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REVIEW

The midkine family of growth factors: diverse roles in nervous system formation and maintenance

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Midkines are heparin-binding growth factors involved in a wide range of biological processes. Originally identified as retinoic acid inducible genes, midkines are widely expressed during embryogenesis with particularly high levels in the developing nervous system. During postnatal stages, midkine expression generally ceases but is often up-regulated under disease conditions, most notably those affecting the nervous system. Midkines are known as neurotrophic factors, as they promote neurite outgrowth and neuron survival in cell culture. Surprisingly, however, mouse embryos deficient for midkine (knockout mice) are phenotypically normal, which suggests functional redundancy by related growth factors. During adult stages, on the other hand, midkine knockout mice develop striking deficits in neuroprotection and regeneration after drug-induced neurotoxicity and injury. The detailed mechanisms by which midkine controls neuron formation, differentiation and maintenance remain unclear. Recent studies in zebrafish and chick have provided important insight into the role of midkine and its putative receptor, anaplastic lymphoma kinase, in cell cycle control in the central and peripheral nervous systems. A recent structural analysis of zebrafish midkine furthermore revealed essential protein domains required for biological activity that serve as promising novel targets for future drug designs. This review will summarize latest findings in the field that help to better understand the diverse roles of midkine in nervous system formation and maintenance.

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Abbreviations
Alk, anaplastic lymphoma kinase; FSGD, fish specific genome duplication; HB-GAM, heparin-binding, growth associated molecule; Mdk, midkine; MFP, medial floor plate; PNS, peripheral nervous system; Ptn, pleiotrophin; RPTP, receptor phospho-tyrosine phosphatase; Shh, Sonic hedgehog

The neural functions of midkine

Midkine (Mdk) and the related heparin-binding growth associated molecule (HB-GAM)/Pleiotrophin (Ptn) are widely expressed, heparin-binding growth factors with a molecular weight of 13 and 18 kDa respectively. They consist of functionally distinct N-terminal and C-terminal domains separated by a highly conserved hinge domain. When overexpressed, they induce pleiotropic effects described in a variety of experimental systems. The growth factor Mdk was originally identified as a retinoic acid inducible gene in embryonic carcinoma cells and initially named MK1 after its discoverers Muramatsu and Kadomatsu (Kadomatsu *et al*., 1988). Later it was renamed to midkine (Mdk), as it was characterized as a cytokine being expressed during midgestation periods of mammalian embryos (Tsutsui *et al*., 1991). Mdk is widely expressed in mouse embryos, with elevated and developmentally regulated patterns in brain and spinal cord, but strongly down-regulated shortly after birth, persisting only in the kidney. It was first demonstrated for Ptn/HB-GAM and then later for Mdk that both growth factors promoted neurite outgrowth (Rauvala, 1989; Maruta *et al*., 1993; Muramatsu *et al*., 1993). In addition, Mdk was shown to have neurotrophic activity in cultured spinal cord and dorsal root ganglion neurons derived from fetal mice (Michikawa *et al*., 1993). Using grid assays, where

