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Sema4d is required for the development of the hindbrain boundary and skeletal muscle in zebrafish

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ABSTRACT

Semaphorin4d (SEMA4D), also known as CD100, an oligodendrocyte secreted R-Ras GTPase-activating protein (GAP), affecting axonal growth is involved in a range of processes including cell adhesion, motility, angiogenesis, immune responses and tumour progression. However, its actual physiological mechanisms and its role in development remain unclear. This study has focused on the role of *sema4d* in the development and expression patterns in zebrafish embryos and the effect of its suppression on development using *sema4d*-specific antisense morpholino-oligonucleotides. In this study the knockdown of *sema4d*, expressed at all developmental stages, lead to defects in the hindbrain and trunk structure of zebrafish embryos. In addition, these phenotypes appeared to be associated with the abnormal expression of three hindbrain rhombomere boundary markers, *wnt1*, *epha4a* and *foxb1.2*, and two myogenic regulatory factors, *myod* and *myog*. Further, a notable increase of cell apoptosis appeared in the *sema4d* knockdown embryos, while no obvious reduction in cell proliferation was observed. Collectively, these data suggest that *sema4d* plays an important role in the development of the hindbrain and skeletal muscle.

1. Introduction

Semaphorins were initially identified 19 years ago as molecular cues for axon guidance growth both in invertebrates as well as humans [1,2]. More than 20 semaphorin genes have been identified in mammals and their protein products are involved in a range of processes, including cell migration, immune responses, angiogenesis and carcinogenesis [3].

Semaphorin4D (SEMA4D), also known as CD100, was originally discovered in the immune system regulation of B cell aggregation and survival and T cell activation [4,5]. *Sema4d* is widely expressed in the mouse nervous system [6,7], and studies have demonstrated that it plays a pivotal role in nerve development. Yet as an axonal guidance cue in central nervous tissue, the functional role of Sema4d remains controversial. For example, the expression of Sema4d has been shown to induce growth cone collapse in primary hippocampal neurons [8], but also to stimulate the axonal outgrowth of embryonic dorsal root ganglion neurons [9]. Cumulative data have suggested that SEMA4D plays a more extensive role in tumour progression. SEMA4D is indeed highly expressed in a wide range of human tumours, such as prostate, colon, breast, oral, head and neck carcinomas and soft tissue sarcomas [10,11], and has been shown to induce cell migration and promote tubulogenesis in endothelial cells, mimicking pivotal events in angiogenesis [12]. Although SEMA4D plays an extensive role in biological events, CD100-deficient mice have exhibited functional defects only in their immune systems, with no apparent abnormalities in other tissues [5]. Currently, the physiological role of SEMA4D in development remains inadequately understood.

This study has investigated the expression of *sema4d* in zebrafish during their embryonic development and has shown that *sema4d* plays an important role in the development of the hindbrain and skeletal muscles. These data might be useful for understanding diseases associated with hindbrain abnormalities or skeletal muscle defects.

2. Materials and methods

2.1. Choice of zebrafish

The phylogenetic tree revealed that *sema4d* protein is highly conserved among such vertebrates, as Mus musculus, humans, Bos taurus, Rattus norvegicus, and the low-grade vertebrate zebrafish (Fig. 1A). Therefore, experiments were performed using zebrafish as the model to investigate the physiological role of *sema4d* in development.

