Molecular mechanisms of floor plate formation and neural patterning in zebrafish

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‘Happy is the person who is able
to discern the causes of things’
Virgil (37 B.C.)

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1. List of Publications

Publications

Winkler, C., Schäfer, M., Duschl, J., Schartl, M. and Volff, J. N. (2003). Functional Divergence of Two Zebrafish Midkine Growth Factors Following Fish-Specific Gene Duplication. *Genome Res.* **13**, 1067-81.

Schäfer, M., Kinzel, D., Neuner, C., Schartl, M., Volff, J. N. and Winkler, C. (2005). Hedgehog and retinoid signalling confines *nkx2.2b* expression to the lateral floor plate of the zebrafish trunk. *Mech Dev.* **122**, 43-56.

Schäfer, M., Rembold, M., Wittbrodt, J., Schartl, M. and Winkler, C. (2005). Medial floor plate formation in zebrafish consists of two phases and requires trunk-derived Midkine-a. *Genes Dev.* **19**, 897–902.

Bollig, F., Mehringer, R., Perner, B., Hartung, C., Schäfer, M., Schartl, M., Volff, J. N., Winkler, C. and Englert, C. Identification and comparative expression analysis of a second *wt1* gene in zebrafish. *Submitted to Dev. Dynamics.*

Schäfer, M., Kinzel, D. and Winkler, C. The lateral floor plate in zebrafish is composed of distinct cell populations that require different Hedgehog and Nkx2.2 activities. *In preparation.*

Lopes, S. S., Müller, J., Carney, T. J., McAdow, R. A., Rauch, J., Schäfer, M., Jacob, A. S., Hurst, L. D., Haffter, P., Winkler, C., Geisler, R., Johnson, S. L. and Kelsh, R. N. Endogenous role for Anaplastic Lymphoma Kinase signaling in neural crest development. *In preparation.*

Schild, K., Giegerich, M. Schäfer, M., Winkler, C. and Krohne, G. The zebrafish lamin B receptor. *In preparation.*

Published Abstracts

Winkler, C., Schäfer, M., Volff, J. N., Duschl, J. and Schartl, M. (2001). Two novel MIDKINE related growth factors in zebrafish with distinct functions during neural development. *Dev. Growth Diff.* **43**, 84.

Schäfer, M., Köppen-Schomerus, K., Volff, J. N., Schartl, M., Wizenmann, A. and Winkler, C. (2003). Comparative functional analysis of secreted Midkine growth factors during floorplate formation in zebrafish and chicken. *Eur J Cell Biol* **82**. Suppl. 53, 133.

2. Summary

The vertebrate spinal cord is composed of billions of neurons and glia cells, which are formed in a highly coordinated manner during early neurogenesis. Specification of these cells at distinct positions along the dorsoventral (DV) axis of the developing spinal cord is controlled by a ventrally located signaling center, the medial floor plate (MFP). Currently, the origin and time frame of specification of this important organizer are not clear.

During my PhD thesis, I have analyzed the function of the novel secreted growth factor Midkine-a (Mdka) in zebrafish. In higher vertebrates, *mdk* and the related factor *pleiotrophin* (*ptn*) are widely expressed during embryogenesis and are implicated in a variety of processes. The *in-vivo* function of both factors, however, is unclear, as knock-out mice show no embryonic phenotype. We have isolated two *mdk* co-orthologs, *mdka* and *mdkb,* and one single *ptn* gene in zebrafish. Molecular phylogenetic analyses have shown that these genes evolved after two large gene block duplications. In contrast to higher vertebrates, zebrafish *mdk* and *ptn* genes have undergone functional divergence, resulting in mostly non-redundant expression patterns and functions. I have shown by overexpression and knock-down analyses that Mdka is required for MFP formation during zebrafish neurulation. Unlike the previously known MFP inducing factors, *mdka* is not expressed within the embryonic shield or tailbud but is dynamically expressed in the paraxial mesoderm. I used epistatic and mutant analyses to show that Mdka acts independently from these factors. This indicates a novel mechanism of Mdka dependent MFP formation during zebrafish neurulation. To get insight into the signaling properties of zebrafish Mdka, the function of both Mdk proteins and the candidate receptor Anaplastic lymphoma kinase (Alk) have been compared. Knock-down of *mdka* and *mdkb* resulted in the same reduction of iridophores as in mutants deficient for Alk. This indicates that Alk could be a putative receptor of Mdks during zebrafish embryogenesis.

In most vertebrate species a lateral floor plate (LFP) domain adjacent to the MFP has been defined. In higher vertebrates it has been shown that the LFP is located within the p3 domain, which forms V3 interneurons. It is unclear, how different cell types in this domain are organized during early embryogenesis. I have analyzed a novel homeobox gene in zebrafish, *nkx2.2b*, which is exclusively expressed in the LFP. Overexpression, mutant and inhibitor analyses showed that *nkx2.2b* is activated by Sonic hedgehog (Shh), but repressed by retinoids and the motoneuron-inducing factor Islet-1 (Isl1). I could show that in zebrafish LFP and p3 neuronal cells are located at the same level along the DV axis, but alternate along the anteroposterior (AP) axis. Moreover, these two different cell populations require different levels of HH signaling and *nkx2.2* activities. This provides new insights into the structure of the vertebrate spinal cord and suggests a novel mechanism of neural patterning.

3. Zusammenfassung

Das Rückenmark von Vertebraten besteht aus Milliarden von Neuronen und Gliazellen, die in einem sehr komplexen Muster während der frühen Neurogenese gebildet werden. Die Spezifizierung dieser Zellen an spezifischen Positionen entlang der dorsoventralen (DV) Achse des Rückenmarks wird durch ein ventrales Organisationszentrum, die mediale Bodenplatte (MFP), kontrolliert. Die Herkunft und der Zeitraum der Spezifizierung dieses wichtigen Organisationszentrums sind zurzeit nicht klar.

In meiner Doktorarbeit habe ich die Funktionen des neuen Wachstumsfaktors Midkine-a (Mdka) im Zebrafisch charakterisiert. *Mdka* und der verwandte Faktor *pleiotrophin* (*ptn*) zeigen ein breites Expressionsmuster während der Embryogenese von höheren Vertebraten und sind offenbar an einer Vielzahl von Prozessen beteiligt. Die exakten *in-vivo* Funktionen sind jedoch nicht bekannt, da knock-out Mäuse keinen embryonalen Phänotyp zeigen. Im Zebrafisch haben wir zwei co-orthologe *mdk* Gene, *mdka* und *mdkb,* sowie ein *ptn* Gen-Ortholog isoliert. Molekulare phylogenetische Analysen ergaben, dass diese Gene durch zwei unabhängige Duplikationen eines Gen-Blocks entstanden sind. Im Gegensatz zu höheren Vertebraten haben *mdk* und *ptn* Gene divergente Funktionen entwickelt, was zu weitestgehend nicht redundanten Funktionen und Expressionsmustern geführt hat. Mittels Überexpressions- und knock-down Analysen konnte ich zeigen, dass Mdka für die Bildung der MFP im Zebrafisch benötigt wird. Anders als bisher bekannte MFP induzierende Faktoren ist Mdka nicht im embryonalen Gastrula-Organisator, dem ‚Shield' oder der Schwanzknospe exprimiert, sondern dynamisch im paraxialen Mesoderm. Durch epistatische Analysen und Mutanten-Experimente konnte ich weiterhin zeigen, dass Mdka unabhängig von diesen Faktoren wirkt. Dies deutet auf einen neuen Mdka abhängigen Mechanismus der MFP-Bildung während der Neurogenese im Zebrafisch hin. Um Einblick in den Signalweg von Mdka im Zebrafisch zu erhalten, wurde die Funktion der *midkine* Gene mit der des potentiellen Rezeptors, der Anaplastischen Lymphom-Kinase (Alk), verglichen. Ein ‚Knock-down' beider Mdk Proteine führte zu einer vergleichbaren Reduktion von Iridophoren wie bei Alk defizienten Mutanten. Demnach könnte Alk ein Rezeptor beider Mdk Proteine während der Zebrafisch-Embryogenese sein.

In vielen Vertebratenspezies wurde neben der MFP eine laterale Bodenplatten (LFP) Domäne definiert. In höheren Vertebraten wurde gezeigt, dass LFP Zellen innerhalb der p3 neuronalen Domäne lokalisiert sind, welche V3 Interneuronen bilden. Es ist zurzeit nicht klar, wie diese Zelltypen angeordnet sind und wie sie während der Embryogenese gebildet werden. Ich habe ein neues Homeobox Gen *nkx2.2b* im Zebrafisch analysiert, welches ausschließlich in der

LFP exprimiert ist. Überexpressions-, Mutanten- und Inhibitorenanalysen haben gezeigt, dass *nkx2.2b* durch Sonic Hedgehog (Shh) aktiviert, durch Retinolsäure und den Motoneuronen induzierenden Faktor Islet-1 (Isl1) aber reprimiert wird. Ich konnte weiterhin zeigen, dass im Zebrafisch LFP und p3 neuronale Zellen auf der gleichen Ebene entlang der DV Achse lokalisiert sind und entlang der anteroposterioren (AP) Achse alternieren. Diese zwei Zellpopulationen benötigen verschiedene Aktivitäten von Hedgehog und *nkx2.2b*. Dies stellt einen neuen Aspekt für den Aufbau des Rückenmarks von Vertebraten dar und deutet auf einen bisher unbekannten Mechanismus der neuronalen Musterbildung hin.

