Dlx5 and Msx2 regulate mouse anterior neural tube closure through ephrinA5-EphA7

Jangwoo Lee,1,3* Amy Corcoran,2 Manjong Han,1 David M. Gardiner3 and Ken Muneoka1

1Department of Cell and Molecular Biology, School of Science and Engineering, Tulane University, New Orleans, Louisiana, 70115, 2University of Illinois at Chicago College of Medicine, Chicago, Illinois, 60612, and 3Department of Developmental and Cell Biology, School of Biological Sciences, University of California at Irvine, Irvine, California, 92697, USA

Homeodomain-containing transcription factors Dlx5 and Msx2 are able to form a heterodimer, and together can regulate embryonic development including skeletogenesis. Dlx5 functions as a transcriptional activator and Msx2 a transcriptional repressor, and they share common target genes. During mouse digit development, the expression domains of Dlx5 and Msx2 overlap at the distal region of the developing terminal phalange, although digit formation and regeneration are not altered in the Dlx5 and Msx2 null mutant embryos. Interestingly, we observed a high rate of defects in neural tube formation in Dlx5 and Msx2 double null mutants. In the absence of both Dlx5 and Msx2, a high occurrence of exencephaly and severe defects in craniofacial morphology are observed. Additionally, Dlx5 and Msx2 expression domain analysis showed overlap of the genes at the apex of the neural folds just prior to neural fold fusion. The expression patterns of ephrinA5 and two isoforms of EphA7 were tested as downstream targets of Dlx5 and Msx2. Results show that EphrinA5 and the truncated isoform of EphA7 are regulated by Dlx5 and Msx2 together, although the full length isoform of EphA7 expression is not altered. Overall, these data show that Dlx5 and Msx2 play a critical role in controlling cranial neural tube morphogenesis by regulating cell adhesion via the ephrinA5 and EphA7 pathway.

Key words: Dlx5, EphA7, ephrinA5, Msx2, neural tube formation.

Introduction

Neurulation is a fundamental process that establishes the neural system during embryonic development. The neural tube is the major structure that forms during neurulation, and represents the anlagen of the brain and spinal cord. The neural tube is formed by the progressive fusion of the neural folds, which are elevated tissue structures along the lateral edges of the neural plate at the dorsal midline of the embryo. Approximately 80 genes are involved in a mammalian neurulation, and neural tube defects (NTDs) result with disruption of any of these genes (Copp et al. 2003). Anencephaly is one such NTD that is caused by a neural patterning defect and results from disrupted tube closure in the cranial region of the embryo. Anencephaly is the resulting phenotype when exencephalic brain tissue gradually degenerates due to the exposure to amniotic fluid (Timor-Tritsch et al. 1996). In humans, the occurrence rate of the NTDs is approximately 1 out of 1000–2000 births in the United States (Copp et al. 2003).

Among the genes that are involved in neurulation, Dlx (distal-less homeobox) and Msx (msh-like homebox) family genes have been reported for their roles in craniofacial embryogenesis, including anterior neural tube formation. Both the Dlx and Msx gene families encode for homeodomain-containing transcription factors. In mammals there are six members of the Dlx family (Dlx1-6) and three members of the Msx family (Msx1-3) (Depew et al. 2005; Ramos & Robert, 2005). The Dlx gene family has been implicated in embryonic development including brain, branchial arches, jaws and limb development (Depew et al. 2005). In a Dlx5 gene null mutant study, it was demonstrated that approximately 24% of mutant embryos showed an exencephaly phenotype at E13.5 (Depew et al. 1999) and this phenotype is more severe in the Dlx5/6 dou-
The two cells will adhere together. This report suggests that regulation of cell adhesion and repulsion processes by ephrinA5/EphA7 plays a critical role in controlling cranial neural tube formation (Frisen et al. 1999).

To date, although many studies unveiled the molecular mechanisms of cranial neural tube patterning from a genetic-based approach, many aspects of the molecular networks of cranial neural tube patterning remain unknown. In this report, we demonstrated that Dlx5/Msx2 double mutants display an increased rate of malformation in cranial neural tube formation as compared to Dlx5 or Msx2 single mutants. Our studies show that the frequency of exencephaly increases incrementally in a Dlx5 mutant background with decreasing Msx2 gene dose. In addition, ephrin-A5 and EphA7-T1 expression, but not EphA7-FL, were downregulated in Dlx5/Msx2 double mutant embryos at the dorsal region of neural tube in association with the failure of neural fold fusion. Our report provides a novel molecular mechanism in which Dlx5 and Msx2 function reciprocally through the regulation of ephrin-A5/EphA7 expression in cranial neural tube closure.