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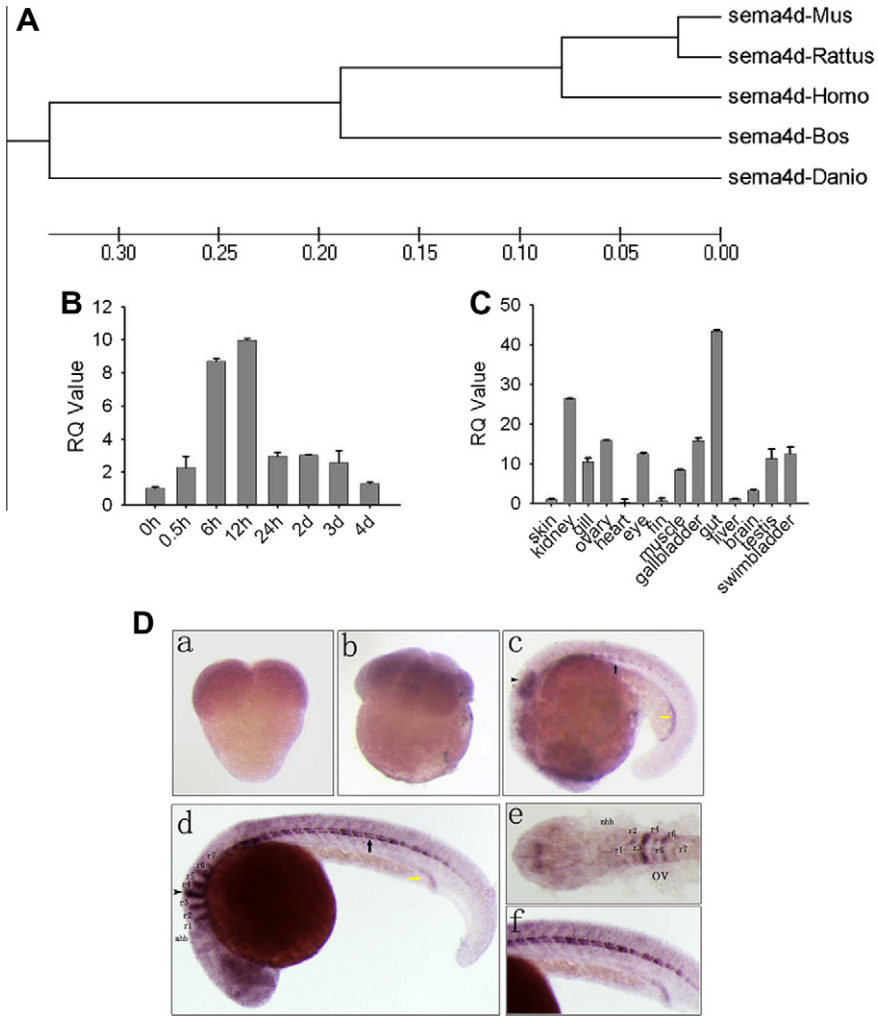

Fig. 1. Alignment of the zebrafish sema4d proteins with other vertebrate counterparts and the expression of zebrafish sema4d. (A) Phylogenetic tree showing the alignment of sema4d using sequences from Mus, Mus musculus; Homo, human; Bos, Bos taurus; Rattus, Rattus norvegicus; and Danio, zebrafish. (B) RT-PCR analysis showing the expression of sema4d during embryogenesis. (C) RT-PCR analysis showing the expression of sema4d in adult tissues. (D) Expression of sema4d in zebrafish. Whole mount RNA *in situ* hybridisation was performed using a sema4d-specific antisense RNA probe on embryos at the 0.75 hpf (a), 1.5 hpf (b), 17 hpf (c), 24 hpf (d). The sema4d expression is restricted to the hindbrain (black arrowhead), spinal cord (black arrow), ICM (intermediate cell mass) (white arrow) and skeletal muscle (c–f). The blue regions indicate positive sema4d expression. mhb, mid-hindbrain; r, rhombomere; ov, otic vesicle. (For interpretation of colour in this figure, the reader is referred to the web version of this article.)

2.2. Maintenance of zebrafish

Zebrafish were kept under a 14-h day/10-h night cycle at 28.5 °C. Fertilised eggs were obtained through mating adult fish from outbred colonies immediately after exposure to light. Embryos were staged according to the hours post-fertilisation (hpf) and morphological criteria [13].

2.3. RNA isolation and real-time PCR

Total RNA was extracted from embryos or adult tissues using TRIzol solution (Life Technologies, Carlsbad, CA). The cDNA was generated using 5 μg of total RNA with TOYOBO reverse transcriptase and oligo-dT (Invitrogen, CA, USA) primers. The PCR reaction was performed using the cDNA as a template. The expression of *sema4d* and *ef1a* was analysed using SYBR green real-time PCR primers (*sema4d*: 5′-TCTGTTCCTGCTGTTCTTCCTGCT-3′, 5′-AGCGATCTGATTGCTGGATCAGT-3′, 187 bp; *ef1a*: 5′-TCAAGCCTGGTATGGTTGTGACCT-3′, 5′-ACGGATGTCCTTGACAGACACGTT-3′, 149 bp). The real-time PCR analysis was performed (CFX-96 Real-time PCR System; Bio-Rad, CA, USA).