4. Introduction - Development of the nervous system in vertebrates

The nervous system is the most complex structure in every vertebrate organism. It coordinates muscle movements, monitors organ activities, processes sensory input and initiates actions. The nervous system consists of the central nervous system, which is composed of the brain, spinal cord and optic nerves and the peripheral nervous system that branches off from the central nervous system. Together, the nervous system is composed of billions of neurons and glia cells, which form a complex network of axonal connections. During early embryonic development these neurons and glia cells are specified in a tightly coordinated manner.

4.1. Induction of the neural ectoderm

The development of the nervous system in vertebrates starts during late blastula stage by induction of the neural ectoderm. Spemann and Mangold have first shown in 1924 in newt embryos that induction of the neural ectoderm occurs by signals of the adjacent mesoderm tissue, which they named Spemann Organizer. The organizer has been independently discovered in other vertebrates and named e.g. the Hensens node in chicken, the primitive node in mice or the shield in zebrafish (Fig. 1). In the last decades, work mostly done in the African clawed frog *Xenopus laevis* led to the identification of the organizer signals.

The ectoderm develops into two different fates during late blastula and gastrula stages. The dorsal part of the ectoderm is induced as neural tissue, while the ventral part develops into epidermis. The epidermal fate is controlled by Bone Morphogenetic Proteins (BMPs), secreted from the ventral mesoderm and the downstream signal transducer Smad (Fig. 1; reviewed in Stern, 2005). In the ventral ectoderm, these factors activate epidermis specific genes like e.g. *lef1* and repress neural specific genes like e.g. *neurogenin* (*ngn*) (reviewed in Gilbert, 2000). In the dorsal ectoderm, in contrast, the activity of BMPs and Smads are blocked by factors secreted from the organizer like e.g. Noggin, Chordin, Follistatin and Xnr3 (Xenopus nodal related3). Thereby neuronal fate is induced (Fig. 1). The factors of the organizer interfere with BMP signaling either by binding to BMP ligands, like e.g. Noggin, Chordin and Follistatin or by binding to the BMP receptor, like e.g. Xnr3 (reviewed in Bainter et al., 2001). Therefore, induction of epidermis is considered as an actively induced fate, while development of neural tissue is the default state of the ectoderm.

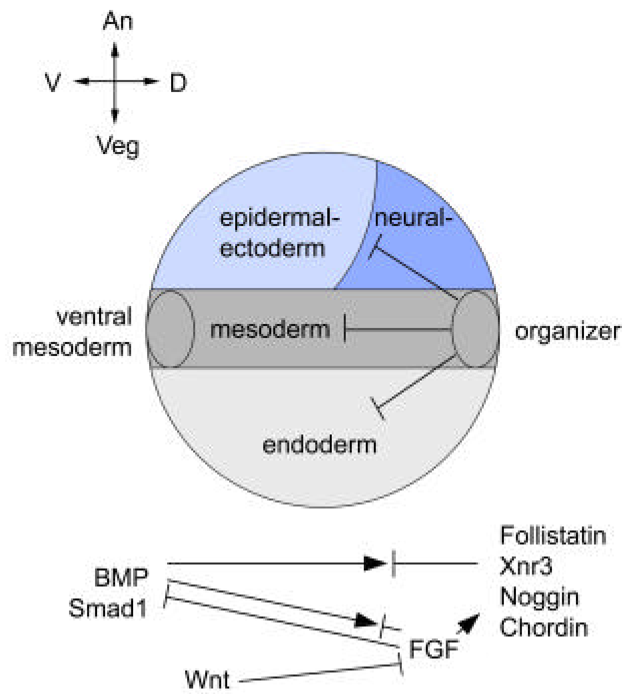

Fig. 1: Model of neural induction in *Xenopus laevis.* During early blastula and gastrula stages, BMPs secreted from the ventral mesoderm induce development of epidermis in the ventral ectoderm (light blue). In the dorsal ectoderm, signals from the dorsal organizer (e.g. Follistatin, Xnr3, Noggin and Chordin) block BMP activity and thereby induce neural fate (dark blue). In addition, FGF induces neural fate by repression of BMP activity and BMP transcription, activation of Noggin and Chordin and direct neural induction. Wnt signaling, in contrast, inhibits FGF activity in the ventral ectoderm and thereby promotes induction of epidermis.

The induction of neural ectoderm is a dynamic process. During gastrulation, cells of the vertebrate embryo undergo movements, which lead to a multilayered body plan. In *Xenopus,* cells of the marginal zone involute at the dorsal blastopore lip into the embryo to form mesoderm and endoderm. Thereby, they pass the organizer. After involution, these cells continue to express organizer specific factors and induce the overlaying ectoderm to develop into neural tissue (Fig. 2).

Xenopus knock-down and rodent explant experiments have recently extended the "default model" of neural induction by adding Fibroblast growth factor (FGF) and Wnt signaling (reviewed in Stern, 2005). These experiments have shown that FGF signaling in the dorsal embryo induces neural fate by inhibition of Smad1, repression of BMP transcription and activation of the BMP antagonists Chordin and Noggin (Launay et al., 1996; Sasai et al., 1996; Streit and Stern, 1999; Faure et al., 2002; Pera et al., 2003). Furthermore, low levels of FGF directly induce neural fate in the dorsal ectoderm (Fig. 1; Lamb and Harland, 1995; Hongo et al., 1999). Wnt signaling, in contrast, is proposed to block FGF signaling in the dorsal embryo and thereby enables BMP expression and consequently induction of epidermis (Fig.1; Lamb and Harland, 1995). In addition to these pathways, also intracellular levels of Calcium (reviewed in Moreau and Leclerc, 2004), the proportion of protein kinase C (PKC) to cAMP (reviewed in Stern, 2005), as well as insulin like growth factor (IGF; reviewed in Munoz-Sanjuan and Brivanlou, 2002) are implicated in neural induction in gastrulating vertebrate embryos.

4.2. Anteroposterior patterning of the neural ectoderm

After induction different regional identities are specified within the neural ectoderm. First an anteroposterior (AP) pattern is formed during late gastrulation and early neurulation. In 1933, Spemann and Mangold have already postulated that the organizer region defines different AP levels of neural tissue by an early and late inducing activity. In the 1950s, Nieuwkoop and colleagues have also postulated a two-step model for neural induction of the organizer. These two steps encompass the early induction of neural ectoderm, which is exclusively of anterior character and the late transformation of neural tissue, which defines posterior identity (reviewed in Chang and Hemmati-Brivanlou, 1998). It has been shown that BMP inhibitors of the organizer mediate early induction of neural ectoderm, while Wnts, FGFs and retinoic acid (RA) control its posterior transformation. During late gastrulation and neurulation, these posteriorizing factors are expressed at high levels around the organizer/blastopore lip and establish an activity gradient along the future posterior to anterior axis (Fig. 2; reviewed in Chang and Hemmati-Brivanlou, 1998). The progressive posterior movement of the organizer/blastopore lip specifies different identities of the neural ectoderm by induction of hindbrain and spinal cord specific genes (reviewed in Chang and Hemmati-Brivanlou, 1998).

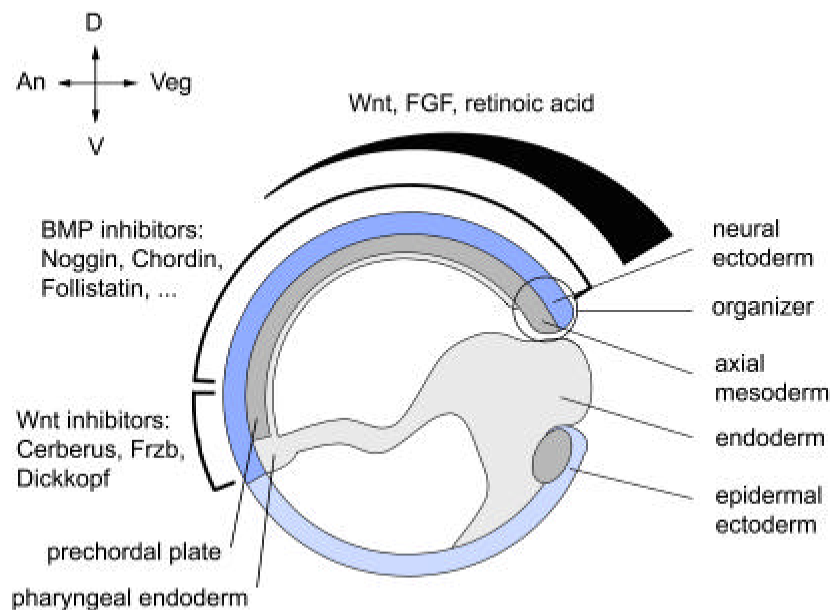

Fig. 2: Model for AP patterning of the Xenopus neural ectoderm. During late gastrulation and early neurulation, Wnt, FGF and RA form an AP gradient highest at the organizer/dorsal blastopore lip. BMP and Wnt inhibitors are expressed underneath the neural ectoderm in a regionally restricted manner. Wnt inhibitors (Cerberus, Frzb and Dickkopf) are secreted from the dorsoanterior endoderm (light gray) and prechordal plate (dark gray), while BMP inhibitors (like e.g. Noggin, Chordin, Follistatin) are expressed in the more posterior axial mesoderm (dark gray). These two mechanisms specify different AP identities of the neural ectoderm. Modified from Gilbert, 2000.