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recombinant Mdk protein was applied in array stripes onto culture dishes, rat brain derived neurons preferentially grew their neurites along the Mdk-coated trails (Kaneda *et al*., 1996). Importantly, this adhesion between neurite surface and Mdk-coated matrix was efficiently blocked when heparin was added to the culture medium. This strongly suggested that extracellular Mdk serves as a growth promoting cue driving matrix adhesion and outgrowth of neurites in a heparin-dependent manner (Kaneda *et al*., 1996). Furthermore, delivery of beads coated with recombinant Mdk to cultures of muscle cells induced clustering of acetylcholine receptors (Zhou *et al*., 1997). Such neurotransmitter receptor clustering in target cells is characteristic of the establishment of neuromuscular junctions and indicates formation of active synapses. Receptor clustering triggered by Mdk thus suggested a possible contribution of Mdk to synapse formation, which was further supported by the detection of endogenous Mdk protein at presynaptic terminals (Zhou *et al*., 1997). In addition, several studies reported that Mdk and Ptn acted as survival factors for neurons (Kikuchi *et al*., 1993; Satoh *et al*., 1993; Hida *et al*., 2003) and that this activity was mediated through inhibition of apoptosis by modulating the MAPK pathway (Owada *et al*., 1999). A more recent study in chicks reported that Mdk controlled proliferation of sympathetic neurons in the peripheral nervous system (PNS; Reiff *et al*., 2011). These authors showed that Mdk was expressed in neuronal, as well as non-neuronal cells of sympathetic ganglia during proliferative stages, and controlled the maturation of sympathetic neuron progenitors derived from the neural crest. Inhibition of Mdk or its putative receptor anaplastic lymphoma kinase (Alk) resulted in reduced proliferation and induced apoptosis, hence further supporting the role of Mdk as survival factor for neurons and a crucial factor for neurogenesis *in vivo* (Reiff *et al*., 2011). So far, the vast majority of studies characterized embryonic neural activities of Mdk in cell culture systems, with some exceptions (see Reiff *et al*., 2011). Therefore, the generation of mutant animal models was expected to provide important new insights by validating cell culture data *in vivo* and determining whether Mdk-deficient animals show any neurological defects.

Studying neural function in the Mdk knockout mouse

To assess the function of Mdks *in vivo*, knockout mice were generated. Despite the widespread embryonic expression of Mdk and Ptn, and potent activities observed in cell culture assays, Mdk-, as well as Ptn-deficient mice develop with no gross phenotypical abnormalities (Nakamura *et al*., 1998; Amet *et al*., 2001). Functional redundancy was therefore postulated to account for the absence of obvious phenotypes (Muramatsu, 2002). Consistent with this hypothesis, a 230-fold up-regulation of Ptn transcription in Mdk knockout mice was observed, notably in an organ-specific manner (Herradon *et al*., 2005). Moreover, the majority of double knockout mice deficient for both Mdk and Ptn die in the early embryonic stages (Muramatsu *et al*., 2006). Only few doubly deficient mice are viable and most of the viable females become infertile. Double knockout mice that are not viable unfortunately die before an assessment of neural abnormalities is possible. Thus, so far no early neural phenotypes have been described in these $Mdk^{-/-}$/$Ptn^{-/-}$ mice.

More thorough analysis of $Mdk^{-/-}$ and $Ptn^{-/-}$ single knockout mice, however, revealed several defects in the nervous system at the molecular and behavioural level during later stages of life. Although embryonic development proceeded phenotypically normal in $Mdk^{-/-}$ mice, expression changes were recorded at postnatal stages (Nakamura *et al*., 1998). Calretinin expression in the dentate gyrus granule cell layer of the hippocampus was up-regulated in postnatal brains, and $Mdk^{-/-}$ mice subsequently also exhibited deficits in memory and increased anxiety, assessed in maze tests (Nakamura *et al*., 1998). This was consistent with findings in $Ptn^{-/-}$ mice, where an increase in long-term potentiation due to lowered induction thresholds was reported in the hippocampus (Amet *et al*., 2001). Furthermore, levels of dopamine and its receptors were reduced in the striatum of $Mdk^{-/-}$ mice (Ohgake *et al*., 2009). Consistent with this, a more recent study identified typical preclinical features of Parkinson's disease in Mdk deficient mice (Prediger *et al*., 2011). These authors observed partial depletion of mesencephalic dopaminergic neurons, reduced dopamine levels in olfactory bulb and striatum together with impaired olfactory discrimination and short-term memory in $Mdk^{-/-}$ mice. This strongly suggested significant deficits in dopaminergic neurotransmission in the absence of Mdk and suggested Mdk deficient mice as a possible model to analyse preclinical stages of Parkinson's disease (Prediger *et al*., 2011). Another important study furthermore showed that morphologically normal, Mdk knockout mice show significant alterations of drug-induced neurotoxicity and addictive behaviour (Gramage *et al*., 2011).