2.4. Whole mount in situ hybridisation

For *in situ* hybridisation, the following genes were used as cRNA probes: *sema4d*, *wnt1*, *epha4a*, *efnb3b*, *hoxb1a*, *mafba*, *foxb1.2*, *mck*, *mylz2*, *smyhc1*, *myod*, *myf5* and *myog*. The cDNAs were amplified using RT-PCR and cloned into pGEM-T plasmids (TIANGEN, Beijing, CHN). Digoxigenin (DIG)-labelled RNA probes were transcribed using RNA DIG labelling mix (Roche, BS, SW) and T7 RNA polymerase or Sp6 RNA polymerase (Ambio, TX, USA). Whole mount *in situ* hybridisation was performed essentially as described elsewhere.

2.5. Microinjection of morpholino-oligonucleotides (MOs)

Morpholino antisense nucleotides (MOs) (Gene Tools, Philomath, OR, USA) were used for gene knockdown. The sequence of the wild-type MO (*sema4d*-MO) was 5′-CATTAAAGAGGCTTTGGGAAGTTCA-3′ and that of the mismatched MO (*sema4d*-mis-MO), containing five nucleotide-mismatches (indicated with lowercase letters), was 5′-CATaAAAcAcGCTTTcGcAAGTTCA-3′. Approximately 500 pL of MOs (500 μM) were injected into one-cell stage embryos [14]. If more than 60% of the MO-injected embryos, excepting those that died,

showed similar characteristics (the number of MO-injected embryos was approximately 60–200 individuals), these phenotypes were considered as consequences of MO injection.

2.6. Western blotting

The embryos were dechorionated using pronase and deyolked in Ginzburg buffer containing protease inhibitor cocktail as described in the zebrafish book [15]. The proteins were extracted from the embryos in lysis buffer containing 1 mM Hepes, pH 7.4, 1 mM ethylenediaminetetraacetic acid, 15 mM $NaCl_2$, 0.5% Triton X-100, Protease Inhibitor Cocktail III (Calbiochem, MA, GE) and 20 mM β-glycerophosphate. The samples were boiled with SDS sample buffer prior to sodium dodecylsulphate–polyacrylamide gel electrophoresis and western blotting. The anti-SEMA4D monoclonal antibody (BD, NJ, USA) was used as a primary antibody, and alkaline phosphatase-conjugated anti-mouse antibody IgG (Jackson ImmunoResearch, PA, USA) was used as a secondary antibody, using the BCIP/NBT system (Sigma, MO, USA) to detect them.

2.7. Immunostaining and TUNEL assay

The anti-phosphohistone H3 (pH3) polyclonal antibody (Eptomics, CA, USA) was used as a primary antibody, and horseradish peroxidase-conjugated goat anti-rabbit IgG (Eptomics, CA, USA) was used as a secondary antibody, using the DAB substrate (Sigma, MO, USA) to detect them. The reagents for the TUNEL assay were purchased from Invitrogen (Invitrogen, CA, USA) and used according to the manufacturer's instructions.

2.8. Statistical analysis

Data from at least three independent experiments were examined by one-way ANOVA followed by the Student-Newman–Keuls test. All Ps were two sided. SPSS v11.5 software was used for all statistical.

3. Results

3.1. Expression of sema4d in zebrafish development

Using real-time PCR and RNA extracted from Zebrafish at different developmental stages revealed that *sema4d* was expressed at all developmental stages from as early as 0 to 96 hpf. As shown in Fig. 1B, the *sema4d* mRNA level increased dramatically after 0.5 hpf, peaked at 12 hpf, decreased sharply and was subsequently maintained at a comparatively low level from 24 to 96 hpf. In adult zebrafish, *sema4d* abundantly expressed in the kidneys, gills, ovaries, eye, trunk muscles, gallbladders, guts, testis, swimbladders and brains, but not in the skin, heart, fin, or livers (Fig. 1C). To visualise *sema4d* expression during embryogenesis in zebrafish, we performed *in situ* hybridisation at various times during development. As shown in Fig. 1D, *sema4d* expression was distributed ubiquitously at 0.75 and 1.5 hpf (Fig. 1D-a and b). At 17 hpf, the *sema4d* expression was aggregated in the head, spinal cord, intermediate cell mass (ICM) and trunk muscle (Fig. 1D-c), and this obvious expression remained until 24 hpf (Fig. 1D-d–f). In the brain, the presence of *sema4d* transcripts was detected in the forebrain, midbrain-hindbrain boundary and hindbrain. Data obtained from previous studies [13] suggested that *sema4d* was particularly expressed along hindbrain rhombomere boundaries (Fig.1D-e), as the morphological segmentation of the zebrafish hindbrain was transiently visible at the 18 somite stage when five prominent bulges along the anterior–posterior extent of the hindbrain, rhombomeres r2–r6, were detectable in the vicinity of the developing otic vesicle, which lay lateral to r5 [16].