Anterior identity of the neural ectoderm is specified by spatially restricted expression of Wnt and BMP inhibitors. During gastrulation, involuting cells express different sets of neural inducing factors in a regionally restricted manner. The first involuting cells of the dorsoanterior pharyngeal endoderm and prechordal plate mesoderm secrete Wnt antagonists like e.g. Cerberus, Dickkopf-1 and Frzb-1 (Fig. 2; reviewed Chang and Hemmati-Brivanlou, 1998; Yamaguchi, 2001). Cells of the later involuting chordamesoderm express BMP antagonists like e.g. Noggin, Chordin and Follistatin. This regionally restricted expression of Wnt and BMP antagonists specifies different regions of identity along the AP axis of the neural ectoderm. In zebrafish, most neural inducing factors have conserved functions, however, the process of neural patterning is not very well understood, compared to *Xenopus*.

4.3. Neurulation and development of neural crest

During gastrulation of the vertebrate embryo, the neural ectoderm gets thicker and extends in length by convergent extension movements and proliferation. Therefore, the neural ectoderm is then named neural plate. At the border between epidermal ectoderm and neural plate, neural crest cells are formed (Fig. 3). Neural crest cells are multipotential cells that develop into a variety of different lineages like e.g. neurons, glia, cartilage, bone and pigment cells. The induction of neural crest cells starts at early gastrula stages by multiple signals, which are not yet fully understood. It is postulated that intermediate levels of BMPs induce neural crest development between neural and epidermal ectoderm. After initial induction by BMPs, neural crest fate is maintained and enhanced by FGF and Wnt signaling (reviewed in Schmidt and Patel, 2005). These signals activate down-stream determining factors like e.g. the zink-finger transcription factor Slug (reviewed in Schmidt and Patel, 2005).

During *Xenopus* neurulation, the neural plate folds to form the rod-like neural tube, the rudiment of the central nervous system. In other vertebrates, either the neural plate folds and invaginates forming a neural grove before closure (e.g. like in anterior chicken) or it forms a solid neural keel and then sinks into the embryo (e.g. like in zebrafish; Fig. 3). Formation of the neural tube generally progresses from anterior to posterior. However, in chicken the neural tube closure starts at the midbrain region and consequently progresses from posterior to anterior into the mid- and forebrain region. In humans, the neural tube closure starts at several points and occurs mostly bidirectional (reviewed in Gilbert, 2000).

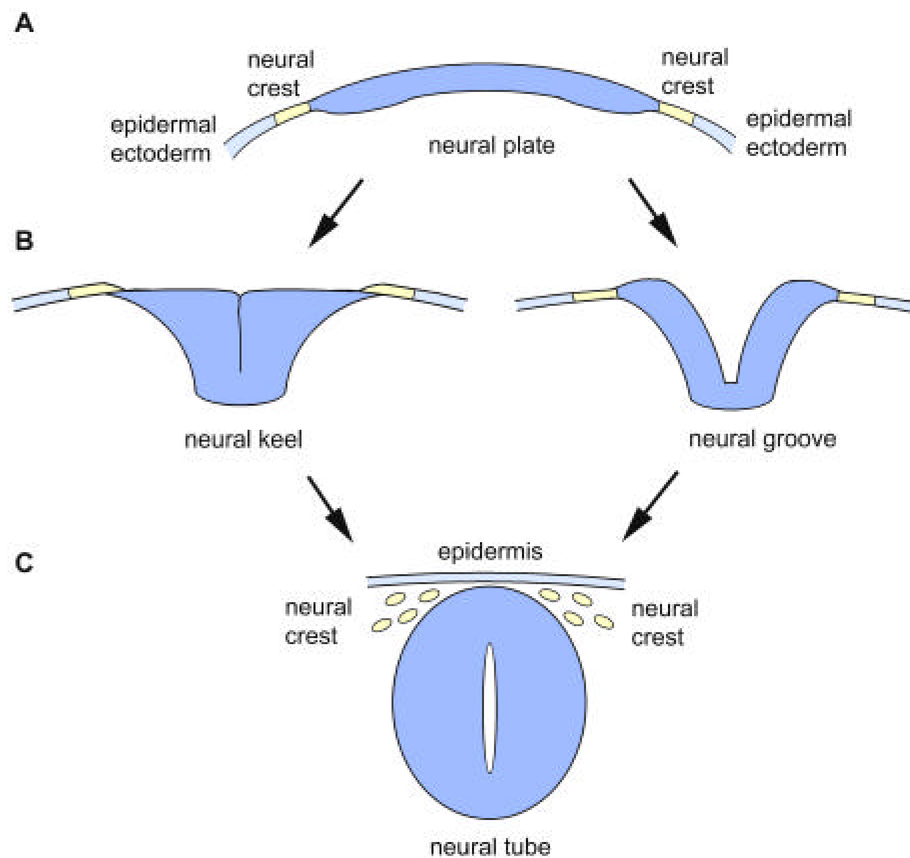

Fig. 3: Formation of the neural tube in vertebrates. (**A**) During late gastrulation, the neural plate has formed, which is flanked by premigratory neural crest cells. (**B**) During neurulation, the neural plate starts to fold to form the neural tube. This either occurs by convergence and formation of a solid neural keel that sinks into the embryo (left side) or by invagination of the neural plate, which subsequently forms a neural groove (right side). (**C**) The folded neural tube is detached from the epidermis, which overlays the neural tube and has a central cavity. During the late phase of neural tube formation, neural crest cells delaminate and start to move to the periphery.

Shortly before neural tube closure, neural crest cells delaminate from the folding neural tube and migrate as undifferentiated cells through the embryo to their target tissues (Fig. 3). During this process, neural crest cells undergo an epithelial-to-mesenchymal transition (EMT). It is postulated that already prior to delamination neural crest cells are restricted in their lineage. Cell culture experiments have shown that BMP signaling specifies neuron and glia fate, while Wnts act antagonistically and specify pigment cell fate (reviewed in Schmidt and Patel, 2005). After delamination, neural crest cells migrate to different target regions of the embryo and differentiate into a variety of different cell types.

4.4. Dorsoventral patterning of the vertebrate neural tube

Dorsoventral (DV) patterning of the neural tube is initiated shortly after neural induction. In the open neural plate, cells along the mediolateral axis acquire different identities. When the neural tube closes during neurulation, lateral cells constitute the most dorsal cells and form the dorsal organizer, the roof plate. Cells at the medial position become the most ventral cells and develop into the ventral organizer, the floor plate (Fig. 4A). Different neuronal subtypes are specified between these two structures, along the DV axis of the neural tube. In the dorsal region, sensory neurons are formed that are responsible for perception of sensory information. In the ventral part, motoneurons are specified that coordinate motor output. Furthermore, several interneuron populations develop along the DV axis in a tightly coordinated manner, which connect motoneurons and sensory neurons.

The specification of neurons starts already at the open neural plate stage, by the inhibitory activity of Delta-Notch signaling. In a process called lateral inhibition, Delta-Notch induces three longitudinal stripes of neuronal cells. These three stripes are located at medial, intermediate and lateral positions in the neural plate. The cells of these stripes are prespecified to develop into neurons and express neuron specific basic helix-loop-helix (bHLH) transcription factors like e.g. *neurogenin* (*ngn*), *neuroD* and *myT1*. Later, they develop into motor neurons, interneurons and sensory neurons, respectively (reviewed in Chang and Hemmati-Brivanlou, 1998; Chizhikov and Millen, 2005).

The specification of these neurons is controlled by different mechanisms in the dorsal and ventral neural tube. Patterning of dorsal neural tube neurons is initiated by BMP signaling from the epidermal ectoderm that first induces development of roof plate cells in the periphery of the open neural plate (reviewed in Chizhikov and Millen, 2005). As the neural tube closes, roof plate cells constitute an internal signaling center, which patterns the dorsal neural tube. This is mediated by secretion of BMP and Wnt proteins that act as morphogens forming a dorsal to ventral gradient in the neural tube (Fig. 4A, B). The activity gradient of BMPs and Wnts induce the differential expression of bHLH genes like e.g. *math, mash* and *ngn*, which define six dorsal neuronal progenitor domains (reviewed in Wilson and Maden, 2005). Wnts further control cell cycle progression of dorsal neuronal progenitor cells (Megason and McMahon, 2002) and seem to act downstream of BMP signaling (reviewed in Wilson and Maden, 2005).