Together, all these findings provide strong evidence suggesting that Mdk and Ptn play important roles in neurotransmission and the maintenance and plasticity of neuronal connections in the postnatal and adult nervous system. Yet, the embryonic neural functions of Mdk remain a mystery. The absence of obvious early neural phenotypes in $Mdk^{-/-}$ and $Ptn^{-/-}$ knockout embryos remains puzzling given the widespread expression of these growth factors during early embryonic stages, and the potent activities of these growth factors on cultured neurons in neurite outgrowth assays. Therefore, alternative animal models were analysed for Mdk loss of function phenotypes, including the zebrafish as a powerful vertebrate model organism.

Characterization of the neural function of Mdk in the zebrafish model

The zebrafish has become a popular vertebrate model for biomedical research and drug discovery (Peterson and Macrae, 2012). It offers several experimental features that make it unique for efficient functional gene analysis during vertebrate organogenesis. These features include the possibility of combining live imaging in intact specimens with powerful genetic approaches, such as large scale mutagenesis. Furthermore, zebrafish produce abundant embryos that are

almost completely transparent and develop rapidly outside the mother. Zebrafish Mdk was first isolated in a cross-species expression cloning screen for neural inducing factors using the *Xenopus laevis* animal cap assay (Winkler and Moon, 2001). In this screen, large cDNA pools from a zebrafish adult brain library containing 25 000 clones were transcribed into mRNA and injected into two-cell stage *Xenopus* embryos. The injected embryos were incubated until the blastula stage, then animal cap tissue was explanted and cultivated *in vitro*. Neural induction was assessed by determining neural marker gene expression in the absence of mesoderm formation. Once a positive cDNA pool was identified, it was subdivided into progressively smaller pools using sib selection. This procedure was repeated until less complex pools and eventually individual clones were identified that showed neural inducing activity. After six rounds of sib selection, a single clone was identified that robustly induced expression of pan neural markers (*Nrp*, *Ncam*) and the cement gland marker *Xag1* in the absence of mesoderm, suggesting that neural induction was direct and that this factor converted naïve ectoderm into neural-fated tissue (Winkler and Moon, 2001). Sequencing of this clone revealed a remarkable sequence homology to human Mdk (67% amino acid identity), and therefore the identified gene was named zebrafish *mdk2*. Shortly after this discovery, cDNA screening identified a second Mdk orthologue in the genome of zebrafish. Sequence alignment and synteny analysis revealed that this second copy was different from Ptn but represented a duplicated Mdk gene copy. This analysis suggested that both zebrafish Mdk genes originated by a fish specific genome duplication (FSGD) that occurred in evolution shortly after the separation of the teleost from the tetrapod lineage approximately 300 million years ago (Figure 1A; Winkler *et al*., 2003). Expression and functional analysis revealed that both zebrafish Mdk genes, now named *mdka* and *mdkb* according to the zebrafish gene nomenclature, underwent significant functional divergence during evolution (Figure 1B,C; Table 1). At this point, the zebrafish Mdk genes represented one of the first examples demonstrating the principle of subfunction partitioning after FSGD, a process that strongly contributed to the rapid evolution and radiation of teleost fishes (Winkler *et al*., 2003; Meyer and Van de Peer, 2005).

Using gene knockdown and overexpression approaches, it was shown that *mdka* and *mdkb* play strictly different roles in patterning the early nervous system in zebrafish. Both zebrafish Mdk genes are expressed in mostly non-overlapping patterns in the developing nervous system (Table 1, Figure 1B,C; Winkler *et al*., 2003). Mdka controls formation of the medial floor plate (MFP), an important organizing centre for the early nervous system, which secretes sonic hedgehog (Shh) and establishes polarity in the developing brain and spinal cord (Schafer *et al*., 2005). A deficiency in *mdka* resulted in the absence of the MFP, while the number of notochord cells was increased. This suggested that Mdka is