3.2. Knockdown of sema4d in developing embryos

When morpholino-oligonucleotides (MOs) designed to suppress *sema4d* (*sema4d*-MO) or mutant MOs (*sema4d*-mis-MO) were injected into fertilised zebrafish eggs to examine the role of *sema4d* in the development of zebrafish embyros and to examine the effect of *sema4d*-MO knockdown using Western blotting significant reductions in *sema4d* expression with *sema4d*-MO at 24 and 72 hpf (Fig. 2A) were observed. Interestingly for our purposes, embryos injected with *sema4d*-MO exhibited a malformed brain structure and curved trunk (Fig. 2B), which was not observed in embryos injected with *sema4d*-mis-MO. At mid-somitogenesis (17 hpf), defects in the brain development of *sema4d* morphants embryos, including a severely distorted mid-hindbrain (Fig. 2B-f) were observed. However, these defects were not obvious in the trunk. At 24 hpf, brain structure became further disorganised, especially in the hindbrain. For example, abnormal-sized ventricles and a poorly defined separation along the dorsal midline, and a notably curved (Fig. 2B-l) trunk appeared. The close focus of this study was on the hindbrain segmentation and trunk muscle development.

3.3. Expression of hindbrain markers in sema4d morphants

Visualizing the entire hindbrain at the mid-somitogenesis stage, which spans the region from the mid-hindbrain boundary to

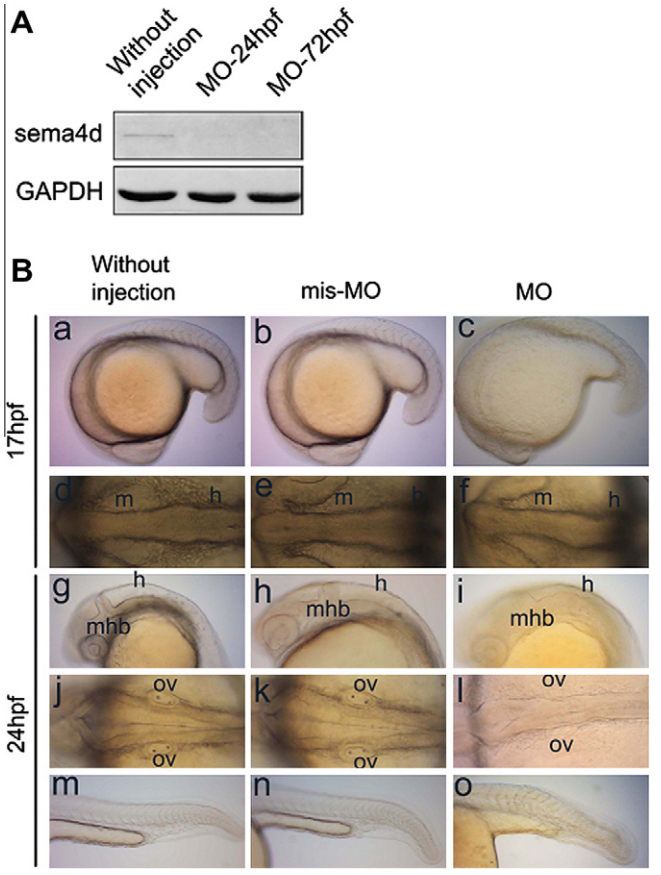

Fig. 2. Effect of sema4d knockdown in zebrafish embryos through sema4d-MO or sema4d mis-MO. (A) Western blot analysis of *sema4d* expression in without injection and sema4d-MO embryos. (B) Live images of lateral (a–c, g–i and m–o) and dorsal views (d–f and h–j) of the developing hindbrain and trunk at 17 hpf (a–c) and 24 hpf (g–o). The early mid-hindbrain, hindbrain regions and trunk are malformed at 17 hpf in sema4d-MO embryos (c and f), and an incomplete ventricle and curved trunk are visible at 24 hpf (i, l and o). H, hindbrain; m, midbrain; mhb, mid-hindbrain boundary; ov, otic vesicle.

