh signal is autocrine or paracrine. It will be particularly important to further investigate the importance of this cross-tissue interaction between these two pathways in vivo. The highly complementary expression profiles of Wnt5a and Shh in developing embryos suggests that Wnt5a may be an important negative regulator of Shh signaling in various tissues, ranging from the heart, to the lungs, to the gut, to the developing limbs (Schleiffarth et al., 2007, Lin et al., 2006, Pepicelli et al., 1998, Cervantes et al., 2009, Fukuda and Yasugi, 2002 and Towers et al., 2012). The importance of this potential interaction is currently unknown. More careful studies may elucidate how these two classical signaling pathways interact during development.

After establishing that Wnt5a could inhibit Shh signaling in multiple cell types, we further determined that Wnt5a activity was leading to the activation of G-protein signaling. In the presence of the G-protein signaling inhibitor, PTX, we observed that the overexpression of Wnt5a no longer altered Shh signaling. Importantly, PTX alone did
not significantly alter Shh signaling suggesting that basal G-protein signaling that would be inhibited by PTX was not interfering with the activation of the Shh signaling pathway. Thus, we concluded that upon activation of the pathway by the Wnt5a ligand, G-proteins are activated. It remains to be determined the nature of this activation. As previously mentioned, Wnt5a typically binds to Frizzled co-receptors to carry out downstream signaling. Interestingly, Frizzled receptors make up a class of 7-transmembrane receptors (Pierce et al., 2002). GPCRs are also 7-transmembrane receptors and a number of groups have hypothesized that the Frizzled receptors may interact directly with G-proteins and may be a legitimate downstream component of the Wnt5a signaling pathway (Malbon, 2004 and Wang et al., 2006). However, there are a number of other groups who disagree with this assertions and suggest there is not significant data showing direct interaction between the Frizzled intracellular domain and G-proteins (Schulte and Bryja, 2007 and Koval et al., 2011). Our work showing that G-proteins are involved with perpetuating the Wnt5a signal does not favor one hypothesis over the other. Therefore, it will be necessary to identify the likely Frizzled involved in the assays we performed, determine whether that particular Frizzled directly interacts with G-proteins, and if not, then identify how these G-proteins are activated.

In further examination of the interaction between Wnt5a and Shh, recent work investigating tooth development has shown that increasing Wnt5a protein levels in the developing tooth germ leads to interesting changes in the spatial distribution and intensity of Shh signaling (Cai et al., 2011). The authors observed that implantation of a Wnt5a-
soaked bead in the tooth germ region of developing embryos lead to a dramatic increase in Shh mRNA expression intensity. Interestingly, this increase in Shh expression was accompanied by the generation of a Shh-negative region surrounding the bead. The authors did not look at markers of Shh signaling so it is unclear whether Wnt5a is able to inhibit the expression of Shh-responsive gene, or if Wnt5a is affecting Shh expression through alternative mechanisms. However, it is clear to us that the interplay between these two signaling pathways is playing an important role during mammalian development. It will be exciting to see what further examination of the two pathways in other tissues elucidates and to what extent the interplay between Wnt5a and Shh has on the development of tissues in which both Wnt5a and Shh play crucial roles.

FIGURE LEGENDS

Figure 27: Wnt5a inhibits Shh signaling in multiple cell types

(A) qRT-PCR quantification of Gli1 expression from Joma1.3, NIH3T3, and HEK293 cells overexpressing Wnt5a for 24-hours. The cells were treated with 0.5ug of recombinant Shh protein for 8-hours to illicit an increase in Gli1 expression. Data normalized to control (no addition of Shh or overexpression of Wnt5a, blue bars). (B) Using a construct where luciferase expression is under the control of Gli-responsive elements, Shh signaling was assayed in the presence of Wnt5a overexpression in the cell types listed. Data normalized to luciferase levels after 8-hour treatment with Shh protein (black bars). *P<0.05 compared to control; **P<0.05 compared to 0.5ug Shh; error bars represent 95% confidence intervals.
Figure 28: Cilia length and number is unaffected by Wnt5a overexpression