**Materials and methods**

**Wildtype and Dlx5, Msx2 mutant mice**

Wildtype mouse embryos used in this study were either outbred CD#1 strain supplied by Charles River Laboratories or wildtype embryos from the breeding of Dlx5 and Msx2 mutant mice. Homozygous Dlx5 or Msx2 mutant embryos were obtained by mating of heterozygotes carrying a targeted deletion of either the Dlx5 gene (Depew et al. 1999) or the Msx2 gene (Satokata et al. 2000). Dlx5/Msx2 double mutant embryos were obtained by double heterozygotes mating. Embryos were collected at embryonic day (E) 9.5 and their genotype was verified by polymerase chain reaction (PCR) with genotype specific PCR primers. Procedures for the care and use of mice for this study were compliant with standard operating procedures (SOPs) approved by the Institutional Animal Care & Use Committee (IACUC) of Tulane University Health Science Center.

**Fetal mouse digit amputation**

To study regeneration in vivo, fetal mouse digit tips were amputated at E14.5. Timed-pregnant mice, which carry E14.5 embryos were anesthetized with sodium pentobarbital (60 μg/g body weight), fentanyl (1.6 μg/animal), and droperidol (80 μg/animal). The pregnant mouse abdomen was opened with a mid-
ventral incision, and fetuses were exposed by incision of the anti-placental uterine wall. Access to the hind-limb was gained through an incision in the extraembryonic membranes and the hindlimb was teased out with a blunt probe. The three central hindlimb digits, digits 2, 3, and 4, were amputated at a distal level, approximately 75 mm from the digit tip. The uterus with attached fetuses was re-positioned within the abdominal cavity, and the abdominal wall of female mouse was closed. Operated fetuses were allowed to develop for 4 days exo utero (Muneoka et al. 1986), after which the hindlimbs were collected for analysis of the digits.

Whole mount skeletal staining
Differential whole mount bone staining of mouse embryos was performed according to the following process. Embryos were isolated at E18.5 and fixed with 95% ethanol (EtOH) overnight. Embryos were then skinned manually, delipidated in acetone, and stained with Alcian Blue 8XG/Alizarin Red S in 5% acetic acid, 95% EtOH. Stained embryos were treated in 1% KOH and cleared by glycerol.

In situ hybridization
Digoxigenin-labeled antisense RNA probes for Dlx5, Msx2, EphrinA5, EphA7-FL, and EphA7-T1 were used to perform in situ hybridization. EphrinA5, EphA7-FL, and EphA7-T1 containing DNA plasmids were kindly provided by Dr Jonas Frisen. Embryos were collected at E 9.5 and fixed in 4% paraformaldehyde at 4°C overnight. Fixed mouse embryos were dehydrated with an ascending series of ethanol (25%, 50%, 75% and 100%), infiltrated in xylene, and embedded in paraffin. Paraffin sections were cut at 5 μm thickness. In situ hybridization was performed according to previous method (Han et al., 2003).

Results
Dlx5 and Msx2 in mouse fetal digit regeneration
The transcriptional repressor Msx1 and cell signaling molecule Bmp4 are co-expressed at the apex of the forming fetal mouse digit and both have been implicated in the control of digit tip regeneration (Han et al. 2003). DLX and MSX proteins can form heterodimers that can regulate gene transcriptions (Zhang et al. 1997). Particularly, the DLX5 and MSX2 have been shown to form a heterodimeric complex that regulates differentiation during skeletogenesis (Newberry et al. 1998). To study the role of Dlx5 and Msx2 in digit regeneration we began by analyzing the expression of Dlx5 and Msx2 on mouse digit at E14.5. Expression of Dlx5 was detected in ectoderm and mesenchymal tissue between the epidermis and condensed cartilage of digit (Fig. 1A). The Msx2 expression pattern is similar to the expression of Dlx5 at the digit tip, but extended proximally (Fig. 1B). Since Msx2 and Dlx5 are co-expressed at the apex of the forming digit, we were interested in whether these two genes were involved in the control of fetal digit regeneration. The Msx2 mutant digit had previously been tested and was found to regenerate normally, thus suggesting that Msx2 was not required for digit tip regeneration (Han et al. 2003). Here we tested the regenerative capacity of Dlx5 mutant digits at E14.5, and as well, we re-tested the Msx2 mutant. We found that the Dlx5 and Msx2 mutant digits possessed a regenerative capacity similar to wildtype digits, thus indicating that neither gene was required for fetal digit tip regeneration (Table 1). To test the role of both Dlx5 and Msx2 in fetal digit regeneration, we generated Dlx5+/− Msx2/−/− double mutant embryos. E14.5 digits from this double mutant were tested for regenerative ability and their regenerative response was undistinguishable from wildtype control digits (Table 1). These studies demonstrate that despite their co-expression at the apex of the forming digit and their known interactions in regulating skeletogenesis, Dlx5 and Msx2 do not appear to play a functional role in fetal digit tip regeneration.