rhombomere 7 (r7), using the markers *wnt1* and *hoxd4a* and *in situ* hybridisation with various segmental markers, including *wnt1*, *hoxd4a*, *mafba*, *epha4a*, *efnb3b*, *hoxb1a* and *foxb1.2*, during different stages of development, such as mis-MO and MO embryos and without injections, in order to examine hindbrain segmentation in more detail, showed *wnt1* expression at the dorsal midline was significantly increased, but was maintained at a normal level at the mid-hindbrain boundary (Fig. 3A-a), and *hoxd4a* was normally expressed in rhombomere 7 of *sema4d*-MO embryos (Fig.3A-b). At 24 hpf, multiple markers were found available to assess the development of individual rhombomeres within the hindbrain, including *epha4a* and *wnt1* for r1, r3, and r5 [17,18], *efnb3b* for r2, r4, and r6 [19], *hoxb1a* for r4 [20], and *mafba*/*valentino* for r5 and r6 [21]. Experiments using these markers showed that the expression of *epha4a* in odd-numbered rhombomeres was strongly increased in *sema4d*-MO embryos, but the expression of *mafba* in r5 was unchanged (Fig.3A-f). In contrast, the normal expression of *efnb3b* in r2, r4 and r6, *hoxb1a* in r4 and *mafba* in r6 was observed in the even-numbered rhombomeres of *sema4d*-MO embryos (Fig. 3A-d–f).

Morphologically visible boundaries formed adjacent rhombomeres during hindbrain development, suggesting, in turn, that boundary formation might depend on the distinct properties of successive odd and even-numbered segments. The first rhombomere boundaries appeared at early somite stages and were well developed at 17–18 hpf, when the expression of these genes could be detected using *foxb1.2* as a marker [21,22]. *Foxb1.2* expression was substantially increased in the hindbrain of *sema4d*-MO embryos (Fig. 3A-g). Collectively, the data suggested complex patterns of gene expression in the hindbrain segments, only some of which depended t on the function of *sema4d*.

3.4. Expression of myogenic markers in sema4d morphants

Using six markers to clarify the mechanism(s) underlying curved trunks, three to clarify and differentiate terminal makers for skeletal muscles, including the skeletal muscle myosin light polypeptide 2 (*mylz2*), slow myosin heavy chain 1 (*smyhc1*), and muscle creatine kinase (*mck*), and three myogenic regulatory factors, including myogenic differentiation (*myod*), myogenic factor 5 (*myf5*) and myogenin (*myog*). *Mylz2*, *smyhc1*, and *mck* were differentiate markers for first muscle, slow muscle, and both slow and first muscles, respectively [23]. *Myod* and *myf5* were found to be expressed in the two lines of adaxial cells lying adjacent to the notochord of somites, while *myog* was found also expressed in these two lines of cells and in the paraxial mesoderm at 12 hpf. *In situ* hybridisation clarified that the expression patterns of *mylz2*, *smyhc1* and *mck* in the morphants were indistinguishable from those in control embryos without injection or injected with *sema4d*-mis-MO at 24 hpf when skeletal muscle differentiation was complete (Fig. 3B-h–j). While the expressions of *myod*, *myog* and *myf5* were not different among the morphants and controls at 12 hpf (Fig. 3B-k–m), the expression of *myod* and *myog* in the morphants was stronger than that in control embryos at 23 hpf (Fig. 3B-n–p). This difference was even more significant at 48 hpf (Fig. 3B-q and r). These sustained *myod* and *myog* expressions were observed in all morphants depicting curved trunks. These data suggest that the abnormal trunk morphogenesis in *sema4d* morphants do not reflect the perturbation of muscle differentiation, but most likely reflect the upregulated expression of myogenic regulatory factors, such as *myod* and *myog*.

3.5. Increased apoptosis in the hindbrain and skeletal muscle of sema4d morphants

Although the hindbrain and trunk undergo at least some level of segmentation in *sema4d* morphant embryos, the hindbrain boundary formation and trunk shape are severely defective as judged by the expression of specific marker genes. We asked whether changes in cell death or cell proliferation in morphant embryos correlate with these developmental defects. Immunostaining with anti-phosphorylated histone 3 (pH3) antibody, which labels

