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Region- and cell type-selective expression of the evolutionarily conserved *Nolz-1/zfp503* gene in the developing mouse hindbrain

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ABSTRACT

Nolz-1/Zfp503, a zinc finger-containing gene, is a mammalian member of the SP1-related *nocA/elb/tlp-1* gene family. Previous studies have shown that *Nolz-1* homologs are important for patterning the rhombomeres in zebrafish hindbrain. We therefore studied the expression pattern of *Nolz-1* in the developing mouse hindbrain. *Nolz-1* mRNA expression was detected in the prospective rhombomere 3, 5 and caudal regions as early as E8.75. After E11.5, *Nolz-1*-positive cells were organized as distinct cell clusters, and they were largely non-overlapped with either *Pax2*-positive or *Phox2b*-positive domains. Most interestingly, we found that *Nolz-1* was specifically expressed by *Phox2b*-negative/Isl1/2-positive somatic motor neurons, but not by *Phox2b*-positive/Isl1/2-positive branchial and visceral motor neurons, suggesting that *Nolz-1* may regulate development of somatic motor neurons in the hindbrain. In addition to be expressed in differentiating post-mitotic neurons, *Nolz-1* was also expressed by progenitor cells in the ventricular zone located in the dorsal part of aqueduct and the alar plates of hindbrain, which suggests a regulatory role of *Nolz-1* in the germinal zone. Taken together, based on its domain- and cell type-selective pattern, *Nolz-1* may involve in regulation of various developmental processes, including regional patterning and cell-type specification and differentiation in the developing mouse hindbrain.

We have previously identified a zinc finger-containing gene *Nolz-1* (Zfp503, Mouse Genome Informatics) in the rodent (Chang et al., 2004). *Nolz-1* is a mammalian member of the SP1-related *nocA/elb/tlp-1* (NET) gene family (Nakamura and Sagerstrom, 2004). Previous studies have shown that members of the NET family across different species are involved in developmental control of different tissues and organs. The *Caenorhabditis elegans tlp-1* gene controls asymmetric cell fate determination in tail morphogeneis (Zhao et al., 2002). The *Nolz-1* homolog *no ocelli* (*noc*) gene in *Drosophila* regulates development of embryonic brain and adult ocellar structure (Cheah et al., 1994). Both *noc* and another homolog *elbow* (*elB*) *genes* are involved in control of the morphogenesis of specific tracheal branches, the formation of wing and leg appendages and the size of the eye-head primordia in *Drosophila* (Dorfman et al., 2002; Luque and Milan, 2007; Weihe et al., 2004). *Nlz2*, the *Nolz-1* ortholog in zebrafish, and its paralogue *nlz1* are involved in closure of the optic fissure and determination of rhombomeres identities and hindbrain segmentation (Runko and Sagerstrom, 2003; Hoyle et al., 2004; Runko and Sagerstrom, 2004; Brown et al., 2009). The chick homolog of *Nolz-1* regulates development of subtypes of motor neurons in the spinal cord (Ji et al., 2009).

Previous studies have shown that *Nolz-1* is expressed in selective tissues in which its homologous genes in other species are also expressed, including the brain, lung, limbs and eyes, which implies a fundamental role of *Nolz-1* in organogenesis during evolution. We and other groups have previously reported that *Nolz-1* expression is

Abbreviations: IV, fourth ventricle; BA, branchial arches; BM, branchial motor neurons; Cb, cerebellum; CLi, caudal linear nucleus raphe; DC, dorsal cochlear nucleus; dmnX, dorsal motor nucleus of the vagus nerve; DPGi, dorsal paragigantocellular nucleus; DTg, dorsal tegmental nucleus; fl, forelimb; Gi, gigantocellular nucleus; GiV, ventral gigantocellular nucleus; hl, hindlimb; ht, heart primordium; IOm, medial nucleus of inferior olive; IOpr, principal nucleus of inferior olive; IRt, intermediate reticular zone; ISH, *in situ* hybridization; LC, locus coeruleus; LDTg, laterodorsal tegmental nucleus; LPGi, lateral paragigantocelullar nucleus; MB (mb), midbrain; MdD, dorsal medullary reticular nucleus; Me5, mesencephalic trigeminal nucleus; MHB, midbrain-hindbrain boundary; nV, trigerminal cranial nerve; nXII, 12th cranial nerve; op, optic vesicles; ot, otic vesicles; PCRt, parvocellular reticular nucleus; po, pons; rmed, rostral medulla; RVL, rostroventrolateral reticular nucleus; PrH, prepostitus hypoglossal nucleus area; s, somites; sc, spinal cord; SC, superior coliculus; SM, somatic motor neurons; Sp5, spinal trigeminal nucleus; Sol, nucleus of solitary tract; ST, striatal primordium; SVZ, subventricular zone; VC, ventral cochlear nucleus; Ve, vesticular nucleus; VM, visceral motor neurons; VZ, ventricular zone.

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enriched in the developing striatum of murine telencephalon (Chang et al., 2004; McGlinn et al., 2008). *Nolz-1* mRNA is also expressed in the developing rat hindbrain (Chang et al., 2004). Because *nlz1* and *nlz2* have been shown to play important roles in patterning rhombomeres of zebrafish hindbrain (Runko and Sagerstrom, 2003; Hoyle et al., 2004; Runko and Sagerstrom, 2004), a natural question is whether *Nolz-1*, by functional conservation, also regulates developing hindbrain in rodents.

On the first attempt to address this issue, we reported in the present study the spatiotemporal expression of *Nolz-1* mRNA in the developing mouse hindbrain. We found that among the three types of motor neurons in the hindbrain, *Nolz-1* was specifically expressed in somatic motor neurons. *Nolz-1* was also expressed in selective nuclei of developing hindbrain. Our study suggests that *Nolz-1* may regulate development of specific types of neurons in distinct hindbrain nuclei during development.