Exencephaly phenotype in Dlx5 and Msx2 double mutant mouse embryo
During our studies on the role of Dlx5 and Msx2 in fetal digit tip regeneration, we noted that the most dramatic phenotype associated with the double mutant embryos was that many embryos displayed exencephaly. It has
Table 1. Regeneration response of fetal digit tips

<table>
<thead>
<tr>
<th>Genotype</th>
<th>WT</th>
<th>Dlx5+/−</th>
<th>Dlx5−/−</th>
<th>Msx2−/−</th>
<th>Dlx5+−/−;Msx2−/−</th>
<th>Dlx5−/−;Msx2−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of regenerated digit</td>
<td>25/27</td>
<td>46/48</td>
<td>17/18</td>
<td>6/6</td>
<td>27/30</td>
<td>30/30</td>
</tr>
<tr>
<td></td>
<td>(92.6%)</td>
<td>(95.8%)</td>
<td>(94.4%)</td>
<td>(100%)</td>
<td>(90.0%)</td>
<td>(100%)</td>
</tr>
</tbody>
</table>

WT, wildtype.

been already reported that 12% or 28% of Dlx5 null mutant embryos displayed an exencephalic phenotype (Acampora et al. 1999; Depew et al. 1999), but that exencephaly was not noted in Msx2 null embryos (Satokata et al. 2000). To characterize the exencephalic phenotype among the different genotypes of embryos developing from a double heterozygote cross, we collected embryos from stages ranging from E9.5 to E18.5 to establish the frequency of exencephaly. In this analysis we obtained and scored embryos with multiple genotypes, including wildtype, Dlx5−/−, Msx2−/−, Dlx5+/−;Msx2+/−, Dlx5+/−;Msx2−/−, Dlx5−/−;Msx2−/−, and Dlx5−/−;Msx2+/− (Table 2). Similar to previous studies, we found that 19% of Dlx5 null mutant embryos displayed exencephaly while Msx2 null mutants had no exencephalic embryos. Dlx5 heterozygote embryos display no exencephaly (Acampora et al. 1999; Depew et al. 1999); however, if the embryos lack either one or both copies of Msx2 they display a low level of exencephaly (Dlx5+/−;Msx2+/−: 7%; Dlx5+/−;Msx2−/−: 9%). Interestingly, in a Dlx5 mutant background, the frequency of exencephaly increased to 39% when one copy of Msx2 is absent, and when both copies are absent the exencephalic frequency jumps to 73%. These studies clearly show that the exencephaly phenotype associated with the Dlx5 mutant is influenced by Msx2 in a synergistic manner.

Since Dlx5 and Msx2 are both transcriptional regulators and are known to interact during skeletogenesis, we next analyzed skull formation of neonates in whole mount skeletal preparations of Msx2 mutants, and of Dlx5−/− and Dlx5−/−;Msx2−/− mutants displaying exencephaly at E18.5. Ossifying frontal, parietal, interparietal, and supraoccipital bones are shown in the calvarium of wildtype embryos at E18.5 (Fig. 2A,B). Skull morphology of the Msx2 null mutant shows that skull size was slightly reduced in comparison to wildtype controls, and that ossification of interparietal and supraoccipital bones is delayed (arrows in Fig. 2E,F). On the other hand the frontal and parietal bones are not affected in the Msx2 mutant. The size of the calvarium of the Dlx5−/− exencephaly phenotype is grossly reduced, and all five fontanelle bones as well as the supraoccipital bone do not form (Fig. 2C,D). Similarly, in the Dlx5 and Msx2 double null mutant embryo the calvarium displays an identical morphology to the Dlx5 mutant (Fig. 2G,H). We also examined the whole mount skull sample of non-exencephaly embryos of Dlx5−/−;Msx2−/− mutants. Although the epidermis of the cranium is intact, frontal, parietal and interparietal bones were only partially developed, and the supraoccipital bones were missing (Fig. 2I,J). While the cranial phenotypes vary depending on genotype, gross morphologies of embryos are not significantly altered from wildtype (Fig. S1). These data show that the primary effect of removing copies of the Msx2 gene on the Dlx5 mutant is associated with the frequency of exencephaly and not the severity of phenotype. This finding, combined with evidence that the expression domains of Dlx5 and Msx2 do not overlap during the embryogenesis of the skull (Kim et al. 1998; Holleville et al. 2003) suggests that the exencephalic defect caused by the double mutation is linked to developmental events that precede skeletogenesis.

