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Arx together with FoxA2, regulates *Shh* floor plate expression

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ABSTRACT

Mutations in the *Aristaless related homeodomain transcription factor* (*ARX*) are associated with a diverse set of X-linked mental retardation and epilepsy syndromes in humans. Although most studies have been focused on its function in the forebrain, ARX is also expressed in other regions of the developing nervous system including the floor plate (FP) of the spinal cord where its function is incompletely understood. To investigate the role of *Arx* in the FP, we performed gain-of-function studies in the chick using *in ovo* electroporation, and loss-of-function studies in *Arx*-deficient mice. We have found that Arx, in conjunction with FoxA2, directly induces *Sonic hedgehog* (*Shh*) expression through binding to a *Shh* floor plate enhancer (SFPE2). We also observed that FoxA2 induces Arx through its transcriptional activation domain whereas Nkx2.2, induced by Shh, abolishes this induction. Our data support a feedback loop model for Arx function; through interactions with FoxA2, Arx positively regulates *Shh* expression in the FP, and Shh signaling in turn activates Nkx2.2, which suppresses *Arx* expression. Furthermore, our data are evidence that Arx plays a role as a context dependent transcriptional activator, rather than a primary inducer of *Shh* expression, potentially explaining how mutations in *ARX* are associated with diverse, and often subtle, defects.

Introduction

Cell type specification is a dynamic process dependent on cell extrinsic and intrinsic signaling programs. The developing spinal cord serves as an excellent model system to study cell type specification. Many studies over past decades have deduced that morphogenic gradients formed by several signaling molecules (*e.g.* sonic hedgehog and retinoic acid) initiate intrinsic transcription networks enabling first the specification of distinct progenitor cells and subsequently maintaining their identity (Davidson, 2002; Jessell, 2000). Understanding how the various factors in this process interact is crucial to unraveling the mechanisms underlying cell fate determination.

Sonic hedgehog (Shh) is a secreted protein with well-established roles in cell fate specification in the ventral spinal cord. It is first expressed in the mesodermally derived notochord and subsequently in the ventral midline of the developing neural tube (*i.e.*, floor plate, FP). When Shh binds the transmembrane receptor, Patched (Ptc), it releases the inhibition of Smoothened (Smo), which then translocates to the cytoplasm and initiates a signaling cascade that results in the nuclear translocation of Gli (Gli1-3) transcription factors. Gli transcription factors bind specific *cis*-elements (GBSs; Gli binding sites) of downstream target genes to activate or repress their transcription (Briscoe et al., 2000; Dessaud et al., 2008). It is known that Shh stabilizes full-length Gli2 and Gli3 proteins in their activator forms (GliA); in the absence of ligand these bi-functional proteins undergo proteolysis and change to repressor forms (GliR) (Dessaud et al., 2008; B. Wang et al., 2000).

Shh signaling functions in a gradient to establish unique cell fates along the dorsal ventral axis of the developing spinal cord. In response to this morphogen gradient, transcription factors in responding cells are either induced or repressed to establish the p0, p1, p2, pMN, p3, and FP domains. In turn, each progenitor domain gives rise to a distinct neuronal (V3, MN, V2, V1 and V0), and non-neuronal (FP) subtypes (Dessaud et al., 2008; Jessell, 2000). Ventral neural tube development is not only dependent on its spatial concentration gradient of Shh, but also the timing and duration of the signaling (Chamberlain et al., 2008; Dessaud et al., 2007, 2008). Increasing levels and durations of Shh signaling direct progenitors to adopt progressively more ventral identities (Chamberlain et al., 2008; Dessaud et al., 2007, 2008). Furthermore, the interpretation of the Shh morphogen gradient into an

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intrinsic transcriptional network, rather than Shh gradient itself, has been shown to be responsible for differential spatial and temporal gene expression (Balaskas et al., 2012). Moreover, identification and characterization of the *cis*-regulatory modules (CRMs) of target genes operating downstream of Shh signaling have clarified how different cells interpret their Shh signaling depending on their relative location in relation to the signaling source (Oosterveen et al., 2012; Peterson et al., 2012).