(A,B) Representative images from immunocytochemistry staining of Joma1.3 cells stained for acetylated-tubulin (AcTub), ninein, and dapi for nuclear visualization. Cells were serum starved for 24hrs and then transfected with either a pcDNA (wildtype) or a Wnt5a-expressing construct (Wnt5a OE) for 24-hours before visualization. (C) Cells represented in (A,B) were counted and the percentage of cells containing a cilia were calculated; n-values represent the total number of cells counted. (D) The cells that contained bona fide cilia were imaged and the cilia length was calculated using Volocity software. The data represents mean cilia length normalized to the wildtype mean length (blue bars). *Error bars represent 95% confidence intervals.* n.s., not statistically significant.

Figure 29: Inhibition of G-protein signaling restores Shh signaling in the presence of Wnt5a overexpression

(A) qRT-PCR quantification of Gli1 expression levels in Joma1.3 cells treated with Shh protein and under Wnt5a overexpression conditions in the presence of the listed inhibitors. The cells were transfected with or without a Wnt5a-expressing construct for 24-hours and then treated with Shh protein and the listed inhibitors for 8-hours. Only PTX fully restores Shh signaling in the presence of Wnt5a overexpression. Data normalized to Gli1 levels under control conditions treated with DMSO. (B) Relative luciferase levels from HEK293 cells transfected with a GLI-responsive luciferase construct with or without Wnt5a overexpression and 8-hour treatment of the listed inhibitors. Luciferase levels were normalized to control transfection and treatment with
DMSO. Error bars represent 95% confidence intervals. DMSO, dimethyl sulfoxide (used as inhibitor control); CsA, cyclosporine A (calcineurin inhibitor); KN93, (CaMKII inhibitor); PTX, pertussis toxin (G-protein inhibitor), JNK, JNK inhibitor II (JNK inhibitor); BIO, 2’Z,3’E-6-bromoindirubin-3’-oxime (GSK-3β inhibitor); BIMI, bisindolylmaleimide I (PKC inhibitor).

Figure 30: Wnt5a requires PKA activity to inhibit Shh signaling

(A) Quantification of PKA activity in HEK293 cells harboring a PKA-responsive luciferase construct. HEK293 cells were transfected with a Wnt5a-expressing construct for 24-hours and then treated with PTX for 8-hours. Relative luciferase levels were normalized to control cells treated with DMSO. (B) Quantification of cAMP levels in HEK293 cells containing a cAMP-inhibiting luciferase reporter, where increases in cAMP levels are quantified by a loss of luciferase activity. Cells were treated similarly to those in (A). Data was normalized to luciferase activity in control cells (blue bar). (C) Relative luciferase levels were determined from HEK293 cells containing a Gli-responsive luciferase construct with or without Wnt5a overexpression and treated with PTX, a PKA inhibitor, or a PKA agonist for 8-hours. Data normalized to control cells treated with DMSO. *P<0.05 compared to control cells treated with DMSO; **P<0.05 compared to cells overexpressing Wnt5a in the absence of inhibitor; error bars represent 95% confidence intervals. DMSO, dimethyl sulfoxide (used as a control); PTX, pertussis toxin (G-protein inhibitor); PKAi, protein kinase A inhibitor fragment 14-22 (PKA inhibitor); PKAa, Sp-adenosine 3’,5’-cyclic-AMP (PKA agonist).
Figure 31: Model depicting the putative model by which Wnt5a inhibits Shh signaling