1. Results and discussion

1.1. Evolutionarily conserved domain-selective expression of Nolz-1 homologs during neural tube patterning

The whole-mount *in situ* hybridization analysis showed that, at the stage of neural tube patterning, a heterogeneously segmented expression pattern of *Nolz-1* mRNA was observed (Fig. 1A–C). A distinct pattern of *Nolz-1* expression was found in the brain vesicles at the turning stage of embryonic day (E) 8.75 (Fig. 1A). *Nolz-1* mRNA was detected in the vesicles of prosencephalon (forebrain primordium), mesencephalon (midbrain primordium) and rhombencephalon (hindbrain primordium). It was of particular interest that high levels of *Nolz-1* mRNA were present at the boundaries between different vesicles, including the forebrain–midbrain boundary and the midbrain–hindbrain boundary (MHB) (single arrow and arrowhead, respectively, in Fig. 1A and B).

In E8.75 and E9.5 rhombencephalon, *Nolz-1* mRNA were detected in the presumptive rhombomeres (r) 3, r5 (double arrows, Fig. 1A and B) and in more caudal regions. This rhombomere-selective expression pattern of *Nolz-1* was persisted through E10.5 (Fig. 1C).

Notably, the preferentially expression of *Nolz-1* mRNA in the MHB region, r3, r5 and caudal hindbrain regions is consistent with the zebrafish *nlz1* and *nlz2* homologous genes expression at early neural tube patterning stages (Hoyle et al., 2004). Taken together, the observation of conserved expression pattern of *Nolz-1* mRNA suggests that Nolz-1 may play an evolutionarily conserved role in regulation of developing MHB and hindbrain segmentation.

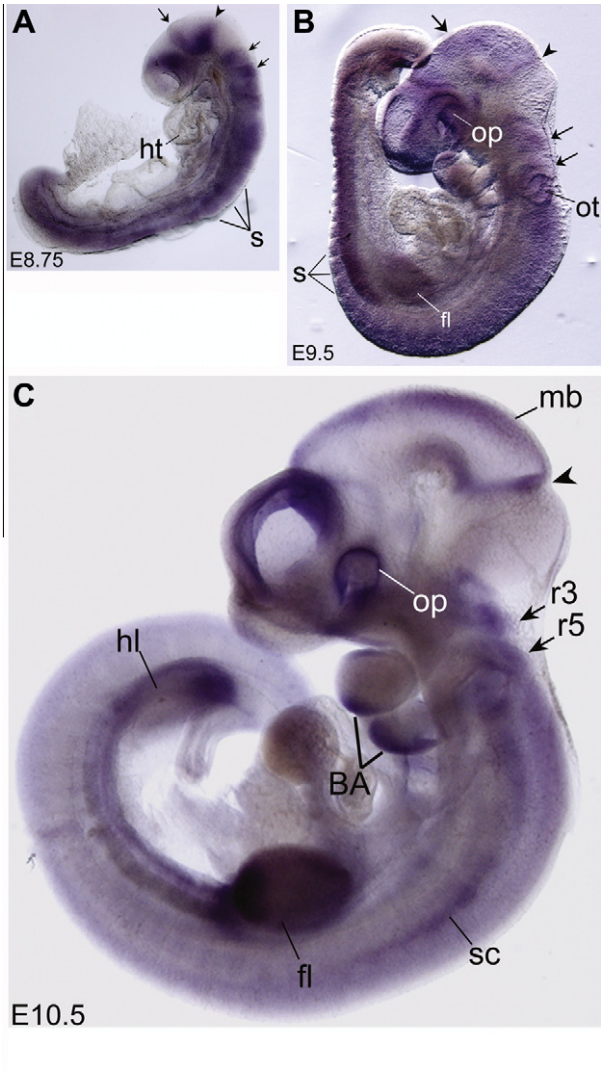

Fig. 1. Domain-selective expression of *Nolz-1* mRNA in neural tube of early developing mouse embryos. Distinct domain-selective expression pattern of *Nolz-1* mRNA is detected in the mouse brains at E8.75 (A), E9.5 (B) and E10.5 (C). High levels of *Nolz-1* mRNA are selectively expressed in the forebrain-midbrain boundary (single arrow), the midbrain–hindbrain boundary (arrowhead), r3 and r5 (double arrows). BA, branchial arches; fl, forelimb bud; hl, hindlimb bud; ht, heart primordium; mb, midbrain; op, optic vesicle; ot, otic vesicle; s, somites, sc, spinal cord.

1.2. Spatiotemporal expression of Nolz-1 mRNA in longitudinal columns and cell clusters in the developing hindbrain at mid-gestational stages

We further performed *in situ* hybridization (ISH) on sections of mouse hindbrains from E10.75 to later stages to analyze the detailed pattern of *Nolz-1* mRNA expression (Figs. 2–4). Interestingly, the rhombomeres-selective expression pattern of *Nolz-1* disappeared after E10.75 when cell migration events began in the developing hindbrain. Instead, dynamic spatiotemporal expression patterns of *Nolz-1* mRNA were observed. *Nolz-1* mRNA expression was more widely distributed in the developing hindbrain except r1 region at E10.75 and E11.5 (Fig. 2A–D).

By comparing the *Nolz-1* expression pattern with the immunostaining pattern of Hoxb1, a marker for r4 (Wilkinson et al., 1989), on adjacent coronal sections of E11.5 hindbrain, we found that *Nolz-1 mRNA* was selectively expressed in r3 and r5, but not in r4 (Fig. 2D and E). *Nolz-1* mRNA was detected in the ventricular zone of r3 and the mantle zones of r3 and r5. Besides, low level of *Nolz-1* expression was expanded rostrally to encompass the ventricular zone of r2 region (Fig. 2A–D). The expression of *Nolz-1* mRNA in both the germinal and the mantle zones of developing hindbrain suggests that *Nolz-1* may regulate different developmental processes during neurogenesis of the developing hindbrain.

It was notable that there were many strong *Nolz-1*-positive cell clusters in the lateral-most parts of E10.75 hindbrain (arrows, insets in Fig. 2A and B). These *Nolz-1*-positive cell clusters were more prominently located at the level of the otic vesicle nearby the vestibulocochelear boundary caps, a region where *Nolz-1* was also expressed at E10.75 (# in Fig. 2B, inset). Later at E11.5, the *Nolz-1*-positive cell clusters were assembled into a *Nolz-1*-positive strip which was expanded rostrally up to the r2 level (double arrows, Fig. 2D).