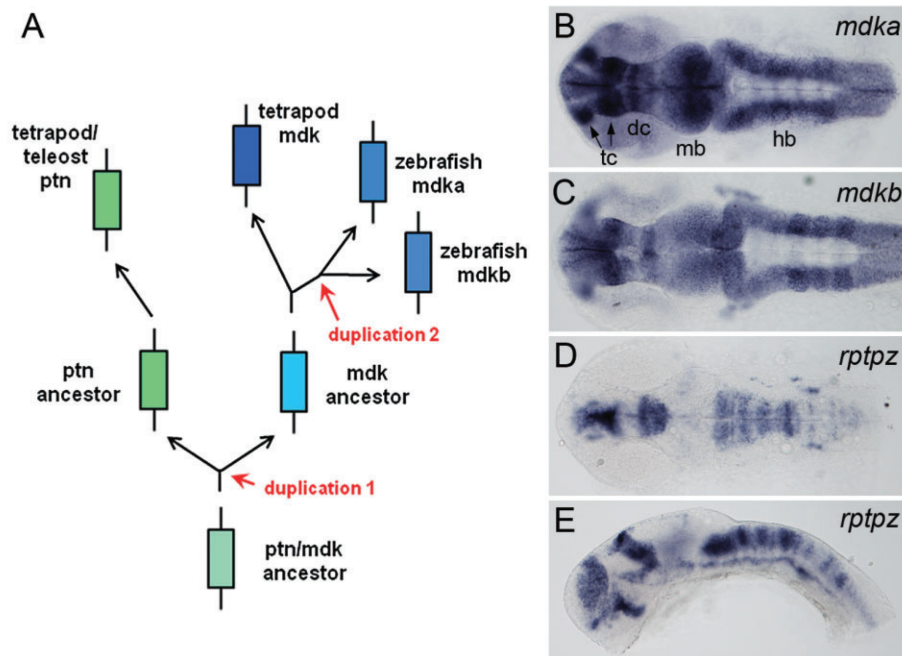

Figure 1

Evolution of the Mdk/Ptn family and divergence of zebrafish *mdka* and *mdkb* after gene duplication. (A) *mdk* and *ptn* arose from a gene duplication that occurred before the separation from tetrapods and teleost fish (duplication 1). The two zebrafish *mdk* co-orthologs arose from a teleost specific genome duplication (duplication 2). There is only one Ptn gene known in zebrafish, but it is possible that duplicated Ptn genes are present in other teleost fish; arrow lengths are not drawn to scale. (B, C) Expression of *mdka* and *mdkb* in the developing zebrafish brain at 24 hours post fertilization (hpf), as analysed by RNA whole-mount *in situ* hybridization. Note expression differences in telencephalon (tc), diencephalon (dc), midbrain (mb) and hindbrain (hb). (D, E) Expression of the putative Mdk receptor *rptpz* in the developing zebrafish brain at 24 hpf. rptpz is expressed in domains directly adjacent to or overlapping with those expressing *mdka* or *mdkb*. B, C, D are dorsal views with anterior to the left, E is a lateral view.

Table 1

Expression, knock-down and overexpression phenotypes of zebrafish and mouse Mdk orthologues during embryogenesis

	Zebrafish *mdka*	Zebrafish *mdkb*	Mouse *Mdk*
RNA expression during early embryogenesis (zebrafish: <24 hpf; mouse: <E9)	Paraxial mesoderm, brain, medial spinal cord; retinal stem and progenitor cells	Neural plate borders, brain, dorsal spinal cord; differentiating retinal neurons	Ubiquitous
Embryonic knock-down phenotype	Expanded notochord, reduced floor plate; reduced proliferation in retina, microphthalmia	Reduced numbers of neural crest cells and sensory neurons	No phenotype
Embryonic overexpression phenotype	Expanded floor plate, reduced notochord, absent somite boundaries; retinal overgrowth, cyclopia	Expanded neural crest and increased numbers of sensory neurons; microphthalmia	Not tested
References	Winkler *et al*., 2003; Schafer *et al*., 2005; Calinescu *et al*., 2009; Luo *et al*., 2012; Lim *et al*., 2013	Winkler *et al*., 2003; Liedtke and Winkler, 2008; Calinescu *et al*., 2009; Lim *et al*., 2013	Kadomatsu *et al*., 1988, 1990; Nakamoto *et al*., 1992