Based on the data presented in Chapter 3 we found that upon Wnt5a binding to its yet to be determined receptor, leads to the activation of certain G-proteins. The activated G-proteins likely induce the activity of adenylyl cyclase leading to the conversion of ATP in to cAMP. The increase in cAMP levels causes the activation of PKA. Once PKA is activated, it is known to phosphorylate Gli2 and Gli3 which are then targeted by the ubiquitin-proteasome machinery and their C-terminal domains are cleaved leading to the repressor forms of each transcription factor (Gli2R and Gli3R). Gli2R and Gli3R can now enter the nucleus and bind to the enhancers of specific Shh-responsive genes, leading to the inhibition of the expression of those genes. Fzd, unknown frizzled receptor; AC, adenylyl cyclase; PKA, protein kinase A; Gli2R and Gli3R, the cleaved repressor forms of Gli2 and Gli3, respectively.
Figure 27: Wnt5a inhibits Shh signaling in multiple cell types
Figure 28: Cilia length and number is unaffected by Wnt5a overexpression
Figure 29: Inhibition of G-protein signaling restores Shh signaling in the presence of Wnt5a overexpression.
Figure 30: Wnt5a requires PKA activity to inhibit Shh signaling
Figure 31: Model depicting the putative mechanism by which Wnt5a inhibits Shh signaling
METHODS

Mating and genotyping mice

\(Dicer^{\text{flox/flox}}\) mice (Harfe et al., 2005) and \(Wnt1-cre\) mice (Danielian et al., 1998) were intercrossed to generate \(Dicer^{\text{flox/flox}}Wnt1-cre\) mice. Genotyping was performed by PCR with these primers: Cre1: 5′AGGTCGTTCAC-TCATGGA3′, Cre2: TCGACCAGTATGTTACCC, Dicer-For: 5′ATTGTTACCAGCG-CTTAGAATTCC, Dicer-Rev: 5′GTACGTCTACAATTTGCTATG3′. \(\text{ROSA26 Reporter (R26R)-YFP}\) mice (Jackson Laboratory, Bar Harbor, ME) were bred with \(Dicer^{\text{flox/flox}}\) mice to generate \(Dicer^{\text{floxP/+R26R-YFP}}\) mice.

Histological analysis

Skeletons from embryos were stained with Alcian blue as described in (McLeod, 1980). Yellow latex cast dye (Connecticut Valley Biological Supply) was injected into the beating left ventricle of wildtype or mutant hearts with a 30-1/2 gauge needle. The hearts were dehydrated and cleared in benzyl benzoate:benezyl alcohol (2:1) to visualize the yellow latex in the vasculature. Pregnant mothers were dissected to obtain E13-14.5 wildtype and mutant embryos, which were fixed in 10% formalin and paraffin embedded. Transverse sections through the heart and brain were stained with hematoxylin and eosin to analyze morphology, 1:500 rabbit-\(\alpha\)-NF-M (Abcam, Cambridge, MA) antibody to visualize neuronal tissue, 1:100 rabbit-\(\alpha\)-GFP (Sigma-Aldrich, St. Louis, MO) antibody to visualize NCC progeny, and 1:500 mouse-\(\alpha\)-SMA, Cy3-conjugated (Sigma) to visualize smooth muscle cells. Apoptotic assays were performed using the TUNEL assay.
kit (Roche, Indianapolis, IN). Proliferation studies were performed using the Phospho-Histone H3\textsuperscript{ser10} Assay (Millipore, Billerica, MA).

**In situ hybridization**

mRNA in situ hybridization of whole-mount embryos was carried out as described (Riddle et al., 1993) with digoxigenin-labeled probes, which were synthesized with digoxigenin labeling mix (Roche, Indianapolis, IN) and T7 or T3 polymerase (Roche). The Msx1, Dlx2, Gli1 and Fgf8 riboprobes have been described (Thomas et al., 1998; Lee et al., 1997; Meyers and Martin, 1999). Briefly, embryos were collected at E10.5, fixed in 4% paraformaldehyde, and dehydrated in 100% methanol. Before hybridization, embryos were rehydrated, treated with 10 \( \mu \)g/ml proteinase K (Sigma-Aldrich) for 15 min, and placed in prehybridization buffer for 2 hours at 70\(^\circ\)C. Probes (0.5 \( \mu \)g/ml) were added, and embryos were hybridized overnight at 70\(^\circ\)C. After a series of washing steps, digoxigenin was detected with an anti-digoxigenin antibody conjugated with alkaline phosphatase (Roche). Color development was seen with BM Purple substrate (Roche), and images were obtained with a Leica microscope.