At the caudal levels of r6-r8, *Nolz-1*-positive longitudinal columns were observed (asterisks, Fig. 2A and B). Two distinct *Nolz-1*-positive cell columns, the medial-most column and the middle

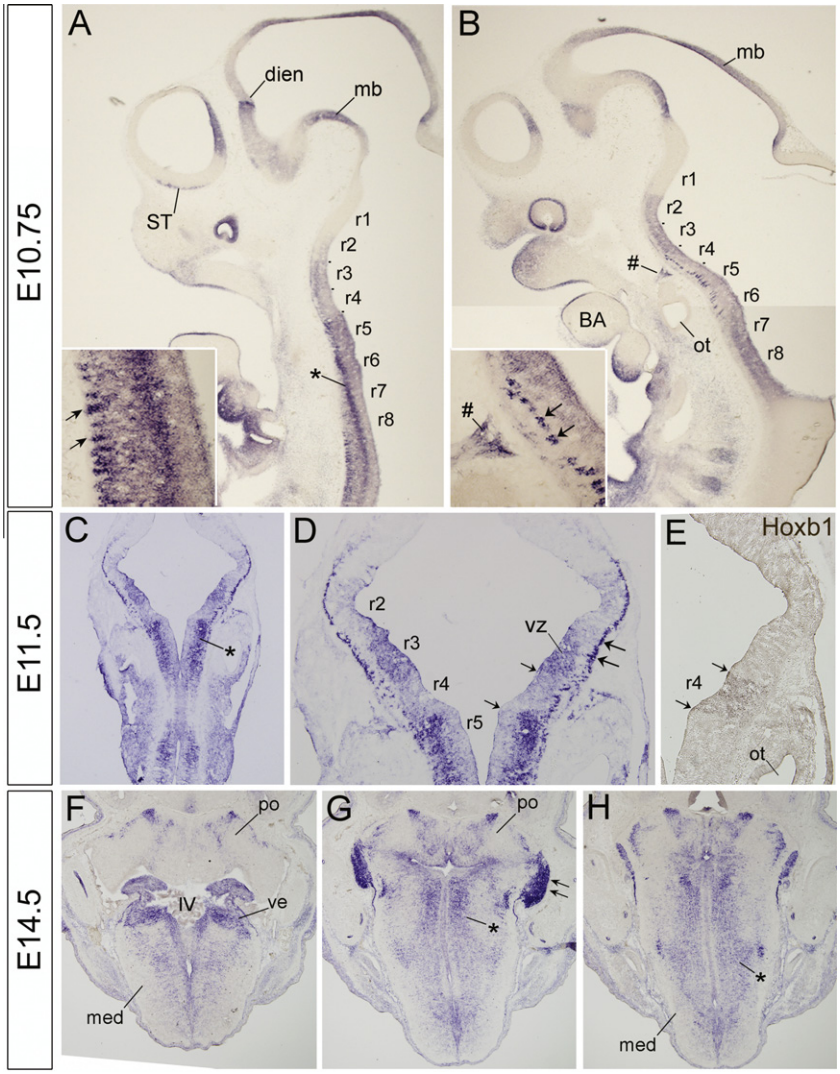

Fig. 2. Spatiotemporal dynamic expression of *Nolz-1* mRNA in developing hindbrain at mid-gestational stages. (A and B) Parasagittal section of E10.75 mouse embryo. (C, D, F, and G) Coronal sections of E11.5 (C and D) and E14.5 (F and G) mouse hindbrain. At E10.75, *Nolz-1* mRNA is broadly expressed in the rhombomeres except r1 region (A and B). Prominent *Nolz-1*-postive longitudinal columns (asterisk in A and C) and distinct *Nolz-1*-positive cell clusters (double arrows in inset of A and in D) extend from r2 to r5 and r6 regions in the hindbrain. *Nolz-1* mRNA is also expressed in the vestibulocochelear boundary cap (# in inset of A). Notably, *Nolz-1* mRNA is detected in the ventricular zone (vz) of r3 but not in Hoxb1-positive r4 region (the region flanked by single arrows in D and E). The longitudinal columns (asterisks in G and H) expression pattern remained in E14.5 medulla (med). High levels of *Nolz-1* mRNA expression are also detected in distinct domains of pons (po) and in the presumptive vesticular nucleus (Ve in F) and cochlear nucleus (double arrows in G). BA, branchial arches; dien, diencephalon; mb, midbrain; ST, stristum primordium; ot, otic vesicle.

column, were identified in the parasagittal sections of E10.75 hindbrain (Fig. 2B; data not shown). These longitudinal *Nolz-1*-positive cell columns were more prominently observed in coronal sections of E11.5 hindbrain (open arrow, arrowhead, Fig. 3B). The middle *Nolz-1*-positive cell column (arrowhead) was extended more rostrally than the medial-most column (open arrow, Fig. 3A and B). Interestingly, anterior to the rostral limit of the longitudinal *Nolz-1*-positive cell columns (about at the r4–r5 level), a *Nolz-1*-negative domain in the medial part of basal (ventral) plate, where presumptive branchial motor (BM) neurons resided, was observed (Fig. 3A; see below) (Pattyn et al., 2000).

Expression of *Nolz-1* mRNA was persistently detected in the longitudinal columns of reticular nuclei at the rostral and caudal levels of medulla (asterisks, Fig. 2G and H) and also in the vesticular nucleus (Ve) at E14.5 (Fig. 2F). Strong *Nolz-1* mRNA was detected in the cochlear nucleus (double arrows, Fig. 2G,), which may be derived from the aforementioned strong *Nolz-1*-postive lateral-most strip at E11.5. In addition to the medulla, a distinct pattern of *Nolz-1* expression was present in the pons (po) of rostral hindbrain (Fig. 2F–H).

1.3. Differential expression of Nolz-1 and Pax2 mRNAs in the developing hindbrain at mid-gestational stages

In the developing mouse brain, *paired box gene 2* (*Pax2*) is prominently expressed in the MHB and hindbrain regions where *Nolz-1* is also expressed (Nornes et al., 1990; Urbanek et al., 1997). It was then of interest to compare the expression pattern of *Nolz-1* mRNA with that of *Pax2* for potential genetic interaction of *Nolz-1* and *Pax2* in hindbrain development.