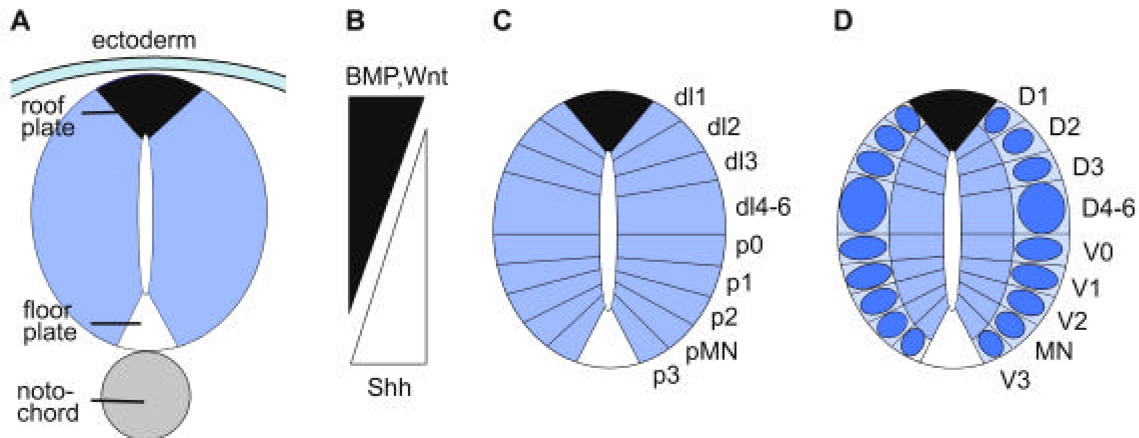

Fig. 4: Dorsoventral patterning of the vertebrate neural tube. (**A**) The specification of different cell types in the neural tube is initiated by signals of the dorsal roof plate, the ventral floor plate and the underlying notochord. (**B**) BMPs and Wnts, secreted from the roof plate and Shh, secreted from the floor plate and notochord act as morphogens that form opposing activity gradients in the neural tube. (**C**) According to different threshold concentrations of these two gradients, distinct neuronal progenitor domains are specified along the DV axis of the neural tube. (**D**) In the periphery, each domain develops a specific subtype of neuron.

Patterning of the ventral neural tube is controlled by Sonic Hedgehog (Shh), secreted from the floor plate and the underlying notochord. Prior to secretion, the Shh protein is processed by autoproteolysis and is lipid modified (Porter et al., 1996; Chamoun et al., 2001). The secreted N-terminal part of Shh (N-Shh) acts as a morphogen and forms a ventral to dorsal activity gradient in the neural tube. N-Shh establishes regionally restricted expression patterns of homeobox genes along the DV axis. This occurs by repression of class I homeobox genes like e.g. *dbx1*, *pax6* and *irx3* and induction of class II homeobox genes like e.g. *nkx2.2* and *nkx6.1* (Fig. 5A; Ericson et al., 1997; Briscoe et al., 1999; Briscoe et al., 2000; Sander et al., 2000; Pierani et al., 2001). The differential expression of these homeobox genes specifies five neuronal progenitor domains along the DV axis of the ventral neural tube. From ventral to dorsal these are the p3, pMN (motoneuron), p2, p1 and p0 domain (Fig. 4C, 5C; Briscoe et al., 2000). Class I and II homeobox proteins of adjacent domains repress each other, which consequently leads to a refinement of boundaries and maintenance of neuronal progenitor domains (Fig. 5B; Briscoe et al., 2000).

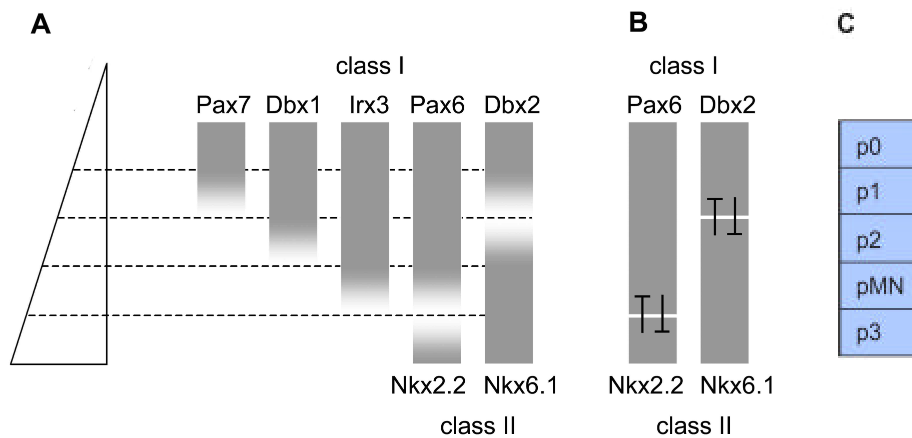

Fig. 5: Specification of neuronal progenitor domains in the ventral neural tube. (**A**) The activity gradient of Shh represses class I (*pax7, debx1, irx3, pax6 and dbx2*) and activates class II homeobox genes (*nkx2.2, nkx6.1*). This establishes regionally restricted expression patterns of homeobox proteins along the DV axis of the neural tube. (**B**) Class I and II homeobox proteins, which are expressed in neighboring cells, repress each other leading to sharp boundaries of expression domains. (**C**) The regionally restricted expression pattern of homeobox proteins defines five neuronal progenitor domains in the ventral neural tube. Modified from Jacob and Briscoe, 2003.

The Shh activity gradient is established by antagonistic activity of the receptor Patched (Ptc) and a DV activity gradient of the downstream transducers Gli, which regulate the range of HH signaling (Meyer and Roelink, 2003; Jeong and McMahon, 2005; Stamataki et al., 2005). Furthermore, BMP and Wnt activity intersect with the Shh pathway and repress Shh signaling (Liem et al., 2000; Robertson et al., 2004). In the ventral neural tube, BMP inhibitors like Follistatin (Fst) secreted from the notochord and adjacent somites block BMP activity and thereby enable the Shh dependent patterning of the ventral neural tube (Fig. 6; Liem et al., 2000). The specification of ventral neural tube cells is also controlled by FGF signaling. FGF has highest expression in the avian node, where it prevents differentiation of stem cells into neuronal cells (Diez del Corral et al., 2003). The activity of FGF has to be blocked in the trunk to allow neuronal differentiation. This is constituted by RA, which is synthesized in differentiated somites and inhibits FGF activity in the trunk neural tube (Fig. 6; reviewed in Wilson and Maden, 2005). The antagonism of RA and FGF acts selectively on the transcription of ventral neural tube specific class I homeobox genes (Pierani et al., 1999; Novitch et al., 2003).

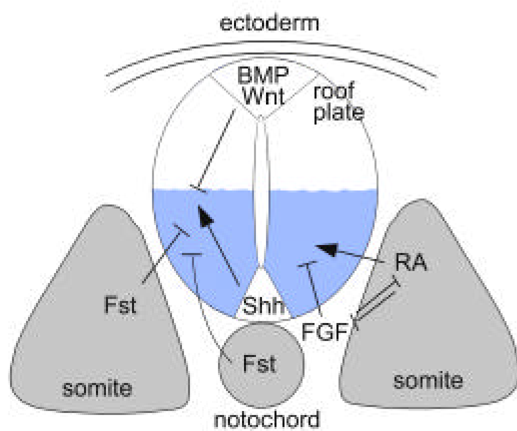

Fig. 6: Induction of neural patterning in the ventral neural tube. Left side: BMP secreted from the roof plate inhibits the ventralizing activity of Shh. To accomplish ventral neural patterning, Follistatin (Fst) secreted from the somites and notochord blocks BMP activity in the ventral neural tube. **Right side:** Differentiation of ventral cells in the neural tube is blocked by FGF signaling. Retinoic acid synthesized in differentiated somites blocks the inhibitory activity of FGF and vice versa.

4.5. Differentiation of the neural tube

The differentiation of distinct neurons and neuronal subtypes along the DV axis of the neural tube is controlled by the combinatorial activity of homeobox and bHLH transcription factors within each neuronal progenitor domain. These activate specific downstream transcription factors, which restrict the fate of neuronal domain cells (reviewed in Briscoe and Ericson, 2001). Consequently, cells in the periphery of each domain stop proliferation and start to differentiate into distinct neuronal subtypes (Fig. 4D).

During subsequent development, these neurons form axons, which project to their target tissue. Motoneurons innervate, for example, muscle, sensory neurons project to the sensory organs and interneurons connect different neuronal subtypes. The guidance of these axons is controlled by complex interactions of attracting and repelling signals. The floor plate, for example, secretes the signaling molecules Shh and Netrin-1, which guide the trajectory of commissural axons from the dorsal neural tube to the floor plate. (Kennedy et al., 1994; Charron et al., 2003). After reaching the floor plate, commissural axons cross the midline of the neural tube and respond no longer to floor plate derived attractant signals. They rather respond to the axonal repellent Slit, which is expressed in ventral midline cells and guides axonal growth at the contralateral side back towards the dorsal neural tube (Stein and Tessier-Lavigne, 2001).