Table 2. Exencephalic phenotype ratio in different mouse genotypes

<table>
<thead>
<tr>
<th>Stage</th>
<th>Dlx5−/−</th>
<th>Msx2−/−</th>
<th>Dlx5+/−;Msx2+/−</th>
<th>Dlx5+/−;Msx2−/−</th>
<th>Dlx5−/−;Msx2−/−</th>
<th>Dlx5−/−;Msx2+/−</th>
<th>Dlx5−/−;Msx2−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>E9.5</td>
<td>1/6</td>
<td>0/13</td>
<td>3/20</td>
<td>1/9</td>
<td>4/11</td>
<td>8/10</td>
<td></td>
</tr>
<tr>
<td>E10.5</td>
<td>1/2</td>
<td>0/5</td>
<td>0/17</td>
<td>0/7</td>
<td>3/8</td>
<td>2/5</td>
<td></td>
</tr>
<tr>
<td>E11.5</td>
<td>0/2</td>
<td>0/2</td>
<td>2/12</td>
<td>2/5</td>
<td>3/7</td>
<td>1/2</td>
<td></td>
</tr>
<tr>
<td>E12.5</td>
<td>0/2</td>
<td>0/5</td>
<td>0/15</td>
<td>0/7</td>
<td>3/5</td>
<td>1/1</td>
<td></td>
</tr>
<tr>
<td>E14.5</td>
<td>N/A</td>
<td>0/12</td>
<td>0/8</td>
<td>2/22</td>
<td>1/5</td>
<td>13/18</td>
<td></td>
</tr>
<tr>
<td>E18.5</td>
<td>1/4</td>
<td>0/1</td>
<td>0/3</td>
<td>0/5</td>
<td>N/A</td>
<td>5/5</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>3/16</td>
<td>0/38</td>
<td>5/75</td>
<td>5/55</td>
<td>14/36</td>
<td>30/41</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(18.8%)</td>
<td>(0%)</td>
<td>(6.7%)</td>
<td>(9.1%)</td>
<td>(38.9%)</td>
<td>(73.2%)</td>
<td></td>
</tr>
</tbody>
</table>

N/A, data not available.
Dlx5 and Msx2 expression patterns during cranial neural tube formation

The cause of the exencephaly phenotype is generally linked to a failure of the anterior neural tube to close properly during neurulation early in embryogenesis (Copp et al. 2003). Double mutant embryos analyzed for exencephaly confirm that the phenotype is present in the early embryo (Table 2), and is associated with the failure of the anterior neural tube to close. To investigate the role of Dlx5 and Msx2 in neural tube closure we first carried out a detailed analysis of gene expression during neurulation. The neurulation process can be divided into four stages: (i) formation of neural plate; (ii) folding of the neural plate to form the neural groove; (iii) elevation of the neural folds; and (iv) closure of the neural folds to form the neural tube (Gilbert 2003). The cranial region of the mouse embryo undergoes its incipient neural groove stage at E8.5. At this time point, Dlx5 transcripts were not detected anywhere in the neural folds (Fig. 3A), whereas Msx2 expression was detected at the edges of the neural folds (black arrowheads, Fig. 3D). At E9.5, neural tube formation is completed in the cranial region of the embryo and both the neural fold elevation stage and neural tube stage can be observed in the same embryo analyzed at different cranial-caudal levels. Dlx5 is transiently expressed at the apex of the neural folds (black arrowheads, Fig. 3B); however, after closure of the neural tube Dlx5 expression is downregulated and is no longer detected in the neural tube (Fig. 3C). During this stage Msx2 transcripts are detected in the apex of neural folds in a region that overlaps the expression domain of Dlx5 (Fig. 3E). After closure of the neural tube Msx2 remains expressed at the point of fusion along the dorsal midline (black arrowheads, Fig. 3F). Summarizing, Msx2 is expressed in the apex of the neural folds and at the dorsal midline of the neural tube during and after fusion to close the anterior neural tube, whereas Dlx5 is transiently upregulated in the apex of the neural folds immediately prior to neural fold fusion and downregulated after fusion (Fig. 3G). In terms of expression domain, Dlx5 and Msx2 expression sites overlap in the apex of the neural folds just prior to fusion (Fig. 3E).