involved in fate decisions between both cell types during gastrulation. Based on these findings, a novel mechanism for floor plate formation in vertebrates was proposed, which implicated that trunk-derived Mdka forces prespecified, organizer-derived axial mesoderm cells into the ventral midline of the developing spinal cord (Schafer *et al*., 2005). *mdkb*, on the other hand, is expressed in a pattern that is mostly complementary to that of *mdka*. At neural plate stages, expression levels are high at the neural plate boundaries separating neural plate ectoderm from non-neural epidermal ectoderm (Winkler *et al*., 2003). At later stages, *mdkb* is strongly expressed in the dorsal spinal cord. Gene knock-down of *mdkb* resulted in the absence of neural crest cells and sensory neurons (Liedtke and Winkler, 2008). This shows that *mdka* and *mdkb* play crucial roles during formation and patterning of the embryonic nervous system in zebrafish.

A Ptn/HB-GAM orthologue has also been identified in the zebrafish genome (Chang *et al*., 2004). It is expressed in a pattern that is strikingly different from that of *mdka* and *mdkb*, but its detailed embryonic function remains unclear (D. Liedtke and C. Winkler, unpubl. data). Like its mammalian counterpart, however, zebrafish Ptn also induced neurite outgrowth in PC12 cells without NGF stimulation as well as enhancing neuritogenesis in zebrafish embryos (Chang *et al*., 2004).

Interestingly and in contrast to higher vertebrates, zebrafish *mdk* and *ptn* are expressed at high levels in the adult brain in spatially restricted domains (Winkler *et al*., 2003; Chang *et al*., 2004). It is tempting to speculate that maintenance of Mdk expression in adult neural tissue can be linked to the capacity of teleosts to efficiently regenerate experimentally induced lesions induced in the CNS (Kizil *et al*., 2012; Reimer *et al*., 2013).

Mdks in neural protection, regeneration and cell cycle control

Mdk and Ptn have been described as neurotrophic factors promoting the survival and function of neurons (Unoki *et al*., 1994; Masuda *et al*., 1995; Prediger *et al*., 2011). They are up-regulated in a variety of neurodegenerative disorders, such as Parkinson's disease (Marchionini *et al*., 2007), and have been assigned protective activities as response to drug-induced neurodegeneration (Gramage and Herradon, 2011). Experimentally induced overexpression of Mdk and Ptn also showed neuroprotective effects in various mouse models for Parkinson's disease and Alzheimer's disease (see Muramatsu, 2011). Conversely, a Mdk deficiency results in a delay of neural degeneration and regeneration of the PNS (Sakakima *et al*., 2009). Injury imposed on the peripheral sciatic nerve in wild-type and Mdk$^{-/-}$ mice revealed a significant delay in nerve regeneration and recovery of the associated soleus muscle when Mdk was absent (Sakakima *et al*., 2009). Most interestingly, a recent report has shown that exogenously applied Mdk even promoted neural regeneration in the injured mouse spinal cord *in vivo* (Muramoto *et al*., 2013). This is especially remarkable, as regenerative processes in the CNS are usually efficiently inhibited by activated glial cells. Noteworthy, endogenous Mdk expression was induced after spinal cord injury (Sakakima *et al*., 2004), suggesting the presence of an intrinsic mechanism in mammals to overcome glial-induced inhibitory processes and allow neuronal axon regeneration.

The positive effect of Mdk or Ptn on regeneration is also documented by several other studies that accumulated over the last decade, most notably for the nervous system. Ptn has been shown to be involved in regenerative processes of the brain (Iseki *et al*., 2002), while Mdk is associated with regeneration in the PNS (Sakakima *et al*., 2009) and the retina (Calinescu *et al*., 2009). The architecture of the retina and organization of its functional circuits are remarkably conserved during vertebrate evolution allowing the use of animal models to study disease-related processes known in humans (Fadool and Dowling, 2008). Intravitreal injection of Mdk protein into rats exposed to constant light flash stimulation for 1 week efficiently protected photoreceptor cells from damage, as recorded at the morphological (Unoki *et al*., 1994), as well as physiological level (Masuda *et al*., 1995), notably however without regeneration of new photoreceptors. In contrast to mammals, zebrafish are able to regenerate lost photo-