Double labeling of *Nolz-1* and *Pax2* showed that the majority of *Nolz-1* expression was complementary to that of *Pax2* expression in several regions of hindbrain at E11.5, E12.75 and E18.5 (Figs. 3 and 4). In the alar (dorsal) plate of E11.5 hindbrain at the level rostral to the otic vesicle, *Pax2* mRNA expression was detected in the intermediate zone (double open arrows) adjacent to the *Nolz-1*-positive ventricular zone (VZ, Fig. 3A and A′, double black arrows). Similar complementary expression pattern *i.e.*, *Nolz-1* in VZ and *Pax2* in adjacent subventricular zone (SVZ), was also observed at E12.75 (Fig. 3G and G′). These results implied that *Nolz-1* may be involved in neurogenesis of some *Pax2*-positive neurons.

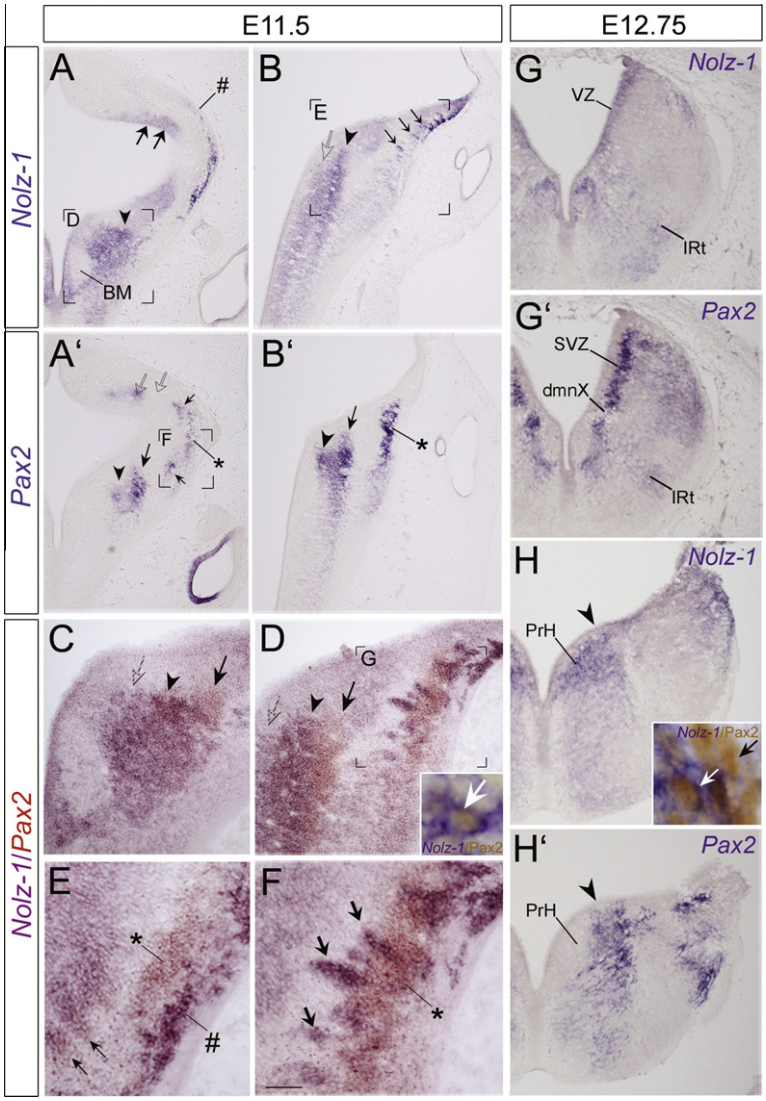

Fig. 3. Differential expression of *Nolz-1* and *Pax2* in developing hindbrain at mid-gestational stages. (A, B, G,H, A′, B′, G′, and H′) Expression of *Nolz-1* and *Pax2* mRNAs in E11.5 (A and A′, rostral levels; B and B′, caudal levels) and E12.75 (G and G′ caudal levels; H and H′, rostral levels) mouse hindbrain. (C–F and inset in H) Double labeling of *Nolz-1* mRNA and *Pax2* mRNA (C–F, at E11.5) or Pax2 protein (insets in D at E11.5 and in H at E12.75). *Nolz-1* is detected in the ventricular zone (double arrows in A; VZ in G) of hindbrain alar plate. In the adjacent coronal sections, *Pax2* is expressed in a domain next to the *Nolz-1*(+) VZ region (double open arrows in A′, SVZ in G′). Note that expression of *Pax2* in SVZ is interrupted by dorsal motor nucleus of the vagus nerve (dmnX, G′). At the most lateral region of pre-optic hindbrain, a *Nolz-1*(+) strip is observed (# in A and E). Medial to the *Nolz-1*(+) strip is a *Pax2*(+) strip (asterisks in A′ and E). Several *Nolz-1*(+) cell clusters associated with the lateral strip (triple arrows in B and F) are extended medially next to the *Pax2*(+) strip. The bracketed region in D is shown at high magnification in F. Additional two *Pax2*-positive domains with clustered cells are observed more medially to the *Pax2*+ strip (small arrows in A′ and E). Note that *Nolz-1* is not expressed in a domain in which the branchial motor neurons (BM in A) may reside. In the medial region of caudal hindbrain, distinct *Nolz-1*(+) longitudinal columns (arrowhead in A, open arrow in B) and *Pax2*(+) longitudinal columns (arrowhead, arrow in A′ and B′) are detected. *Nolz-1*(+) and *Pax2*(+) columns are overlapped in the middle column (arrowheads in C, D, H, and H′) where cells co-expressing *Nolz-1* mRNA and Pax2 protein are detected (white arrows in insets in D and H; black arrow indicates the single Pax2-positive cell). At E12.75, this *Nolz-1* and Pax2 co-expression domain is lateral to the *Nolz-1*(+) prepositus hypoglossal nucleus area (PrH). Expressions of *Nolz-1* and *Pax2* are also detected in the prospective pre-intermediate reticular zone (IRt in G and G′). Scale bar in F for C and E-F is 50 μm.