Shortly after differentiation of neurons, the neural tube also forms glia cells, which are important for support, maintenance and nutrition of neurons. One important subtype of glia cells are oligodendrocytes that form myelin sheaths around neurons, which are essential for signal transmission. Oligodendrocytes originate from the pMN domain of the ventral neural tube, which in a first phase develops motoneurons and in a second phase oligodendrocyte precursor cells (OLPs). This process is controlled by the Shh downstream bHLH genes *olig1*

and *olig2,* which presumably first initiate transcription of motoneuron specific genes like e.g. *islet-1* and later OLP specific genes like e.g. *plp* and *dm20* (reviewed in Marti and Bovolenta, 2002). The later differentiation of OLPs is independent of Shh and controlled by the homoebox transcription factors Nkx2.2 and Sox10 (reviewed in Rowitch, 2004). Mature oligodendrocytes migrate into the lateral and dorsal regions of the central nervous system and are later equally distributed.

4.6. Development of the vertebrate brain

During early neurulation, the anterior neural plate bulges and forms the brain. Three functional compartments named primary vesicles form along the AP axis: the prosencephalon (forebrain), the mesencephalon (midbrain) and the rhombencephalon (hindbrain; Fig. 7). The prosencephalon consists of the dorsal telencephalon, which develops olfactory lobes, hippocampus and cerebrum as well as the ventral diencephalon, which forms retina, epiphysis, thalamus and hypothalamus (Fig. 7; reviewed in Gilbert, 2000). The mesencephalon is not further subdivided (Fig. 7). It consists of fiber tracts that connect anterior and posterior brain. The rhombencephalon consists of repetitive units, termed rhombomeres (Fig. 7). The most anterior rhombomere forms the cerebellum, while the more posterior rhombomeres constitute the medulla (reviewed in Gilbert, 2000). Each rhombomere forms individual ganglia projecting to different targets, for example individual branchial arches (reviewed in Kandel et al., 2000).

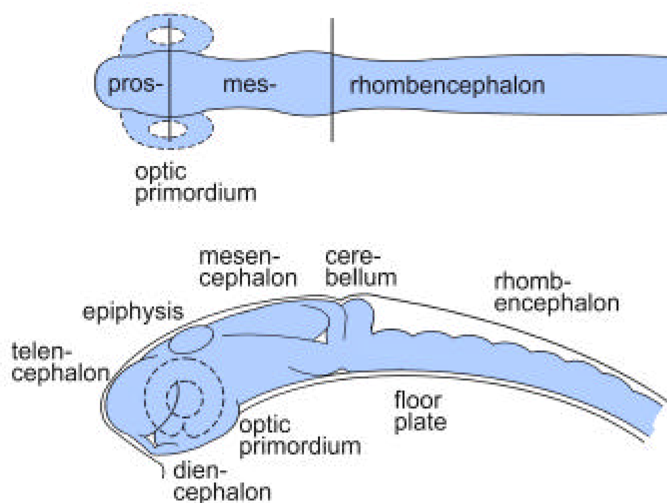

Fig. 7: Structure of the embryonic brain of a one day old zebrafish embryo. Upper scheme: dorsal view of the brain that consists of the pros-, mes- and rhombencephalon. On each side of the forebrain the eye primordium is located, in which the retina is neural derived but not the lens. **Lower scheme:** Lateral view of the brain. The prosencephalon consists of the dorsal telencephalon and ventral diencephalon as well as the dorsal epiphysis. The middle brain structure is the mesencephalon. Posteriorly, the rhombencephalon is located, which consists of the cerebellum and segmental rhombomeres. The mes- and rhombencephalon are underlaid by the floor plate. Modified from Westerfield, 2000.

4.7. Mechanisms of medial floor plate formation in vertebrates

In the floor plate of most vertebrate species, expression analysis have led to the definition of the inner located floor plate cells as medial floor plate (MFP) and the flanking cells as lateral floor plate (LFP; Placzek et al., 1991; Placzek et al., 1993; Marti et al., 1995; Odenthal et al., 2000; Charrier et al., 2002). The mechanisms of MFP formation have been controversially discussed over the last few years and are still not fully understood. Two mutually exclusive models have been originally proposed based on experiments in chicken (see Le Douarin and Halpern, 2000 and Placzek et al., 2000). One model predicts that the MFP is specified in the median neural plate by vertical signals from the underlying notochord. Thus, MFP induction occurs in the embryonic trunk when notochord cells have been fully differentiated (Fig. 8). This model is based on cell explantation and grafting experiments in chicken like e.g. the transplantation of a notochord to an ectopic position aside of the neural tube, which induces a secondary MFP (Placzek et al., 1990; van Straaten and Hekking, 1991; Yamada et al., 1991; Placzek et al., 1993). The second model proposes that the MFP originates from a population of midline precursor cells that also forms notochord and hypochord. This model predicts that the induction of midline precursor cells occurs in the organizer and MFP cells intercalate into the open neural plate. This is mainly based on cell lineage experiments of quail-chicken chimeras (Fig. 8; Catala et al., 1996; Le Douarin et al., 1998; Teillet et al., 1998).

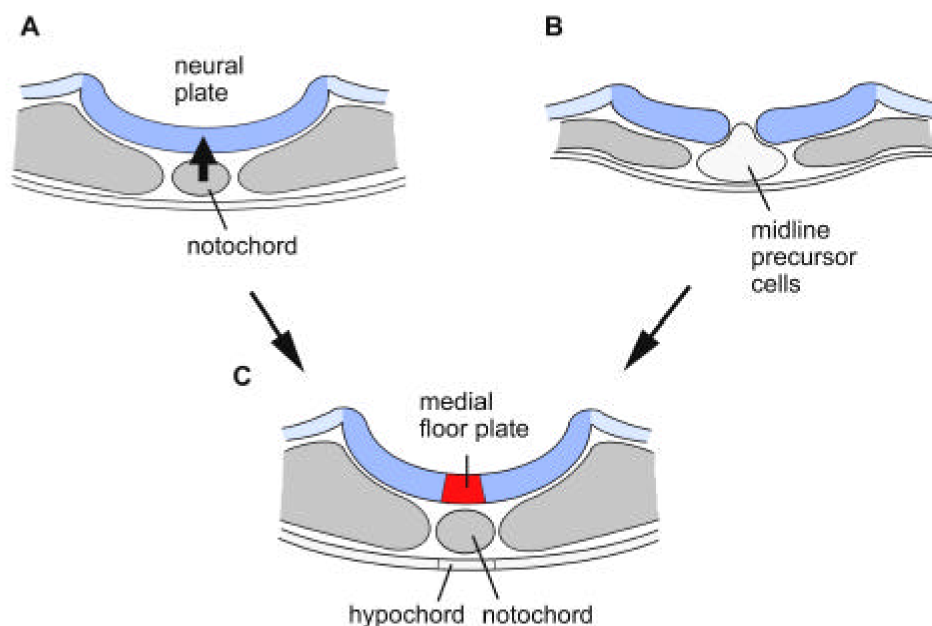

Fig. 8: Two controversial models for MFP formation in vertebrates. (**A**) The first model proposes that vertical signals from the differentiated notochord induce MFP development in the median neural plate of the trunk. (**B**) The second model predicts that a pool of midline precursor cells is specified in the organizer to develop into MFP, notochord and hypochord. MFP cells then intercalate into the open neural plate. (**C**) Finally, in the ventral part of the infolding neural plate, MFP cells have been formed by one of the two speculated processes.

Insights into the mechanisms of MFP formation came from analyses of different other vertebrate model organisms. In zebrafish, lineage tracing experiments have shown that both MFP and notochord cells originate from the zebrafish organizer, the shield (Shih and Fraser, 1995). Furthermore, a mutant deficient for the mesoderm inducing factor *no-tail* (*ntl*, *brachyury*; Schulte-Merker et al., 1992) has been identified, in which MFP forms in the absence of a differentiated notochord (Halpern et al., 1997). This indicated that MFP formation occurs independent of notochord-derived signals. In *ntl* and other mutants, the MFP forms at the expense of the notochord, indicating that midline precursor cells are specified to develop either into MFP or into notochord (Halpern et al., 1997; Appel et al., 1999; Amacher et al., 2002). Together, these data have provided a strong line of evidence that MFP formation occurs according to the second proposed model. In mice, however, it has been clearly shown that MFP and notochord derive from separate origins and do not share the same linage. Thus in mice, MFP obviously forms as proposed by the first model (Jeong and Epstein, 2003). These data have shown that both mechanisms of MFP formation exist in vertebrates.

Besides the cellular mechanisms, also the signals controlling MFP induction are apparently different in the vertebrate species. In higher vertebrates, knock-out mutants and explantation experiments have shown that the morphogen Sonic hedgehog (Shh) induces formation of the MFP (Chiang et al., 1996; Wijgerde et al., 2002). In zebrafish, in contrast, MFP induction is independent of Shh but controlled by factors of the organizer, most notably Cyclops (Cyc; Nodal-related 2; Sampath et al., 1998; Tian et al., 2003). Based on the data from zebrafish, the role of Nodal signaling in formation of the MFP has also been investigated in higher vertebrates. In mice, it has been shown that MFP formation is independent of Nodal signaling in the node (Vincent et al., 2003). However in chicken, Nodal signaling controls MFP formation at hindbrain level. Development of the trunk MFP, in contrast, requires Shh (Patten et al., 2003). Thus in chicken, MFP induction is controlled by two different signals along the AP axis. Although different mechanisms and factors have been found during MFP development in the different model organisms, it still remains unclear, whether these occur exclusively in each model organism and reflect species-specific differences or whether the MFP forms differently during distinct phases of embryonic development.