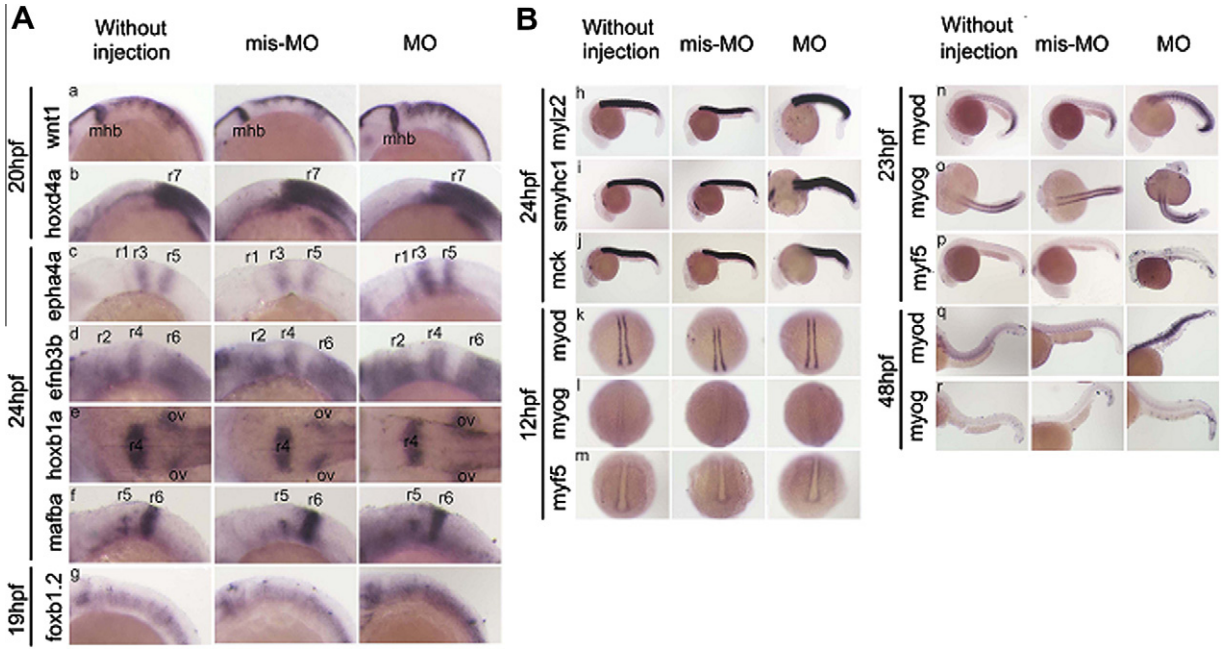

Fig. 3. *In situ* hybridisation analysis of hindbrain boundaries markers and terminal differentiation markers of skeletal muscle and myogenic regulatory factors. (A) Lateral (a–d, f and g) and dorsal views (e) of control and morphant embryos, as indicated, stained through whole mount *in situ* hybridisation. Expression of *wnt1* and *hoxd4a* at 20 hpf (a and b). Expression of *epha4a* (c), *efnb3b* (d), *hoxb1a* (e), and *mafba* at 24 hpf (f), and *foxb1.2* at 19 hpf (g). (B) Expression of *mylz2*, *smyhc1* and *mck* in sema4d morphants, control embryos injected with sema4d-mis-MO and without injection embryos at 24 hpf (h–g). Expression of *myod*, *myog*, and *myf5* in sema4d morphants, control embryos injected with *sema4d*-mis-MO and without injection embryos at 12 hpf (k–m), 23 hpf (n–p) and 48 hpf (q and r). The embryos are shown in lateral view, anterior toward the left (h–g and n–r). The embryos are shown in dorsal view, anterior toward the top (k–m). Mhb, mid-hindbrain boundary; ov, otic vesicle; r, rhombomere.