Furthermore, in the lateral differentiated regions at the level anterior to and around the otic vesicle, non-overlapping expression pattern of *Nolz-1* and *Pax2* mRNAs was also found (Fig. 3A, A′ and E). A *Pax2*-positive and *Nolz-1*-negative lateral strip was located immediately medial to the lateral-most *Nolz-1*-postive strip. Some *Nolz-1*-positive cell clusters (arrows) were extended into, but non-overlapped with, the Pax2-postive lateral strip (asterisk, Fig. 3D and F) in the caudal part.

At the levels caudal to the otic vesicle, there were two prominent *Pax2*-positive longitudinal columns in the basal/medial plate of E11.5 hindbrain (Fig. 3B′). The double labeling analysis showed that, the short one located medially was overlapped with the aforementioned *Nolz-1*-positive middle column (arrowhead, Fig. 3C and D). In contrast, *Nolz-1* expression was absent in the other strong *Pax2*-postive column located laterally (arrow, Fig. 3C and D). Double labeling of *Nozl-1* mRNA and *Pax2* protein identified many *Nolz-1*(+)/Pax2(+) cells in the overlapping domain at E11.5 (inset, Fig. 3D). At E12.75, *Nolz-1*(+)/Pax2(+) cells were also detected in a similar medial domain which was laterally to the *Nolz-1*-positive prospective prepostitutus hypoglossal neucleus (PrH; Fig. 3H, inset). *Nolz-1*-postive and *Pax2*-positive overlapped domain included the prospective pre-intermediate reticular zone (IRt, Fig. 3G and G′).

Like *Nolz-1*, *Pax2* is also a *trans-dev* gene (genes implicated in transcriptional regulation and early development) whose tissue-specific expression is conserved under regulation of evolutionarily