4.8. Characterization of the lateral floor plate in vertebrates

The identification of a MFP and LFP in many vertebrate species is based on the spatially restricted expression of floor plate marker genes (Placzek et al., 1991; Placzek et al., 1993; Marti et al., 1995; Odenthal et al., 2000; Charrier et al., 2002). In the MFP, all floor plate

genes are coexpressed, while in the LFP only a few distinct floor plate genes are found. For example in zebrafish, *shh* and *netrin-1* are restricted to the MFP, while *foxa2* and *fkd4* are more broadly expressed, also in the flanking LFP (Odenthal et al., 2000). In chicken and mice, LFP cells are apparently positioned within the p3 neuronal domain, which is characterized by expression of the class II homeobox genes *nkx2.2* and *nkx2.9* and development of V3 neurons (Fig. 9; Briscoe et al., 1999; Briscoe et al., 2000; Charrier et al., 2002). Therefore it is currently unclear, whether LFP cells are floor plate or neuronal cells (Placzek and Briscoe, 2005).

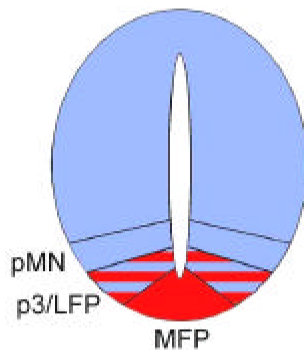

Fig. 9: Structure of the ventral neural tube in vertebrates. The medial floor plate (MFP) is positioned most ventrally in the neural tube. Lateral floor plate (LFP) cells are positioned laterally to the MFP, within the p3 neuronal progenitor domain. p3 neuronal cells and LFP cells are dorsally flanked by the pMN domain.

The zebrafish has provided significant insights into the organization of the LFP. In contrast to higher vertebrates, the zebrafish MFP and LFP are both only one cell in diameter and thus constitute clearly defined spatially restricted domains. The zebrafish was therefore the first model organism, in which MFP and LFP have been described (Odenthal et al., 2000). Moreover in zebrafish, MFP and LFP cells can be distinguished by their origin and signals of induction. MFP cells derive from the shield and are induced by shield-derived factors like e.g. Cyc (Shih and Fraser, 1995; Sampath et al., 1998; Schauerte et al., 1998). LFP cells, in contrast, are neurectodermal cells, which are induced later by Shh, secreted from the MFP and notochord (Odenthal et al., 2000; Schäfer et al., 2005a [1]). These distinct differences resulted in the definition of a node derived MFP and a neuroectodermal LFP also in chicken (Charrier et al., 2002). However, like in higher vertebrates also in zebrafish the structure of the LFP is not clear. Within the zebrafish LFP a specific type of GABAergic interneurons named Kolmer-Agdhur (KA) neurons have been identified, which project ipsilaterally into the ventral fasciculus (Bernhardt et al., 1992). This indicates that also in zebrafish the LFP contains neuronal cells. It remains to be resolved how these different cell types are organized and specified during early embryonic development.

[1] own contributions are underlined

4.9. The zebrafish as a model to study embryonic development

The zebrafish has been established in the last 20 years as an important model organism for vertebrate development, most notably by researchers in Oregon, Boston (USA) and Tübingen (Germany). The zebrafish (*Danio rerio*) is a tropical fish originating from Pakistan and India with an adult size of 3-4 cm and characteristic longitudinal stripes. The major advantage of the zebrafish as a model organism is the simultaneous application of embryological and advanced genetic techniques. Embryological methods like e.g. transplantation of cells are easy to perform, as zebrafish embryos are very robust and cell movements can be easily monitored due to a total transparency of embryos (reviewed in Westerfield, 2000). Genetic methods are limited by the lack of an established gene knock-out technology through homologous recombination. Nevertheless, a huge number of defined mutant lines has been generated in recent years by reverse genetics. Several large-scale mutagenesis screens using the chemical mutagen ethylnitrosurea (ENU) and extensive screening for developmental defects have been performed so far (Driever et al., 1996; Haffter et al., 1996). These screens can be done efficiently, as zebrafish embryos can be obtained in large numbers, have a short generation time of three month and adult fish are easy to maintain in relatively small facilities (Haffter and Nusslein-Volhard, 1996). Moreover in zebrafish, gain of function and transient gene knock-down analysis can be efficiently performed by ubiquitous mRNA overexpression as well as antisense morpholino oligonucleotide gene knock-down (Nasevicius and Ekker, 2000). This is possible by a well-established microinjection technique and the access of a huge number of embryos at earliest developmental stages. Furthermore, due to the transparency of zebrafish embryos, expression analysis by in-situ hybridization can be easily performed to investigate genetic interactions. Thus, in zebrafish novel genes controlling functionally important developmental processes can be rapidly identified and functionally analyzed. Altogether, this shows that the zebrafish provides many important advantages for the analysis of vertebrate development and complements other established model organisms and experimental systems.

4.10. Duplicated genes in zebrafish

Zebrafish belong to the teleost fish that diverged from the tetrapod linage about 450 million years ago. In recent years, it has been shown that many genes in teleost fish have two co-orthologous copies, while only one ortholog is present in tetrapods (Wittbrodt et al., 1998). For example, *hox* genes, which specify cells along the AP axis, have been found in four clusters in mice, but seven clusters have been identified in zebrafish (reviewed in Key and

Devine, 2003). It has been predicted that the reason for this was a fish specific whole-genome duplication event that occurred after divergence of the ray-finned fish (including zebrafish) from the lobed-finned fish (Amores et al., 1998). Genome duplication seems to be an important event during evolution of new organisms. The duplication of the entire genome can lead to genes with new functions without loosing important ancestral genes (reviewed in Taylor et al., 2003). It has been proposed that the genome duplication in the rayed fined lineage is responsible for the extreme radiation of the teleost fish with approximately 25,000 species (Amores et al., 1998; Meyer and Schartl, 1999; Volff, 2005).

In zebrafish, many duplicated genes are functionally redundant (Wittbrodt et al., 1998). However, mutations in co-orthologous genes have resulted in three other scenarios. In most cases, duplicated genes became inactivated by mutations and got lost over time. On the other hand, genes could have also acquired new functions. As duplicated genes were free of selective pressure, one copy could have easily diverged and acquired mutations, which resulted in a neo-functionalization of one copy. The function of the ancestral gene could have also been split in the co-orthologous genes, which resulted in a sub-functionalization (Furutani-Seiki and Wittbrodt, 2004; Hoegg et al., 2004).

The sub-functionalization of genes in zebrafish can provide a significant advantage if the homologous gene in mammals cannot be functionally analyzed. One prominent example is the functional analysis of the *shh* gene. In mice, knock-out of *shh* leads to severe defects during early development, thus the function of Shh is hard to investigate (Chiang et al., 1996). In zebrafish, on the other hand, there are two *shh* co-orthologs, named *shh* and *tiggy-winkle hedgehog* (*twhh*), which most likely underwent a sub-functionalization (Zardoya et al., 1996). Mutants in one of the *shh* co-orthologs only show a part of the mouse phenotype and thus enable a functional analysis of the gene. Knock-down of both *shh* co-orthologs, on the other hand, leads to the same severe phenotype as observed in mouse knock-outs (reviewed in Furutani-Seiki and Wittbrodt, 2004). Although functional redundancy of duplicated genes in zebrafish often impedes a functional gene analysis, this example shows that neo- and sub-functionalization can provide significant new insights into the molecular mechanisms of developmental processes.

4.11. *Midkine* and *pleiotrophin* genes in vertebrates

Another example of duplicated genes in teleosts are *midkine* genes that have two co-orthologs in zebrafish named *mdka* and *mdkb* but only one ortholog in birds and mammals (Winkler et al., 2003). Midkine (Mdk) is a secreted heparin-binding growth factor, which has

neurotrophic activities in cell culture assays and mediates e.g. neurite outgrowth, nerve cell migration and neuron protection (reviewed in Kadomatsu and Muramatsu, 2004). Moreover, Mdk seems to be involved in neurodegenerative diseases like e.g. Alzheimer's disease (reviewed in Kadomatsu and Muramatsu, 2004). Mdk has originally been identified in a screen for retinoic acid induced genes in embryonal carcinoma cells (Tomomura et al., 1990). In mammals, Mdk shares 50% identity with the related factor Pleiotrophin (Ptn). Together, both factors constitute a new family of heparin-binding proteins, which are structurally related. They consist of functionally distinct C- and N-terminal domains and have 10 highly conserved cysteine residues (reviewed in Kadomatsu and Muramatsu, 2004). Both factors have been implicated as important factors during carcinogenesis as they show anti-apoptotic activity. They also promote growth, survival, transformation and angiogenic response of tumor cells. Moreover, Mdk and Ptn are upregulated in a variety of different tumors (reviewed in Muramatsu, 2002; Kadomatsu and Muramatsu, 2004). *In-vitro* analyses have furthermore shown a broad range of other activities during tissue repair, vasculogenesis, chondrogenesis, neurogenesis etc.