Specification of the most ventral cell type, the non-neuronal FP cells, is thought to be a sequential process. Initially, the presumptive FP cells, in response to notochord-derived Shh, express a set of transcription factors (*e.g.* FoxA2, Nkx2.2 and Nkx6.1) that are also expressed in adjacent progenitor cells (p3). Later the developing FP cells also begin expressing Shh and Arx, whereas Nkx2.2 expression is down-regulated and no longer detected in the presumptive FP but continues to be expressed in the adjacent p3 domain. Unlike other ventral neuronal subtypes, where high levels and longer duration of Shh signaling predict more ventral identities, FP specification involves a biphasic response to Shh signaling. Initially, high levels of Shh signaling are required for FP specification (Ribes et al., 2010); however, maintenance of the FP is Shh signaling independent, although Shh continues to be expressed by FP cells. If Shh signaling is maintained during this time instead of down-regulated, FP cells convert their identity to ventral neural progenitors (Ribes et al., 2010).

Despite the down-regulation of Shh signaling in FP cells, Shh itself is not down-regulated, suggesting that the FP cells must maintain adequate levels of Shh production for the generation of other ventral cell types, and for functions such as a commissural axon chemoattraction (Bourikas et al., 2005). Paradoxically, the transcription factor FoxA2 is responsible for inducing Shh expression, while it simultaneously down-regulates Shh signaling to maintain FP identity and inhibit p3 fate.

Two enhancer regions have been identified in the regulatory regions of *Shh* that are responsible for spinal cord FP specific expression: ***S**hh* **F**loor **P**late **E**nhancer 1 and 2 (SFPE1 and 2) (Epstein et al., 1999; Jeong and Epstein, 2003). SFPE1 activity is controlled in a FoxA2-independent manner. In contrast, SFPE2 activity is regulated by two elements, a Homeobox transcription factor Binding Site (HBS) and a FoxA2 binding site. Both are required for the full activity in the FP (Epstein et al., 1999; Jeong and Epstein, 2003). To date the homeodomain transcription factor(s) that binds to SFPE2 has not been identified.

The *aristaless related homeodomain transcription factor* (*Arx*) is the vertebrate homolog of Drosophila *Aristaless* (Miura et al., 1997). It is expressed in the developing brain including the cerebral cortex, basal ganglia, hypothalamus, thalamus, midbrain, and hindbrain (Colombo et al., 2004; Miura et al., 1997). Its expression is first detected at the 3 somite stage (~E8) in mouse embryos and it persists through early postnatal life (Colombo et al., 2004). Mutations in *ARX* have been linked to morphological brain anomalies as well as multiple neurologic deficits in patients (Friocourt and Parnavelas, 2010; Kato et al., 2004; Kitamura et al., 2002; Mégarbané et al., 2011; Olivetti and Noebels, 2012; Sherr, 2003; Shoubridge et al., 2010; Strømme et al., 2002). *Arx*-deficient mice have intermediate progenitor cell proliferation defects in the forebrain resulting in small brains (Colasante et al., 2013; Kitamura et al., 2002). They also show aberrant migration and differentiation of interneurons in the ganglionic eminence and neocortex (Fulp et al., 2008; Kitamura et al., 2002; Marsh et al., 2009; Nasrallah et al., 2012). Furthermore, loss of *Arx* in mice, through conditional gene abrogation, results in structural brain anomalies, epilepsy, and neurocognitive phenotypes (Colasante et al., 2013; Fulp et al., 2008; Kitamura et al., 2002; Marsh et al., 2009).

Arx is also expressed in FP cells of the developing spinal cord; however its function in the FP has not been explored. Based on the observations that (1) Arx is expressed in FP cells during the period of Shh induction and (2) it is a homeodomain transcription factor, we hypothesized that Arx binds to the HBS of SFPE2 and induces *Shh* expression. To test our hypothesis, we performed both gain-of-function and loss-of-function experiments using the chick embryo and *Arx* deficient mice. We find Arx indeed binds the SFPE2 site and induces *Shh* expression in the presence of FoxA2. Furthermore, our data demonstrate that FoxA2 induces *Shh via* its activation domain, while Nkx2.2 represses FoxA2-induced *Arx* expression. These results support a model where Arx and FoxA2 participate in a feedback loop with Shh signaling, establishing a robust method to regulate the dynamic expression of *Shh* required for its multiple functions during spinal cord development.