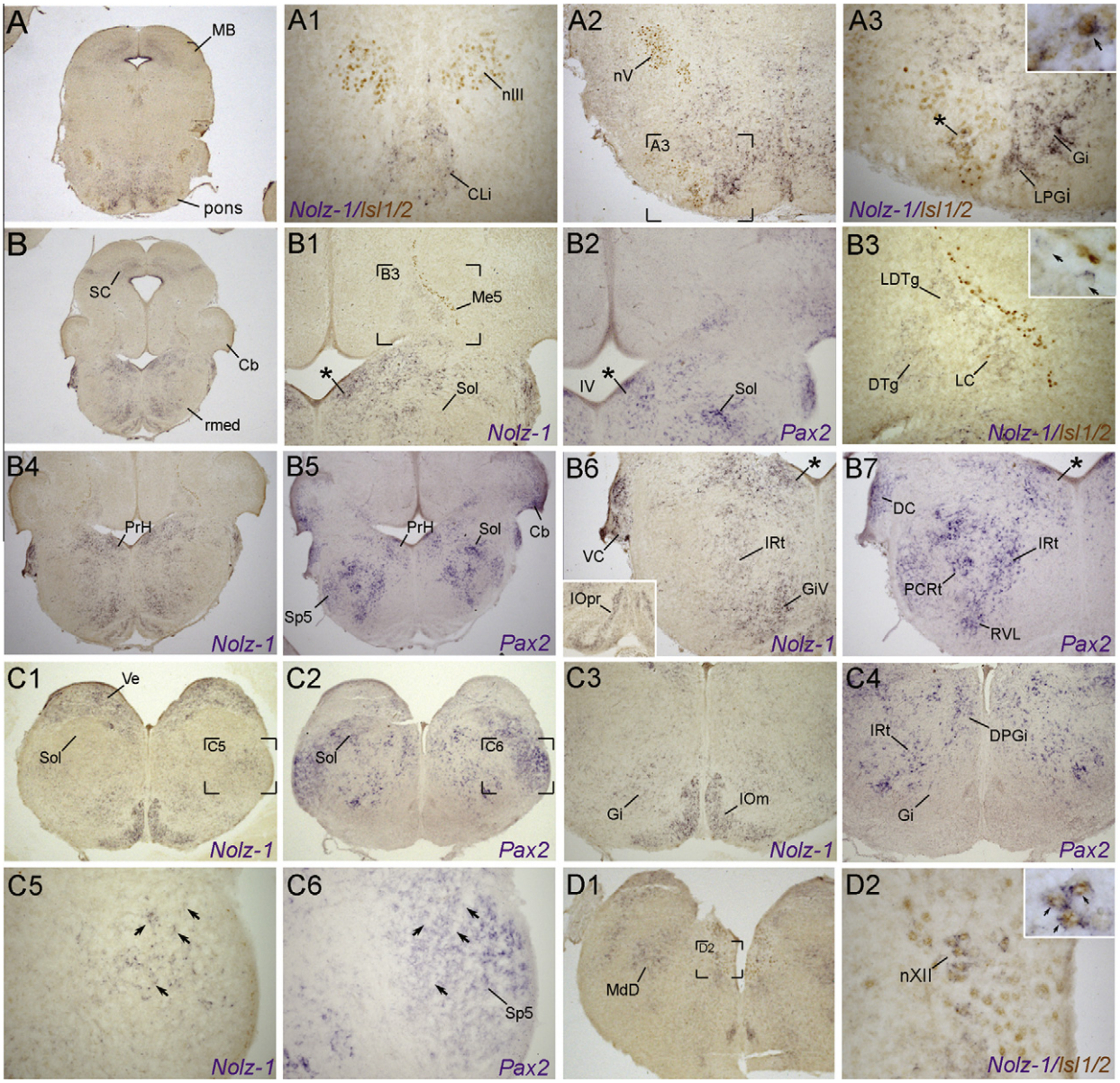

Fig. 4. Expression of *Nolz-1* mRNA in E18.5 hindbrain. Double labeling of *Nolz-1* mRNA with Isl1/2 protein or Pax2 mRNA on adjacent coronal sections was performed. Region-selective expression pattern of *Nolz-1* mRNA is detected in the midbrain (MB, A), pons, rostral medulla (rmed, B) and caudal medulla (C1). In the midbrain region, Nolz-1 is expressed in the superior coliculus (SC), ventricular zone of dorsal aqueduct, caudal linear nucleus raphe (CLi, A1), laterodorsal tegmental nucleus (LDTg), dorsal tegmental nucleus (DTg) and locus coerulus (LC) (B1, high magnification in B3). In the pons, strong *Nolz-1*(+) cells are present in the ventromedial part, including the lateral paragigantocelullar nucleus (LPGi) and the gigantocellular nucleus (Gi) (A2, high magnification in A3). No *Nolz-1* expression is detected in the Isl1/2(+) trigerminal motor neucleus (nV). A few *Nolz-1* and Isl1/*2* double-labeled cells are present in the Isl1/2(+) cell clusters laterally to the LPGi (asterisk in A3, arrow in inset). At this stage, many *Nolz-1*(+) cells are found in the regions expressing none or low levels of *Pax2*, including the prepostitus hypoglossal nucleus (PrH, in B4 and B5; see also asterisks in B6 and B7), vesticular nucleus (Ve, C1 and C2), medial nucleus and principal nucleus of inferior olive (IOm and IOpr, respectively; B6 and B7, inset in B6, C3 and C4), Gi (C3 and C4) and ventral Gi nucleus (GiV, B6 andB7). The tendency of avoiding *Pax2*(+) areas by *Nolz-1*(+) cells, and vice versa, were also found in the solitary nucleus (Sol, C1 and C2), rostroventrolateral and parvocellular reticular nucleus (RVL and PCRt, respectively, B6 and B7) and dorsal paragigantocellular nucleus (DPGi, C1–C4). Note that, although some *Nolz-1*(+) cells are detected in *Pax2*(+) regions such as the intermediate reticular zone (IRt, B6 and B7, C3 and C4) and spinal trigeminal nucleus (Sp5, C1 and C2, high magnifications in C5 and C6), these *Nolz-1*(+) cells appear to be embedded in *Pax-2*-negative domains (arrows in C5 and C6). In the cochlear nucleus, *Pax2* is expressed in the dorsal part, whereas *Nolz-1* is expressed in both the dorsal and the ventral cochlear nucleus (DC, VC, B6 and B7). *Nolz-1* expression in SM neurons of the 12th cranial nerve (nXII) is remained at this stage (D1, high magnification in D2 and inset; arrows in inset indicate *Nolz-1* and Isl1/2 double-labeled somatic motor neurons).

conserved *cis*-, elements during development (Woolfe et al., 2005; McEwen et al., 2006; Woolfe and Elgar, 2007). Previous study in zebrafish has shown that *nlz1* expression in hindbrain and MHB is dependent on *pax2a*, which suggests that *nlz1* is downstream target gene of *pax2a* (Hoyle et al., 2004). However, recent study of closure of optic fissure in zebrafish shows that Nlz1 and Nlz2 are capable of binding to specific conserved *cis*-element in *Pax2* promoter, and Nlz acts as transcriptional repressor on *Pax2* promoter in ARPE19 human retina epithelium cell line (Brown et al., 2009). Consistent with this report, our study showed that the majority of *Nolz-1* expression was complementary to *Pax2* expression in the developing hindbrain.

1.4. Expression of Nolz-1 mRNA in the midbrain and hindbrain nuclei at E18.5

At E18.5, *Nolz-1* mRNA was expressed in selective nuclei in the midbrain and hindbrain regions (Fig. 4; Table 1). In the midbrain

Table 1
Expression of Nolz-1 and Pax2 mRNAs in E185 mouse hindbrain

Hindbrain regions	Expression level	
	Nolz-1	*Pax2*
Nucleus of ambiguus (Amb)	-	-
Caudal linear nucleus raphe (CLi)	+	-
Cochlear nucleus, dorsal (DC)	+++	++
Cochlear nucleus, ventral (VC)	++	-
Dorsal motor nucleus of the vagus nerve (DmnX)	-	-
Gigantocellular nucleus (Gi)	+++	-
Gigantocellular nucleus, ventral (GiV)	++	-
Hypoglossal nerve 12th cranial nerve (nXII)	++	-
Inferior oliver, principal nucleus (IOpr)	+++	-
Inferior oliver, medial nucleus (IOm)	+++	-
Intermediate reticular zone (IRt)	+	+++
Locus coerulus (LC)	+	++
Mesencephalic trigeminal nucleus (Me5)	-	-
Nucleus of solitary tract (Sol)	-	++
Paragigantocellular nucleus, dorsal (DPGi)	-	++
Paragigantocellular nucleus, lateral (LPGi)	++	-
Parvocellular reticular nucleus (PCRt)	-	+++
Prepositus hypoglossal nucleus (PrH)	++	+
Reticular nucleus, dorsal medullary (MdD)	++	-
Reticular nucleus, rostroventrolateral (RVL)	+/-	+++
Spinal trigerminal nucleus (Sp5)	+	+++

Signal intensity: +++ strong; ++ moderate; + weak; - none.

region, *Nolz-1* expression remained in the superior colliculus (SC; Fig. 4B). *Nolz-1* was also expressed in the caudal linear nucleus raphe (CLi) (Fig. 4A1). Moreover, low levels of *Nolz-1* were detected in the laterodorsal and dorsal tegmental nucleus (LDTg, DTg) and the locus coeruleus (LC) (Fig. 4B1 and B3). Notably, in addition to the differentiated regions, *Nolz-1* mRNA was also detected in the neuroepithelium surrounding the dorsal part of aqueduct (Fig. 4A and B).

In the rostral hindbrain region, *Nolz-1* mRNA was detected in the lateral paragigantocellular nucleus (LPGi), the gigantocellular nucleus (Gi) (Fig. 4A2 and A3). In the medulla, *Nolz-1* was expressed in PrH and Ve (Fig. 4B4 and B6, asterisk) as observed at E12.75 and E14.5. Moreover, *Nolz-1* mRNA was also detected in the ventral Gi (GiV), principal nucleus of inferior olive (IOpr; Fig. 4B4 and B6, inset), medial nucleus (IOm; Fig. 4C1 and C3) and dorsal medullary reticular nucleus (MdD; Fig. 4D1). Scattered *Nolz-1*-positive cells were also found in the intermediate reticular zone (IRt; Fig. 4B6) and the spinal trigeminal nucleus (Sp5; Fig. 4C5). Interestingly, double labeling of *Nolz-1* mRNA and Isl1/2, a marker for hindbrain motor neurons, showed that, in the nucleus of the hypoglossal cranial nerve (nXII), most Isl1/2-posiitive somatic motor (SM) co-expressed *Nolz-1,* mRNA (Fig. 4D2, inset).