EphrinA5 and EphA7 are regulated by Dlx5 and Msx2 together

The developmental expression of Dlx5 and Msx2 suggests that the exencephaly phenotype associated with the double mutation may be linked to a synergistic interaction between these two genes during a transient period when they are both expressed at the apex of the neural folds during neural fold fusion. Since both Dlx5 and Msx2 are transcriptional regulators that must regulate morphogenetic events by affecting the expression of structural genes, we explored potential downstream target genes that might be linked to the exencephaly phenotype. In a previous report, it has been demonstrated that ephrinA5 and its receptor EphA7 participate in cranial neural tube morphogenesis via cell attraction and cell repulsion, and that
embryos that possess a defect in this signaling pathway can display exencephaly (Holmberg et al. 2000). To explore whether Dlx5 and Msx2 have a regulatory role on the expression of ephrinA5, EphA7-FL, and EphA7-T1, we performed gene expression pattern analysis of ephrinA5, EphA7-FL, and EphA7-T1 on WT, Dlx5−/−, Msx2−/−, and Dlx5−/−;Msx2−/− mouse embryos at E9.5 (two embryos were used in each genotype for the gene expression pattern analysis). In wildtype embryos, the expression domain of ephrinA5 and EphA7-FL are almost identical to each other in that expression is restricted to the outer layer of the neural tube (Fig. 4A,E). The expression domain of EphA7-T1 is in the dorsal two-thirds of the neural tube (Fig. 4I, the ventral margin of EphA7-T1 expression domain indicated by arrows), and the domain is broader than ephrinA5 and EphA7-FL. In Dlx5 null mutant embryos, expression of ephrinA5 and EphA7-FL appeared similar to wildtype embryos (Fig. 4B,F). However, EphA7-T1 expression domain was expanded to the ventral region of the neural tube in Dlx5−/− mutants, although the intensity of expression did not appear to be significantly changed (Fig. 4J). In Msx2 null mutant embryos, expression of EphA7-FL was not changed (Fig. 4G), whereas ephrinA5 expression was slightly decreased (Fig. 4C). The expression domain of EphA7-T1 in Msx2 mutant was expanded to the ventral region, but the expression level was not altered (Fig. 4K). In Dlx5 and Msx2 double null mutant embryos displaying exencephaly the expression of ephrinA5 was decreased all over the neural tissue, particularly in the region of Dlx5/Msx2 co-expression at the apex of the neural folds (Fig. 4D). EphA7-FL expression was not modified in the apex of neural folds in Dlx5−/−;Msx2−/− embryos (Fig. 4H), whereas EphA7-T1 transcripts were largely absent throughout the neural tissue including the apex of the neural folds (Fig. 4D,L). These results show that the expression of the ligand, ephrinA5, and one of its receptors, EphA7-T1, is regulated by the combined activity of Dlx5 and Msx2 during anterior neural tube closure.

**Discussion**

In the mouse, neural tube closure initiates at three different points along the cranial-caudal axis. The primary initiation point (closure 1) is located at the hindbrain/cervical boundary, and closure then proceeds in both cranial and caudal directions. The second neural tube closure (closure 2) initiation point is located at the forebrain/midbrain boundary. The last neural tube closure (closure 3) initiation point is located at the extreme rostral end of the embryo, and closure proceeds in the caudal direction (Copp et al. 2003). In this study we focused on the role of Dlx5 and Msx2 in cranial neural tube closure (closure 2) and found that Dlx5 and Msx2 are co-expressed at the apex of the neural folds during neural tube formation. Using Dlx5 and Msx2 genetically disrupted mice, we confirm a low frequency of exencephaly in the Dlx5 mutant (Depew et al. 1999), and found that the frequency of exencephaly...
Expression of EphA7-T1 and Msx2 detected outer layer of the neural tube. This expression is not altered in the double mutant embryo shows significantly decreased expression of EphA7-T1 in E9.5. (A–D) Expression of Msx2 in wildtype (A), Dlx5–/– (B), Msx2–/– (C), and Dlx5–/–;Msx2–/– (D) mice embryos at E9.5. Transcripts of Msx2 are detected broadly in the dorsal side of neural folds and becomes more severe with the sequential removal of Msx2 alleles. Since Dlx5 is a known transcriptional activator and Msx2 is a transcriptional repressor known to heterodimerize and/or compete with Dlx5 for DNA binding to antagonize Dlx5 activity (Zhang et al. 1997), the observed genetic interaction between Dlx5 and Msx2 in the double mutant poses a bit of a conundrum that requires further investigation. A similar synergistic interaction between Dlx5 and Msx1 has been reported in association with frontal bone development (Chung et al. 2010). Based on these results the simple interpretation that the loss of a transcriptional repressor would phenotypically cancel the loss of a transcriptional activator seems unlikely.