The molecular basis of Mdk and Ptn signaling that leads to the diverse *in-vitro* activities has been approached in recent years. Several receptors have been identified to bind Mdk and Ptn in *in-vitro* binding assays. This includes the receptor-type protein tyrosine phosphatase PTPζ (Maeda et al., 1996; Maeda et al., 1999), Anaplastic lymphoma kinase (Alk; Stoica et al., 2001; Stoica et al., 2002) and LDL receptor-related protein (LRP; Muramatsu et al., 2000). It is suggested that the downstream signaling of Mdk and PTP involves the PI3 kinase-MAP kinase-Erk pathway (Souttou et al., 1997; Owada et al., 1999; Qi et al., 2001; Stoica et al., 2001). Furthermore, an activation of the Jak-Stat pathway and internalization and nuclear translocation of Mdk via LRP have been observed (Ratovitski et al., 1998; Shibata et al., 2002; Dai et al., 2005). However, the functional interaction of different receptors and downstream components and the specific biological activity remain to be resolved. Most importantly, the receptor of Mdka and Ptn *in-vivo* has not been identified so far.

During murine embryogenesis, Mdk and Ptn are widely expressed, including the nervous system (Mitsiadis et al., 1995). To analyze the function of Mdk and Ptn, knock-out mice have been generated, which show no early embryonic phenotype (Nakamura et al., 1998; Amet et al., 2001). Double knock-out of both factors, on the other hand, resulted in early embryonic lethality (Muramatsu, 2002). These analyses have indicated that in mice Mdk and Ptn are functionally redundant (Muramatsu, 2002). Therefore, the *in-vivo* function of Mdk and Ptn during embryogenesis remains unknown.

4.12. Aim of the PhD thesis

The floor plate is an important signaling center, which plays a major role in specification of neurons and glia cells along the dorsoventral (DV) axis of the ventral neural tube, as well as guidance of outgrowing axons. Recently, two different domains have been defined in the floor plate, named the medial floor plate (MFP) and the lateral floor plate (LFP).

The origin and timing of specification of MFP cells is a matter of ongoing debates. In zebrafish, specification of MFP cells during gastrulation is well characterized, however, it is unclear how the MFP forms during later stages of embryonic development. In our group, we have recently isolated a novel secreted growth factor, Midkine-a (Mdka). In higher vertebrates, Mdk shows a variety of *in-vitro* activities, however, the *in-vivo* function is unknown. First preliminary analyses have indicated that the *mdka* gene is involved in MFP formation in zebrafish. The aim of my PhD thesis was to analyze the function of Mdka during MFP formation at stages after gastrulation and to investigate the regulation and interaction of Mdka with known MFP inducing factors.

Currently, also the receptor of Mdk in fish and higher vertebrates is not known. Therefore, I have started to investigate whether Mdka functions through a putative candidate receptor, Anaplastic lymphoma kinase (Alk).

The second project of my PhD thesis was to analyze the mechanisms of LFP formation in zebrafish. The organization and the specification of the LFP are presently not fully understood. We have recently isolated a homeobox gene *nkx2.2b*, which is exclusively expressed in the zebrafish LFP. During my PhD thesis, I have analyzed the expression, regulation and function of *nkx2.2b*. To obtain further insight into the structure and mechanisms of LFP formation, I have investigated the expression profile of the LFP and regulation by the signaling molecule Shh.

5. Results and Discussion

5.1. Evolution of *midkine* and *pleiotrophin* genes in the teleost lineage

5.1.1. Phylogenetic and divergence analysis of Midkine and Pleiotrophin in vertebrates

In the last few years many genes have been identified that have two or more copies in teleosts but only one copy in mammals. This has led to the suggestion that more genes exist in fish compared to mice and human (Wittbrodt et al., 1998; Furutani-Seiki and Wittbrodt, 2004). Two controversial models have been proposed to explain the mechanisms responsible for this phenomenon. One model proposes a whole-genome duplication event that occurred 300-450 Million years ago in the ray-finned lineage and resulted in subsequent loss of many gene co-orthologes (Amores et al., 1998). The other model suggests rapid local gene duplication events that occurred more recently in the ray-finned lineage (Robinson-Rechavi et al., 2001a; Robinson-Rechavi et al., 2001b). Although the first model has been strongly supported in recent years (Taylor et al., 2003; Van de Peer et al., 2003; Hoegg et al., 2004), it is still not exactly clear how duplicated genes in teleosts have evolved.

We have recently isolated two functional *midkine* genes named *mdka* and *mdkb* in zebrafish (Winkler and Moon, 2001; Winkler et al., 2003). Phylogenetic analyses have indicated one additional fish species, the rainbow trout that also has two *mdk* co-orthologes. In mammals and birds, in contrast, only one *mdk* gene exists. Zebrafish *mdka* and *mdkb* have been located by radiation hybrid mapping to linkage groups 7 and 25, respectively. Both linkage groups have synteny to the human chromosome Hsa11, which contains the human *mdk* gene (Postlethwait et al., 2000, Woods et al., 2000). Interestingly, at least four other genes have been reported so far that also have duplicates on the same zebrafish linkage groups like e.g. *pax6a*/*pax6b* or *islet2*/*islet3* (Woods et al., 2000; Taylor et al., 2001). Divergence and phylogenetic analyses have indicated a large block duplication containing these genes in the ancestral fish lineage. This is consistent with the model of an whole-genome duplication in the rayed fin lineage and stands in contrast to the model of single random gene duplication events.

In contrast to the duplicated *mdk* genes, we have found only one *ptn* gene in different fish species. Zebrafish *ptn* was mapped to linkage group 4, which has synteny to the human chromosome Hsa7p that contains the human *ptn* gene (Woods et al., 2000). Comparison of flanking genes of *mdk* and *ptn* in different fish species and humans suggested that *mdk* and *ptn* themselves have also evolved by a large block duplication. This, however, has occurred before divergence of the fish and the tetrapod lineage, at least 450 million years ago. The duplicated genes of this block have either been maintained like *mdk/ptn* or only one co-

ortholog was kept like it is the case for *shh* and *eng2*, which are linked to *mdk* in zebrafish and *ptn* in mammals. This suggests that *shh* and *eng2* genes from humans are possibly not orthologous but co-orthologous to *shh* and *eng2* in fish.

5.1.2. Expression and function of Midkine and Pleiotrophin in zebrafish

After duplication, most gene co-orthologs become inactive and get lost over time. However, they can also undergo a neo-functionalization, in which they acquire new activities or a sub-functionalization, in which ancient gene expression and activity is split in the co-orthologous genes. To analyze how *mdk* and *ptn* genes evolved after duplication in zebrafish, we investigated expression and function of both genes during embryogenesis. In mice, *ptn* and *mdk* are both widely expressed during midgestation and functional redundancy has been proposed based on gene knock-out studies (Fan et al., 2000). In zebrafish, in contrast, we found a very restricted and mostly non-overlapping expression of *mdk* and *ptn* genes. Expression of *ptn* was only detected in the ventral diencephalon of the forebrain as well as in rhombomeres 5 and 6 of the hindbrain (Fig. 10; unpublished data M. Schäfer, D. Liedtke and C. Winkler). The two *mdk* genes, in contrast, are very dynamically expressed in several brain regions and in the embryonic trunk and overlap with *ptn* only in hindbrain rhombomeres. This shows that in zebrafish *ptn* has retained only part of the expression compared to higher vertebrates, indicating mostly non-redundant functions of *mdk* and *ptn*.

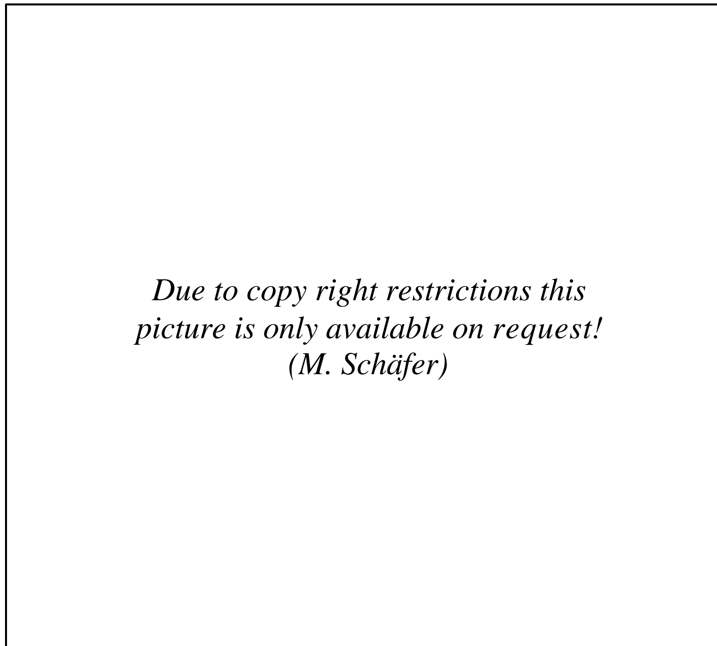

Fig. 10: Expression of *mdka*, *mdkb* and *ptn* in the head of a 12 somite (s) stage embryo. (**A,B**) *Mdka* shows strong expression in the telencephalon, caudal midbrain, MHB and caudal rhombomeres. (**C,D**) *Mdkb* is expressed in the telencephalon and entire rhombencephalon. Note complementary expression of *mdka* and *mdkb* in midbrain and rostral hindbrain. (**E,F**) *ptn* is weakly expressed in the diencephalon of the forebrain and rhombomeres 5 and 6. Arrowheads in A-D demarcate the boundary of *mdka* and *mdkb* expression at the MHB. A,C,E is dorsal view, B,D,F is lateral view. MDB, mid-diencephalon boundary; MHB, mid-hindbrain boundary; r, rhombomere; te, telencephalon.