1.5. Differential expression of Nolz-1 and Pax2 mRNAs in the hindbrain nuclei

Analysis of coronal sections of E18.5 hindbrain showed that *Pax2* mRNA was not expressed in the LPGi, Gi and ventral Gi (GiV), Ve, IOm, IOpr, MdD, in which *Nolz-1* mRNA was detected. *Pax2* was detected in many *Nolz-1*-negative regions, including the rostroventrolateral and parvocellular reticular nucleus (RVL, PCRt; Fig. 4B7), the dorsal paragigantocellular reticular nucleus (DPGi, Fig. 4C4) and the nucleus of solitary (Sol; Fig. 4B2 and B5) where *Phox2b* was also expressed (data not shown).

For other *Pax2*-positive regions, including IRt and Sp5, some scattered *Nolz-1*-positive cells were found (Fig. 4B6, B7, C5 and C6). Note that *Nolz-1*-positive cells were found in the *Pax2*-negative domains of Sp5 nucleus (arrows, Fig. 4C5 and C6). In the *Nolz-1*-positive PrH region, scattered *Pax2*-positive cells were detected. Note that *Pax2* expression was absent in PrH at E12.75 (Fig. 3H and H′). In the cochlear nucleus nearby the otic vesicle, *Nolz-1* mRNA was expressed in both the dorsal and ventral cochlear nucleus (VC), whereas *Pax2* mRNA was expressed only in the dorsal cochlear nucleus (DC, Fig. 4B6 and B7).

It was of interest that *Nolz-1* mRNA expression was not detected in the primordium of cerebellum in which *Pax2* mRNA expression was detected (Fig. 4B4 and B5). Taken together with the findings of predominantly non-overlapping pattern of *Nolz-1* and *Pax2* at E11.5 and E12.75, *Nolz-1* and *Pax2* were preferentially expressed by different types of cells in the developing hindbrain.

1.6. Specific expression of Nolz-1 mRNA in somatic motor neurons of hindbrain motor columns

The aforementioned results showed that *Nolz-1* was not expressed in the domain of presumptive nucleus of BM neurons, but Nolz-1 was expressed in SM neurons in nXII nucleus in E18.5 medulla. There are three types of motor neurons, SM, BM and visceral motor neurons (VM) in the hindbrain. To study if *Nolz-1*, was

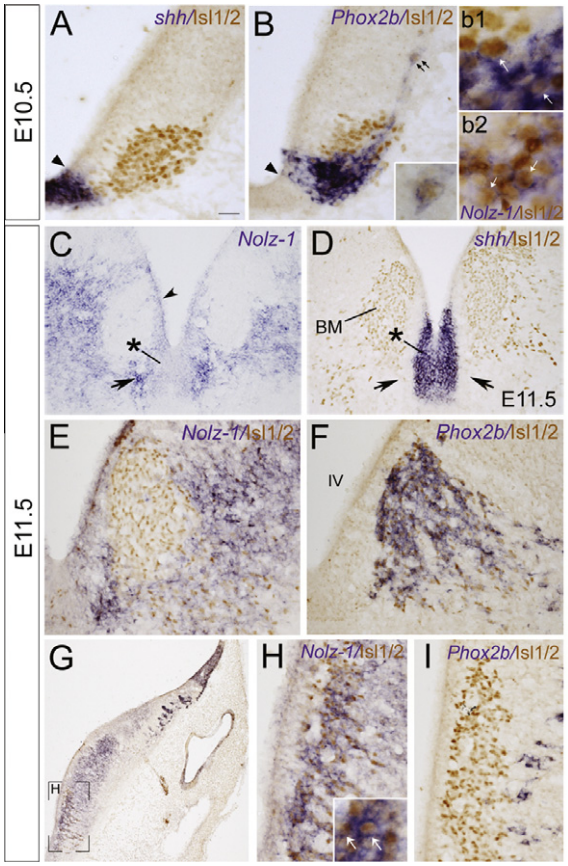

Fig. 5. Selective-expression of *Nolz-1* mRNA in somatic motor neurons of developing hindbrain at mid-gestational stages. (A, B, b1, and b2) Double labeling of Isl1/2 protein and *shh* (A), Isl1/2 and *Phox2b* (B and b1) and Isl1/2 and *Nolz-1* mRNA (b2) were performed in adjacent horizontal sections of E10.5 mouse hindbrain. Within the ventral part of Isl1/2(+) motor neurons domain, many *Phox2b*(+)/Isl1/2(+) branchial motor neurons (BM)/visceral motor neurons (VM) neurons are present (B, see b1 for high magnification, white arrows), whereas at the dorsal part, many *Nolz-1*(+)/Isl1/2(+) cells are detected (white arrows in b2). Double arrows in B indicate the caudally migrating facial BM/VM motor neurons (see inset for high magnification). Arrowheads in A, B indicate the boundary of *shh*-positive midline region. (C–F) Expression pattern analysis on E11.5 adjacent coronal hindbrain sections (C, *Nolz-1* mRNA; D, *shh* mRNA/Isl1/2 protein, E: *Nolz-1* mRNA/Isl1/2 protein) show that *Nolz-1* is not expressed in the Isl1/2-positive region. Double labeling of *Phox2b* mRNA and Isl1/2 on the adjacent section show that this *Nolz-1*-negative domain is *Phox2b*(+)/Isl1/2(+) BM motor column (F). (G–I) *Nolz-1* is expressed in *Phox2b*(-)/Isl1/2(+) somatic motor (SM) neurons (H and I), and the majority of *Nolz-1*(+)/Isl1/2(+) SM neurons (white arrows, inset in H) are present in the medial part of hindbrain at the level caudal to the optic vesicle. Scale bar in A for A and B is 50 μm.

expressed in specific class of hindbrain motor neurons, we performed double ISH and immunohistochemistry of *Nolz-1* and Isl1, a marker for all three types of cranial and hindbrain motor neuron (Guidato et al., 2003). Because the K5 Isl1 antiserum that we used for immunostaining recognized Isl1 and Isl2 proteins, we referred the immunoreactivity detected by K5 antiserum as Isl1/2 immunoreactivity (Fig. 5b2, E, G and H). We also compared the expression pattern of *Nolz-1* mRNA with that of *Phox2b* mRNA, a marker for both BM and VM neurons, but not SM neurons, on adjacent sections (Fig. 5E–I) (Pattyn et al., 2000).