receptor neurons (Vihtelic and Hyde, 2000), but also other tissues, including neurons and axons in the CNS (Reimer *et al.*, 2013). This opens the possibility of analysing Mdk's function during regeneration *in vivo* using the zebrafish model. Regeneration of damaged tissues involves re-entry of quiescent progenitor cells into the cell cycle. Interestingly, both zebrafish Mdk genes were strongly up-regulated in retinal stem cells during regeneration of photoreceptor cells as they re-entered the cell cycle (Calinescu *et al.*, 2009). A more recent study has now shown that zebrafish Mdka controls the cell cycle during neurogenesis of photoreceptors (Luo *et al.*, 2012). Both zebrafish Mdks, *mdka* and *mdkb*, are dynamically expressed in the zebrafish retina, albeit with different patterns (Calinescu *et al.*, 2009). While *mdka* is expressed in retinal stem and progenitor cells (i.e. cells of the ciliary marginal zone and Muller glial cells), *mdkb* is expressed in newly post-mitotic cells shortly after they have exited the cell cycle to differentiate into various retinal neurons such as ganglion and amacrine cells (Calinescu *et al.*, 2009). Expression of *mdkb* is excluded from progenitor cells. Gene knockdown studies in zebrafish embryos showed that *mdka* is required for cell cycle exit during formation of retinal photoreceptors. A deficiency in *mdka* results in microphthalmia caused by delayed neuronal differentiation as fewer progenitor cells exit the cell cycle (Luo *et al.*, 2012). In contrast, ectopic overexpression of *mdka* accelerated the cell cycle and resulted in transient retinal overgrowth. Using various cell cycle markers, Luo *et al.* (2012) concluded that *mdka* levels regulate the progression from S- to M-phase of the cell cycle. Although corresponding data for *mdkb* are still lacking, this suggests that the two zebrafish Mdk co-orthologues have evolved distinct expression patterns reflecting possibly divergent activities in controlling cell cycle kinetics of neuronal progenitors. The tightly controlled regulation of two zebrafish Mdk genes in stem cells versus differentiated cells has important implications for the situation in other vertebrates. In mammals, only one Mdk gene exists. It thus remains open whether Mdk in mammals has similar functions in regulating cell cycle exit and differentiation of stem or progenitor cells. It will be interesting to find out whether the strictly distinct roles of the two zebrafish orthologues during cell cycle exit are reproduced by the single Mdk protein in mammals through interaction with different co-factors or receptors. As Mdk genes are highly expressed in the adult brain of zebrafish under normal conditions (Winkler *et al.*, 2003), as well as under disease conditions in mammals, it is furthermore tempting to speculate that Mdk might be involved in the differentiation of neural progenitor cells in the embryonic and adult CNS of zebrafish and mammals, similar to the situation found in embryonic photoreceptors.