Functional redundancy among members of the Dlx or Msx families has been previously demonstrated (Robledo et al. 2002; Lallemand et al. 2005). Within the Dlx family, Dlx5 and Dlx6 are very similar in their expression pattern, homology of the amino acid sequences (Merlo et al. 2000; Zerucha et al. 2000), and they have redundant function in limb development and cranial neural tube formation (Robledo et al. 2002). Similarly, in the Msx family, redundancy has also been demonstrated in the regulatory function of Msx1 and Msx2 in limb development and cranial neural tube formation (Lallemand et al. 2005; Han et al. 2007). Therefore, the functional redundancy among the Dlx transcriptional activators and the Msx transcriptional repressors can account for the dose-related effects of Msx2 in a Dlx5 mutant background. Thus, the functional redundancy within Dlx and Msx family members, the established functional antagonism between Dlx5 and Msx2, and the co-expression of Dlx5 and Msx2 at the tips of the neural fold prior to fusion suggest that the interaction between Dlx and Msx proteins play a primary regulatory role in controlling neural tube closure. This conclusion is further supported by gain of function studies in which Msx2 overexpression also results in a low frequency of exencephalic embryos (Winograd et al. 1997).

Our Dlx5/Msx2 mutant studies identified that the expression of the ephrinA5 gene and transcripts of the truncated form of its receptor gene, EphA7-T1, are downregulated during neural tube formation. These data provide evidence of molecular networking between Dlx5/Msx2 and ephrinA5/EphA7 in cranial neural tube morphogenesis (Fig. 5A,B); however, the detailed molecular mechanisms of this network are not clear at this time. The expression domains of Dlx5 and Msx2 are restricted to the very tip of the neural folds, whereas transcripts of ephrinA5, EphA7-FL, and EphA7-T1 are detected broadly in the dorsal side of neural folds and...
the neural tube. These non-overlapping expression domains suggest an indirect regulatory interaction possibly involves downstream signaling between cells of the neural fold. However, it is clear that the ephrinA5 gene is a downstream target in this pathway, whereas the EphA7 gene is not. It is also important to note that the frequency of neural tube closure defect in the Dlx5/Msx2 double mutants is four times greater than the ephrinA5 null mouse, thus indicating additional downstream targets modulating neural tube closure.

Transcripts for the EphA7 gene encode for a full-length receptor (EphA7-FL), and two tyrosine kinase domain truncated isoforms, EphA7-T1 and EphA7-T2, which are the products of alternative splicing (Ciossek et al. 1995; Valenzuela et al. 1995). Since Dlx5/Msx2 regulates expression of a truncated isoform of EphA7, but not the full length isoform, Dlx5/Msx2 must regulate the mechanism by which EphA7 RNA is differentially spliced. To date, there is no evidence for regulation of pre-mRNA alternative splicing by Dlx or Msx transcription factors. However, modulation of the 5’-splice site by the transcription factor c-Myb has been reported (Orvain et al. 2008). Further investigations into this novel molecular network in which Dlx5/Msx2 regulates neural fold morphogenesis by controlling differential cell adhesion via ephrinA5/EphA7 interactions is necessary for our understanding of cranial neural fold fusion.

With respect to the developing digit we find that despite overlapping expression domains at the digit tip, an interaction between Dlx5 and Msx2 is not functionally linked to either digit tip formation or regeneration. Since other Dlx and Msx family members are co-expressed in similar domains (Robledo et al. 2002; Han et al. 2003) it is reasonable to conclude that functional redundancy may be masking any phenotypic defect. However, the discovery of a Dlx5/Msx2 link to the control of ephrinA5/EphA7 activity during neural tube closure is suggestive that this signaling network may be conserved during digit formation and regeneration. The role of ephrin/Eph signaling in mammalian digit regeneration has not been explored in detail; however, differential cell adhesion is known to play a critical role both during limb development and limb regeneration (see Wada 2011) making further exploration of this molecular network primed for future studies.

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