At E10.5, Isl1/2-positive motor neurons were detected in the ventral domains neighboring the *Shh*-expressing midline in transverse sections of hindbrain (Fig. 5A). At this stage, both of BM/VM and SM progenitor cells are generated in the same pool but are located separately at the ventral and the dorsal parts, respectively (Dauger et al., 2003). Indeed, our double staining of *Phox2b* and Isl1/2 detected many *Phox2b*(+)/Isl1/2(+) BM/VM neurons in the ventral part of the Isl1/2-positive domain. Some of the dorsolaterally migrating *Phox2b*(+)/Isl1/2(+) BM/VM neurons were also identified at this stage (double arrows, Fig. 5B, inset). Interestingly, at the dorsal part of the Isl1/2-positive domain, many *Nolz-1*(+)/Isl1/2(+) cells were found, suggesting that *Nolz-1* is expressed in the dorsally born and early differentiated SM neurons (Fig. 5b2).

Double labeling analysis on E11.5 coronal sections at r4–r5 levels was further performed. The results showed that *Nolz-1* mRNA was not expressed in the domains containing *Phox2b*(+)/Isl1/2(+) BM/VM neurons, but instead was expressed in their surrounding regions (Fig. 5C–F). In contrast, many *Nolz-1*(+)/Isl1/2(+) cells arranged in a longitudinal column manner were found in the medial part of caudal hindbrain (Fig. 5G and H). In fact, most, if not all, Isl1/2-positive cells co-expressed *Nolz-1*, but not *Phox2b* in this region (Fig. 5H and I).

In summary, our study suggests that *Nolz-1* is not expressed by BM/VM neurons, but is specifically expressed by SM neurons in the presumptive hypoglossal nucleus which was presumably derived from the aforementioned medial-most longitudinal column.

2. Conclusions

In the present study, we reported the spatiotemporal expression of *Nolz-1* in the developing mouse hindbrain. *Nolz-1* was initially expressed at the MHB and then in the prospective r3, r5 and caudal regions. Later at E11.5, *Nolz-1*-positive cells were organized as distinct cell clusters. Importantly, we found that *Nolz-1* was specifically expressed in somatic motor neurons, but not in *Phox2b*-positive branchial and visceral motor neurons, suggesting that *Nolz-1* may be involved in determination and/or differentiation of somatic motor neurons in the developing hindbrain.

3. Experimental procedures

3.1. Whole-mount and sections in situ hybridization

Embryos of time-pregnant ICR mice (Nation Yang-Ming University and National Laboratory Animal Center, Taipei, Taiwan) were collected for *in situ* hybridization analysis. The protocol of animal use was approved by the Institutional Animal Care and Use Committee in National Yang-Ming University, and was conformed to the NIH Guide for the Care and Use of Laboratory Animals. The whole-mount and sections *in situ* hybridization were performed according to Ding et al. (1998) and Chang et al. (2004), respectively. Digoxygenin (Dig)- or FITC-labeled antisense and sense probes were synthesized by *in vitro* transcription (Roche Diagnostics), The probes used were *Nolz-1* (NM145459: 2640–3744 bp), *Pax6* (NM013627: 1703–2019 bp), *Phox2b* (BC079610: 1249–2857 bp), *Ngn2* (NM009718: 1696–2055 bp), *Pax2* (kindly provided by Dr. P. Gruss of the University of Göttingen; Nornes et al., 1990) and *Shh* (BC063087: 442–1084 bp). The hybridized probes were detected by incubation with alkaline phosphatase (AP)-conjugated sheep anti-digoxigenin (Dig) antibody and colorimetric reacted using nitroblue tetrazolium chloride (NBT)/5-bromo-4-chloro-3-indolyl-phosphate (BCIP) as substrates (Roche Diagnostics).

3.2. Double in situ hybridization

Double *in situ* hybridization was performed according to Okada et al. (2004). The brain sections were hybridized with Dig-labeled *Nolz-1* probes and FITC-labeled *Pax2* probes. Double *in situ* hybridization for *Nolz-1* and *Pax2* were visualized by colorimetric detection system (Roche Diagnostics). The brain sections were simultaneously hybridized with Dig-labeled *Nolz-1* probe and FITC-labeled *Pax2* probes. Alkaline phosphatase-conjugated antibodies against Dig or FITC (Roche Diagnostics) were sequentially applied, and NBT/BCIP and iodonitrotetrazolium (INT)/BCIP were used as substrates.

3.3. Double in situ hybridization and immunohistochemistry

After being processed for *Nolz-1* or *Phox2b in situ* hybridization, the brain sections were then processed for Isl1/2 or Pax2 immunohistochemistry as previously described (Chang et al., 2004). Following incubation with rabbit anti-Pax2 polyclonal antibody (1:500; Covance) or K5 rabbit anti-Isl1/2 polyclonal antibody (1:2000; kindly provided by Dr. S.L. Pfaff of the Salk Institute), the brain sections were sequentially incubated with biotinlyated goat anti-rabbit secondary antibody (1:500, Vector Laboratories), Elite avdin-biotin complex (Vector Laboratories) and DAB substrate with several intervening rinses of 0.1 M PBS. The biotin–tyramide amplification system was used to amplify the immunoreactive signals (Yau et al., 2003). For Hoxb1 immunohistochemistry, rabbit anti-Hoxb1 polyclonal antibody (1:500; Covance) was used.

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