The structural basis of Mdk function

Mdk and Ptn are proteins rich in basic aminoacids, with evolutionarily highly conserved cysteine residues. In zebrafish, the duplicated *mdka* and *mdkb* genes encode proteins that share 65% sequence identity (62.3% and 58.2% identity to human Mdk, respectively; mature protein without signal peptide), but show significant functional divergence during nervous system formation (Table 1; Schafer *et al.*, 2005; Liedtke and Winkler, 2008) and in the developing retina (Luo *et al.*, 2012). This divergence offers a unique opportunity to analyse *in vivo* the effects of single amino acid substitutions in the two related sequences on protein structure and ultimately, biological function. The NMR structures of individual N-terminal and C-terminal domains of human Mdk have been previously reported (Iwasaki *et al.*, 1997), and the C-terminal domain is a popular therapeutic target (see Muramatsu, 2011). Recently, the NMR structure of the full-length zebrafish Mdkb protein was obtained (Lim *et al.*, 2013). Site-directed mutagenesis revealed residues that are critical for structural features, heparin-binding and biological activity. The highly conserved hinge region separating the N- and C-terminal domains has previously been proposed to confer flexibility to the full-length protein. The study by Lim *et al.* (2013) surprisingly showed that the hinge domain itself binds to heparin and is essential for coordinating simultaneous binding of N- and C-terminal domains to heparin. This opens the possibility that the hinge region, so far basically ignored as a potential drug target, could be used for future drug design and therapeutic interventions. The present zebrafish study also demonstrated that certain aspects of Mdkb's biological activity in embryos were not strictly dependent on heparin binding. This observation could also lead to possibly novel drug design strategies that aim at biologically relevant residues directly, rather than at heparin-binding domains. Other than previously observed in cell culture studies (Maeda *et al.*, 1999), expression of the C-terminal domain alone does not exert any biological effect *in vivo* in zebrafish embryos. Instead, hinge and N-terminal domains together with the C-terminal domain are required to confer efficient heparin-binding and biological activity. The present structure-function analysis for Mdk by Lim *et al.* (2013) thus demonstrates the value of simple *in vivo* models to analyse the full range of biological effects of Mdk variants. The zebrafish model furthermore offers unique opportunities for *in vivo* drug screening of potential Mdk inhibitors. At present, studies are ongoing to determine the full-length structure of zebrafish Mdka. A comparison of Mdka and Mdkb structures and their corresponding biological activities is likely to identify individual motifs responsible for distinct structural configurations. These in turn are expected to be responsible for the divergent biological activates observed, for example through interaction with different receptor complexes and activation of specific signal transduction pathways.

The receptors mediating the neural functions of Mdk

Previous studies performed mainly in cell culture suggested that Mdks bind to several cell surface receptors. Mdk and Ptn both induce phosphorylation of the receptor tyrosine kinase Alk and its downstream kinase PI3K suggesting at least an indirect interaction between Mdk and Alk (Stoica *et al.*, 2001; 2002). Other putative transmembrane Mdk receptors include the receptor protein tyrosine phosphatase ζ (RPTPζ; Maeda *et al.*, 1999), integrins (Muramatsu *et al.*, 2004) and low-density lipoprotein-receptor related protein (Muramatsu *et al.*, 2000). Whether Mdk binds and activates Alk directly or

indirectly through RPTPζ, and whether Mdk and Ptn exhibit similar binding kinetics remains under discussion (Perez-Pinera *et al*., 2007).

In zebrafish, experimentally induced overexpression of Alk leads to activation of the MEK/ERK pathway, increased cell proliferation in the CNS and aberrant neurogenesis leading to mispositioning of differentiated neurons (Yao *et al*., 2013). Interestingly, a knockdown of Alk in early zebrafish embryos did not affect formation of neural progenitor and stem cells, but prevented their differentiation into mature CNS neurons suggesting that cell cycle exit of the neural progenitors is disturbed. This is consistent with findings in the PNS in chicks (Reiff *et al*., 2011) and suggests that Alk is a general regulator of neurogenesis in the CNS and PNS of vertebrates. This proposed role for Alk in cell cycle exit is also remarkably similar to that proposed for Mdk function in the zebrafish retina (Luo *et al*., 2012), providing possible support for an expected Mdk-Alk interaction. Importantly, however, the overall knockdown phenotypes for Alk and Mdk in zebrafish are strikingly different. Knockdown of *mdka* and *mdkb* leads to patterning defects in the zebrafish neural plate as early as during gastrulation (Schafer *et al*., 2005; Liedtke and Winkler, 2008), while inhibition of Alk only affects neurogenesis at much later stages when patterning is already completed (Yao *et al*., 2013).