**Flow sorting and miRNA microarray**

Embryos from \( Wnt1^{Cre} \) or \( Dicer^{fl/fl} Wnt1^{Cre} \) mice intercrossed with \( R26R-YFP \) mice were collected at E10.5 and E11.5, dissected, and trypsinized. The cells were spun at 2000 rpm, and the pellet was resuspended in PBS and filtered through a 40-\( \mu \)m membrane (Millipore). Selection by FACS was based on YFP expression. YFP\(^+\) (10,000–
30,000 cells per embryo) and YFP− (between 50,000-100,000 cells per embryo) cells were collected, total RNA was isolated (Trizol, Invitrogen, Carlsbad, CA), and 1 µg of RNA for each cell population was used for miRNA microarray hybridizations (Exiqon, Denmark). Array data were analyzed using R/Bioconductor Bioinformatic software (Gentleman et al., 2004) and the marray package. GenePix flagged spots were removed and only unflagged mmu–miR probes were used for normalization and subsequent analysis. Cy5/Cy3 signals were calculated for each array and normalized by loess normalization (Yang et al., 2002).

Pharyngeal arch culture

PAs were cultured according to the modified Sanger method (Trowell, 1954). Briefly, PAs from mutant and control embryos were dissected at E9.5 and incubated with 2 U/ml dispase to dissociate the epithelial layer from the mesenchymal cells. The epithelial layer was further permeabilized with fine tungsten needles, and the PAs were placed on a nitrocellulose membrane (Millipore) supported by a wire frame. The apparatus was placed in BGJb medium (Gibco, Carlsbad, CA) supplemented with 50 U/ml penicillin/streptomycin and kept at 37ºC for 48 hours.

Dlx2 rescue experiments were carried out in cultured PAs co-transfected with 66 pmol 2′O-methyl oligoribonucleotide mimics (miR-452 or miR-513; Dharmacon, LaFayette, CO) and 33 pmol Block-iT AlexaFluor-red fluorescent control oligo (Invitrogen) with Lipofectamine 2000 (Invitrogen). After 48 hours, PAs were fixed in 4%
paraformaldehyde and dehydrated in 100% methanol for in situ hybridization or in Trizol for quantitative RT-PCR.

Affi-Gel beads (Bio-Rad) were incubated in 200 ng/µl recombinant Wnt5a protein (R&D Systems, Minneapolis, MN) or bovine serum albumin (Invitrogen) for 2 hours at 37°C. During incubation, the PA1s from E10.5 Ptch1-LacZ (Jackson Laboratory) mouse embryos were dissected and prepped for explant culture as described, excluding the dispase treatment. Beads were then transplanted into the mesenchyme with fine tungsten needles and cultured for 36 hours. The PAs were then fixed in 4% paraformaldehyde and stained for β-galactosidase (β-Gal) activity. Similar experiments were done in wildtype embryos assaying Dlx2 mRNA expression in response to Wnt5a-soaked beads.

**Quantitative-RT-PCR**

RNA was prepared from PAs transfected with miR-452 mimic and Block-iT Alexa Fluor-red (Invitrogen) or Block-iT alone, using Trizol (Invitrogen). For miRNA qRT-PCR, cDNA was reverse transcribed from 10 ng of total RNA using the Taqman microRNA reverse transcription kit (Applied Biosystems, Foster City, CA). miR-16 and RNU6 served as endogenous controls. For mRNA qRT-PCR, cDNA was reverse transcribed from 500 ng of total RNA using the Taqman SuperScript II reverse transcription kit (Invitrogen). Gapdh served as endogenous control. All mRNA probes were purchased from Applied Biosystems.
**In vivo antagonir injections**

Wildtype FVB mice (Jackson Laboratory) were mated and plugs were identified; noon of the day of plug discovery was considered E0.5. At E8.5, pregnant mothers were anesthetized with isofluorane. A small incision was made in the abdomen, and the uterus was carefully pulled through the incision to reveal the ovaries. A NanoFil syringe (World Precision Instruments) fitted with a 35-gauge beveled needle was filled with PBS (control) or 3 µg/ml miR-452 antagonir (Dharmacon) diluted in PBS. Using a microsyringe pump (World Precision Instruments), 3 µl was injected into the embryonic space through the intact deciduum of each embryo. After all embryos were injected, the uterus was carefully placed back into the pregnant mother, and the incision was closed with sutures. The embryos were allowed to develop to E11.5 or E16.5 and harvested for in situ hybridization, qRT-PCR, or histological analysis.