Materials and methods

Mice

Arx mutant mice (Fulp et al., 2008) were bred and maintained on C57Bl/6 background in according with an approved IACUC protocol at the Children's Hospital of Philadelphia and Brigham and Women's Hospital/Harvard Medical School. $Arx^{-/y}$ mouse embryos were generated by mating $Arx^{F/+}$ with $EIIa^{cre}$ male (The Jackson Laboratory stock no. 003724). All genotypings were performed as previously described (Fulp et al., 2008).

DNA constructs

Arx, *FoxA2*, *Nkx2.2* (human sequence for *NKX2.2 was used but is referred throughout as Nkx2.2*) and each deletion mutant, used for *in ovo* electroporation, were cloned into the *pCIG* vector (Megason and McMahon, 2002) that expresses eGFP under IRES, after PCR-amplification with the oligonucleotides as following: ArxF (5′-CG GAATTCCACCATGAGCAATCAGTACCAGGAAGAG-3′), Arx61F (5′-CG GAATTCCACCATGGAAAAAGCCATGCAAGGCTCCCCC-3′), Arx220F (5′-CGGAATTCCACCATGGGCGCCGAGGACGACGAGG-3′), Arx471mycR (5′-ACTTCAACGCGTCTACAGATCTTCTTCAGAAATAAGTTTTTGTTCCGCTGCT CCTAGAAAAGTGCTCAGACC-3′), ArxmycR (5′-ACTTCAACGCGTCGAGC-TACAGATCTTCTTCAGAAATAAGTTTTTGTTCGCACACCTCCTTCCCCGTGCT G-3′), FoxA2FLAGF (5′-CGGAATTCCACCATGGATTACAAGGATGACGAC-GATAAGCTGGGAGCCGTGAAGATGGAA-3′), FoxA2R (5′-ACCGACGCGTT-TAGGATGAGTTCATAATAGGCCTGGAGTACACTC-3′), FoxA2F52 (5′-CGG AATTCCACCATGGATTACAAGGATGACGACGATAAGGGCGGCGGTTCCGG-CAACAT –3′), FoxA2R-418 (5′-AACCGACGCGTTTAGGAACCATAGCCCCCT GGGTAGTGC-3′), FoxA2D372-383F (5′-CCACCTGAAGCCCGAGCACCAT-TACTCGTCCGAGCAGCAACATCACCA-3′), Nkx2.2F (5′-CGGAATTCCAC-CATGGATTACAAGGATGACGACGATAAGATGTCGCTGACCAACACAAAGA CGG-3′), Nkx2.2R (5′-AACCGACGCGTTCACCAAGTCCACTGCTGGGCCT-3′), Nkx2.2F113 (5′-CGGAATTCCACCATGGATTACAAGGATGACGACGAT-AAGGACAATGACAAGGAGACCCCGGGC-3′) and Nkx2.2R187 (5′-AA-CCGACGCGTTCACCGGGCGCGCTTCATCTTGTAG-3′). Arx MT is R3-32H, which does not bind to DNA due to mutation in homeodomain of Arx (Cho et al., 2012). The *FoxA2* and *Nkx2.2* constructs include a *FLAG*-tag embedded in the 5′ end of the oligonucleotide sequence. The *FoxA2ΔA* (52-418) deletion construct lacks the transcription activation domain (Pani et al., 1992). The *FoxA2ΔI*, internal deletion mutant which excludes amino acid 372–387 (TLE/Groucho binding site), was cloned into EcoRI and MluI of pCIG vector as described previously (J.C. Wang et al., 2000). *Nkx2.2HD* (aa113–187) (dominant negative mutant which contains only homeodomain) was constructed into pCIG as previously described (Watada et al., 2000). The $Ptc^{\Delta loop2}$, *SmoM2*, and ΔN-*Gli3* constructs were all previously described (Lei et al., 2004; Lek et al., 2010; Tenzen et al., 2006). The deleted Arx DNA fragments, used for immunoprecipitation experiment, were subcloned into both EcoRI and XbaI digested pM vector (Clontech) after PCR amplification; ArxF