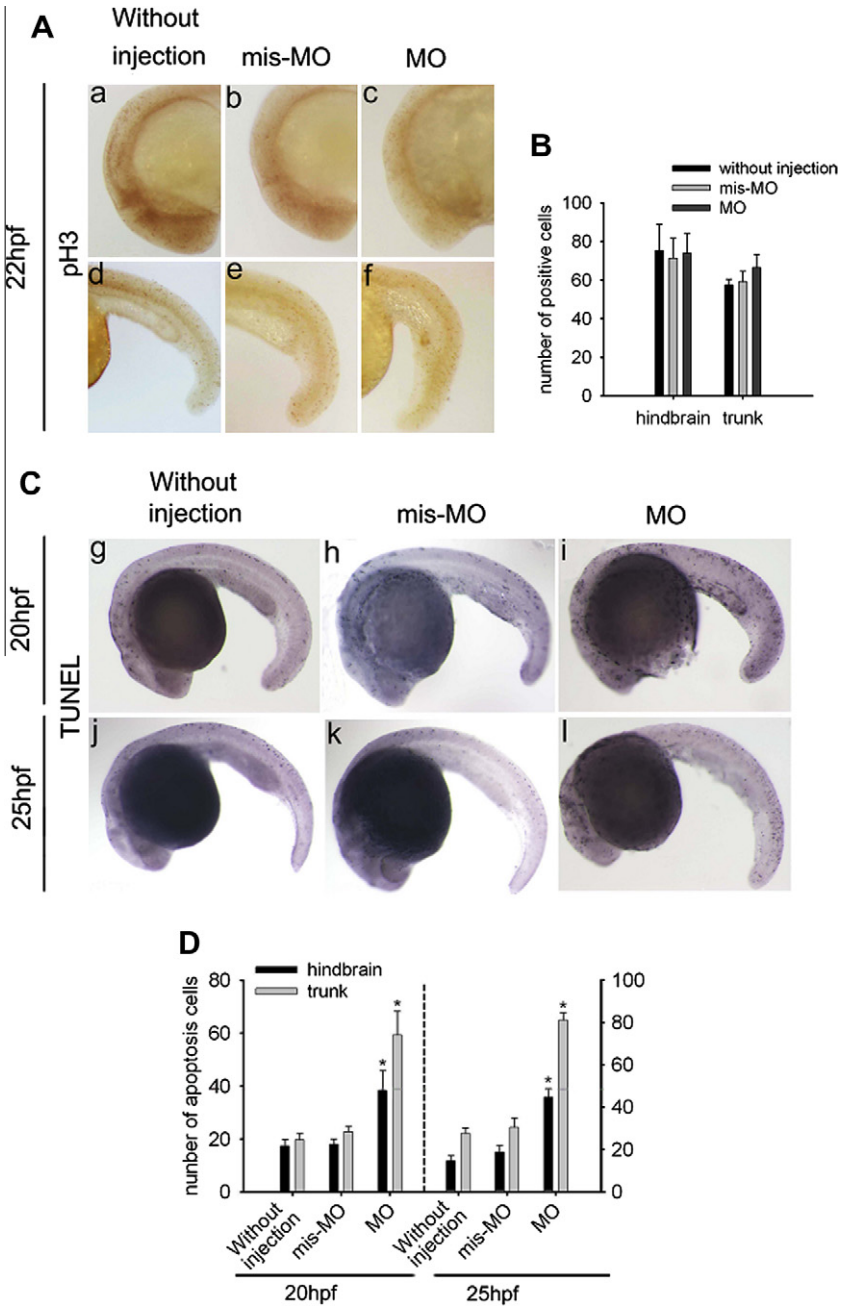

Fig. 4. Increased cell apoptosis in the hindbrain of sema4d morphants. (A) Staining of pH3 positive cells in control and morphant embryos. (B) Quantification of pH3 positive cells. (C) Analysis of cell death using a TUNEL assay in control and morphant embryos. (D) Quantification of apoptotic cells. Five embryos were counted for each condition; the error bars indicate the standard deviation based on five samples. pH3 staining, $p = 0.6$; TUNEL assay, at 20 hpf, $p = 0.00$; at 25 hpf, $p = 0.00$. $^{\star}p < 0.01$.

mitotic cells, was performed at 22 hpf, to assess differences in cell proliferation of hindbrain and trunk between morphants and control embryos. No obvious differences were observed among these embryos, indicating that hindbrain segmental disorganisation and curved trunk in *sema4d* morphants were not the consequences of cell proliferation (Fig. 4A and B). Characterizing apoptotic cells at 20 and 25 hpf using a TUNEL assay in morphants and control embryos, the number of positive cells were shown to be greatly increased at 20 and 25 hpf (Fig. 4C), and the differences between the morphants and control embryos were highly significant at both stages ($p = 0.00$) (Fig. 4D). These, in turn, led to the conclusion that apoptosis of cells in the hindbrain and trunk were severely affected in the embryos of *sema4d* morphants.

4. Discussion

Semaphorins are a large class of proteins that function throughout the nervous system to regulate axonal growth. In zebrafish, *sema5a* is a bifunctional axon guidance cue for vertebrate motor axons *in vivo* [24]. Sema3 signalling from boundaries link hindbrain segmentation to the positioning of *fgf20a*-expressing neurons that regulate neurogenesis [25]. Several studies have shown that semaphorin protein families also function outside of the nervous system. Previous studies have shown that soluble class III semaphorins were involved in the development of cardiac and skeletal muscles [26,27]. In mice, Sema4c participated in myogenic differentiation *in vivo* and *in vitro* through the p38 MAPK pathway

[28]. This study has shown that *sema4d* played an important role in the development of skeletal muscle and hindbrain boundary organisation.