**Cell culture**

Joma1.3 cells were cultured as described (Maurer et al., 2007). Briefly, cells were plated on cell culture dishes coated with 1 mg/ml fibronectin (Roche) in a 1:1 mixture of Dulbecco’s modified Eagle’s medium and F12 medium (Gibco) containing 1% N2 supplement (Invitrogen), 2% B27 supplement (Invitrogen), 10 ng/ml epidermal growth factor (Invitrogen), 1 ng/ml fibroblast growth factor (Invitrogen), 100 U/ml penicillin-streptomycin (Invitrogen), and 10% chick embryo extract (Stemple and Anderson, 1992). To maintain cells in an undifferentiated state, 200 nM 4-OHT (Sigma-Aldrich) was added fresh to the medium.
Luciferase assays to determine putative targets were performed with undifferentiated Joma1.3 cells. An ~500-basepair fragment containing the predicted miR-452 binding site for each predicted mRNA target was cloned into pMIR-Report luciferase reporter vectors (Applied Biosystems). For the luciferase assays for the mutant Wnt5a binding site, oligos for 50 bp surrounding the wildtype Wnt5a binding site (mouse chromosome 14: 29338052-74), or mutant binding site (corresponding to bases 3-6 in the seed of miR-452, mutated from CAA to TCG) were annealed together to form concatamers consisting of five-consecutive binding sites. These were then cloned into pMIR-Report luciferase reporter vectors. All assays were performed in triplicate in 12-well plates with Joma1.3 cells transfected with Lipofectamine 2000 (Invitrogen). After 24 hours in culture, cells were harvested, and luciferase intensity was measured with the Luciferase Dual-Reporter assay (Promega) and normalized to Renilla.

For Shh responsiveness assays, hsa-Wnt5a cDNA (Open Biosystems, Huntsville, AL) was cloned into pEF6-V5 expression vectors (Invitrogen). Joma1.3, NIH3T3, or HEK298 cells were transfected with Lipofectamine 2000 and the pEF6-V5-Wnt5a expression construct (200ng/mL). After 24 hours in culture, 0.5 μg/ml recombinant Shh-N protein (R&D Systems) was added to the medium, and the cells were cultured for 24 hours. Cells were then harvested, RNA was isolated, and qRT-PCR was performed to determine relative gene expression levels. For assays including pharmacological inhibitors and agonists, the inhibitors or agonists were added at the same time as the Shh-N protein at the following concentrations: DMSO, 30 uM (Sigma); CsA, 1 uM (Sigma); KN93, 5 uM (Sigma); PTX, 200 ng/mL (Invitrogen); JNK, 30 uM (EMD Biosciences); BIO, 1 uM.
(Sigma); BIMI, 10 uM (Sigma). Cells were then harvested and for expression assay, qRT-PCR was performed to determine relative gene expression levels. For luciferase-based assays, the following luciferase reporter assay kits were used following the manufacturers’ recommendations: for Gli responsive assays, Cignal GLI-Luciferase Assay Kit (SABiosciences); for PKA responsive assays, Cignal CRE-Luciferase Assay Kit (SABiosciences); for cAMP level quantification, GloSensor cAMP Luciferase Assay Kit (Promega).

Western blots were either performed using lysate from Joma1.3 cells or PA explant culture with or without transfection of 66 pmol miR-452 mimic and immunoblotted with goat anti-Wnt5a antibody (R&D Systems) and quantified using the LI-COR Odyssey Imaging System (LI-COR Biosciences, Lincoln, NE).
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