While Alk has been proposed as a potential receptor for Mdk in mammals, a direct interaction of Mdk with Alk in zebrafish or *Drosophila* remains unclear. Notably, the physiological ligand for Alk in *Drosophila*, Jelly Belly (Jeb; Englund *et al*., 2003; Lee *et al*., 2003) shares no sequence or structural homology with the Mdk homologue in *Drosophila* (Miple) or vertebrate Mdk, and is not capable of activating mouse Alk (Yang *et al*., 2007). Also in zebrafish, a direct binding of Mdk to Alk appears highly unlikely. Similar to *mdk* and *ptn*, *alk* and *rptpζ* are also expressed strongly in the developing CNS of zebrafish, often with overlapping patterns (Figure 1D,E; Winkler *et al*., 2003; Yao Sheng, data not shown; Yao *et al*., 2013). Notably, however, there is significant sequence divergence found in the extracellular domain of Alk in vertebrates. While Mdk is highly conserved at the amino acid level between zebrafish and human (62.3% amino acid identity for Mdka), as is the kinase domain of Alk (89.6%), the identity in the extracellular ligand-binding domain of Alk is strikingly low (25.3%). This profound sequence divergence in the Alk ligand-binding domain argues against a direct Mdk-Alk interaction, which is consistent with the different effects observed after knockdown of both components in zebrafish embryos *in vivo*. This suggests that Mdk might bind to other receptors in the developing nervous system. While this could argue against a direct interaction of Mdk and Alk in vertebrates in general, it does not exclude the possibility that Mdk regulates Alk indirectly, for example through binding to RPTPζ. This idea has been proposed earlier based on cell culture experiments (Perez-Pinera *et al*., 2007). Interestingly, zebrafish *rptpζ* shows a spatially and temporally highly dynamic expression profile, which is in remarkable contrast to the more widespread expression of *alk* (Figure 1D,E; Yao *et al*., 2013). It is therefore tempting to speculate that RPTPζ mediates the regionally restricted effects observed after constitutive Mdk or Alk overexpression.

Clearly, more studies are needed to elucidate the developmental roles of Mdk-receptor complexes and gain insight into the composition of probably multicomponent receptor complexes. It appears likely that the Mdk receptor is an assembly of several proteins including Alk, Lrp6, RPTPζ, integrins and others, as proposed earlier (Sakaguchi *et al*., 2003). Interestingly, a recent study has shown that *Drososphila* Alk controls the activity of Gli transcriptional activators and repressors (Popichenko *et al*., 2013). Gli factors on the other hand are known as direct downstream targets of Shh signalling, which is crucial for establishing the early nervous system in vertebrates (Jacob and Briscoe, 2003). This opens the attractive possibility that Mdk-Alk signalling cross-talks with Shh signalling components during embryogenesis to establish distinct neural fates in the embryonic brain and spinal cord.

Conclusion and outlook

The Mdk growth factors are widely expressed during embryogenesis, but in adult tissues show elevated levels mostly under disease, injury and repair conditions. They have been assigned neurotrophic and neuroprotective roles, in particular in context of drug-induced neurotoxicity, nerve injury and neurodegeneration at later stages of life. Thus, while multiple roles in the postnatal and adult nervous system have been firmly established, their role during embryonic neurogenesis remains less clear. Functional redundancy with other growth factors has been made responsible for the fact that Mdk-deficient embryos develop normally with no gross morphological abnormalities. Such redundancy could also be relevant at adult stages and thus could mask additional aspects of Mdk function that have remained unknown so far. Until now, no attempts have been reported to generate conditional mouse mutants where Mdk and Ptn are simultaneously inactivated in individual tissues and/or at distinct stages. This could solve the problem of early lethality of double knockout mice and reveal so far unidentified cell type-specific Mdk functions.

A detailed analysis of the endogenous roles of Mdk during neurogenesis and neural differentiation using conditional knockout mice or alternative animal models such as zebrafish is expected to provide a better understanding of the many roles of Mdk during nervous system formation and function. Assigning individual functional aspects to particular structural features of this relatively small growth factor will help to establish Mdk and Ptn as better drug targets, for example to enhance their neuroprotective capacities. Of particular importance for drug design will be the description of structural changes that are induced after Mdk has bound to its different cellular receptors. A better understanding of the mechanisms involved in Mdk-receptor interaction at a structural and functional level will provide the basis for design of tailored drugs that interfere with Mdk activity in a variety of pathological conditions ranging from neurodegenerative diseases to inflammation and cancer.

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Conflict of interest

The authors declare to have no conflict of interest.

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