Sema4d knockdown in zebrafish clearly resulted in hindbrain malformation. During hindbrain development, the segmentation of the vertebrate hindbrain into rhombomeres is a key step in the development of the complex pattern of differentiated neurons from a homogeneous neuroepithelium. Many of the transcription factors important for establishing the segmental plan, assigning rhombomere identity and maintaining boundary have already been identified, such as the Eph family of receptor tyrosine kinases and their ligands, the ephrins, Hox group 1–4 proteins, Krox20 and Valentino/Mafb. *In situ* hybridisation demonstrated that *sema4d* morphants did not show abnormal expression of gene markers in even-numbered rhombomeres, but showed the upregulated expression of *wnt1* and *epha4a* in odd-numbered rhombomeres. These data suggest that *sema4d* might be involved in a complex gene regulatory network comprising Eph-ephrin, Wnt and potentially Notch signalling in hindbrain development. Rhombomere organisation is the foundation of the survival neurons in the hindbrain. Foxb1.2 is expressed at the onset of gastrulation and has been used as a molecular marker for the neurectoderm in mutations affecting the dorsoventral axis in zebrafish. Here *sema4d* morphants showed the upregulated expression of *foxb1.2*, suggesting that *sema4d* might affect the growth of commissural axons juxtaposed to segmental boundaries.

In addition to the abnormal hindbrain, we have shown that *sema4d* morphants developed a curved trunk, which was associated with the sustained expressions of *myod* and *myog* at a late development stage (48 hpf). Trunk skeletal muscles were derived from a primary myotomal component of somites, and established as paraxial mesoderms adjacent to the neural tube and notochord. A complex network comprising basic helix-loop-helix (bHLH) transcription factors, known as muscle regulatory factors (MRFs), regulates the activation of myogenesis. Among the MRFs, the myogenic master transcription factor *myod*, has been detected in adaxial cells adjacent to the notochord as early as 7–7.5 hpf during zebrafish embryogenesis. The *myod*-expressing cells expand in an anterior-to-posterior wave at 14.5 hpf, followed by a marked reduction in *myod* expression at 24 hpf [29]. The sustained expression of *myod* and *myog* in *sema4d* morphants might result from the downregulation of upstream regulators, such as Pax3 [30], or an undetermined mechanism of *myod* and *myog* regulation. Because *myog* is considered a direct downstream target of *myf5* and *myod*, the sustained expression of *myog* is most likely due to the elevated expressions of *myod*. Notably, Sema4C promotes terminal myogenic differentiation in a p38 MAPK-dependent manner, which activates the myogenin promoter during myogenic differentiation [28]. From these data, it is tempting to speculate that *sema4d* might be involved in regulating p38 MAPK signalling to activate *myod* and *myog* expressions. Although additional studies are needed to clarify the mechanism(s) by which *sema4d* functions in muscle development, our findings here provide insight into the potential roles of *sema4d* in myogenesis.

Although the *sema4d* morphants exhibited malformed hindbrains and curved trunks, the formation of hindbrain boundaries and skeletal myogenesis are normally accomplished at early stages. Therefore, *sema4d* might not be involved in cell specification or differentiation, but rather function in the maturation or proliferation of those cells. During embryonic development, neural stem cells generate rapidly amplifying neural progenitors, which are responsible for the proper formation of the nervous system. In addition, resident progenitor cell populations, maintaining the balance between self-renewal and differentiation, are essential for muscle growth as development proceeds. *Sema4D*, originally considered as a negative regulator of axon guidance, is primarily expressed by oligodendrocytes in the postnatal brain. Studies have shown that the *Sema4D* might act as an intrinsic inhibitory regulator of oligodendrocyte differentiation through the induction of apoptosis [31]. However, we observed that the loss of *sema4d* function resulted in an obvious increase in the number of apoptotic cells in the malformed hindbrain and curved trunk of zebrafish embryos. The loss of *sema4d* function might lead to the failure of neural and skeletal progenitor cell differentiation and induce neuronal and muscle apoptosis.

Taken together, we have shown that *sema4d* plays a crucial role in hindbrain boundaries and skeletal muscle development. These findings will be helpful for the elucidation of the molecular mechanisms underlying the hindbrain formation and skeletal muscle development.

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