The two *mdk* co-orthologes in zebrafish have also mostly non-overlapping expression patterns. In the head region, *mdka* and *mdkb* are coexpressed in the telencephalon and caudal rhombomeres. However, exclusive expression was found in the midbrain, mid-hindbrain boundary (MHB) and eye primordia, where only *mdka* is expressed, as well as in the rostral rhombomeres, where only *mdkb* is detectable (Fig. 10, unpublished data M. Schäfer, D. Liedtke, C. Winkler). In trunk and tail, *mdka* is expressed in the ventral neural tube, excluding the floor plate and in the somites. *Mdkb* is expressed in the roof plate of the neural tube, dorsal to *mdka* (Winkler et al., 2003).

Functional analyses mostly revealed the differential activities of *mdk* genes. In the forebrain, where *mdka* and *mdkb* are overlappingly expressed, ubiquitous overexpression of both genes by mRNA microinjection leads to similar defects in morphology. In both cases, a shortening of the brain was observed with severe reduction of the diencephalon and consequently a proximal fusion of eye primordia (Fig. 11, unpublished data D. Liedtke, M. Schäfer, C. Winkler). This shows that both factors have the same activity during forebrain development, when ectopically expressed.

Due to copy right restrictions this picture is only available on request! (M. Schäfer)

Fig. 11: Overexpression of *mdka* and *mdkb* interferes with forebrain development. A,E,I control embryos, B,F,J *mdka* RNA injected and C,G,K *mdkb* RNA injected embryos at 24 hours post fertilization (hpf). (**A-C**) lateral view of embryonic head regions showing shortening of the head and severe reduction of rostral diencephalons in *mdka* (B) and *mdkb* (C) RNA injected embryos. (**D-F**) Frontal view of embryos with merged eye primordia after injection of *mdka* (E) and *mdkb* (F) RNA.

In the embryonic trunk, however, where *mdk* genes have no overlapping expression, distinct and significantly different activities of both genes were observed. As reported earlier, *mdkb* overexpression resulted in expansion of premigratory neural crest markers, consistent with its expression in the dorsal neural tube (Winkler and Moon, 2001). *Mdka* overexpression, in contrast, had no effect on neural crest formation but resulted in expansion of the MFP. Moreover, a loss of somite boundaries and strong reduction of early and late somite markers was observed (Winkler et al., 2003). This mostly correlates with the expression of *mdka* in

these tissues. Thus, although both genes were ubiquitously overexpressed in the developing embryo, different effects were observed. These distinct activities of *mdk* genes could be best explained by a differential interaction with so far unknown cofactors and/or binding to different receptors.

In addition to distinct expression patterns in the developing embryo, also in the adult fish we found mostly non-overlapping expression of *mdk* genes within the brain. *Mdka* was detected at the surface of the telencephalon and optic tectum. *Mdkb*, in contrast, was found in the telencephalon and cerebellum, as well as in the dorsal medulla oblongata. Also in the hypothalamus, both *mdk* genes were expressed in different subregions. However, in human and mice, *mdk* is only barely detectable in any adult tissue, except the kidney (Kadomatsu et al., 1990). This shows that the expression patterns of zebrafish *mdk* genes seem to be significantly different from *mdk* in higher vertebrates.

Altogether, our data indicate that *mdk* genes have undergone functional divergence after ancient gene duplication. The mostly non-overlapping expression and strictly different activities during neural crest, MFP and somite formation indicate a possible sub-functionalization. As expression of both *mdk* genes was found in the adult brain, while this is not the case in higher vertebrates, also a neo-functionalization could be possible. However, the exact mechanisms cannot be clearly determined since no data about the ancestral unduplicated gene in fish are available.

5.2. Midkine-a (Mdka) controls medial floor plate formation in zebrafish

The MFP is an organizing center, located most ventrally in the neural tube of vertebrates. It specifies neuronal and glia cells along the DV axes of the ventral neural tube and guides the trajectory of outgrowing axons. The mechanisms of MFP formation are currently controversially discussed (see Strähle et al., 2004; Placzek and Briscoe, 2005). One model suggests that the MFP is induced in the median trunk neural plate by signals from the underlying notochord (Placzek et al., 2000). The alternative model, in contrast, postulates an early specification of MFP cells within the organizer. According to this model, MFP cells derive from a population of midline precursor cells that also develop into notochord and hypochord (Le Douarin and Halpern, 2000).

In zebrafish, mutant analysis and lineage tracing experiments have shown that MFP cells originate from a population of midline precursor cells, which are induced in the embryonic organizer, the shield (Shih and Fraser, 1995; Appel et al., 1999; Tian et al., 2003). Therefore, this strongly supports the second proposed model. Induction of MFP cells in the pool of

midline precursor cells occurs during gastrulation by shield-derived factors like e.g. Cyclops (Cyc, Nodal-related 2; Sampath et al., 1998; Tian et al., 2003), No-tail (Ntl, Brachyury; Halpern et al., 1997; Amacher et al., 2002) and Delta-Notch signaling (Appel et al., 1999). However, while the mechanisms and factors of MFP induction during gastrulation are well characterized, it is unclear how MFP formation occurs during later stages (Fig. 12). It is predicted that after gastrulation initially induced MFP precursor cells persist in the tailbud and differentiate into MFP, hypochord and notochord. However, it is not clear how the allocation of these cells in the pool of midline precursor cells is controlled.

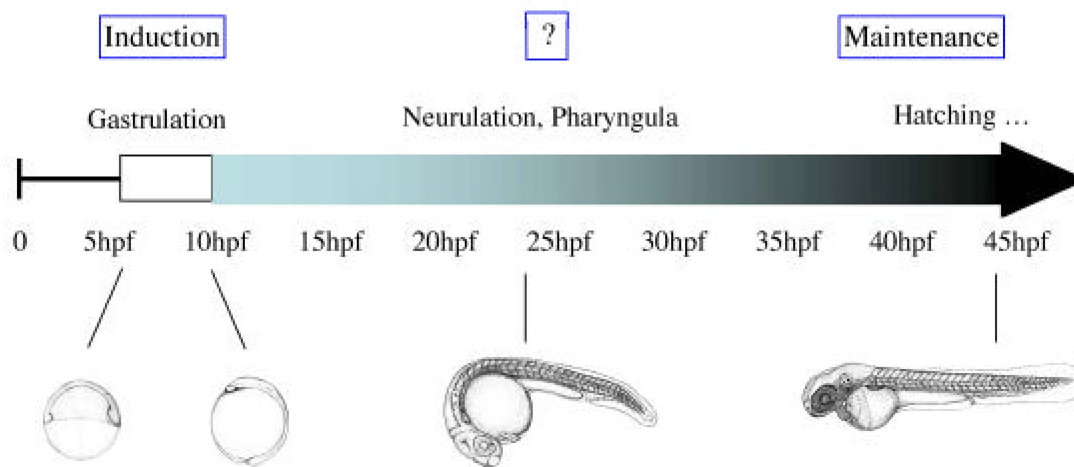

Fig. 12: Different phases of MFP development in zebrafish. MFP formation in zebrafish occurs in three different phases. During gastrulation, between 5hpf and 10hpf post fertilization MFP cells are initially induced. During the hatching period, after around 40hpf post fertilization, MFP cells are maintained (Albert et al., 2003). The mechanism of MFP formation between these two stages, during neurulation and pharyngula stage is currently unknown. During this stage the zebrafish embryo rapidly grows and also the MFP elongates dramatically.

We have analyzed the role of Mdka during formation of the MFP in zebrafish (Schäfer et al., 2005b). *Mdka* expression starts at the end of gastrulation, as revealed by RT-PCR indicating that *mdka* is not involved in development during early gastrulation. To analyze the function of Mdka during MFP formation, we performed an overexpression approach by injection of mRNA and a knock-down approach by injection of antisense morpholino oligonucleotides. Overexpression of *mdka* resulted in a strong expansion of the MFP, as shown by broader expression of several MFP marker genes. In these embryos the hypochord was similarly increased, while the notochord was significantly smaller or completely failed to form. Vice versa, knock-down of Mdka interfered with MFP formation and resulted in significant gaps in

the trunk MFP. The hypochord was similarly reduced, while on the other hand the notochord was increased in cell density.

To investigate the mechanisms of Mdka dependent MFP formation, we performed proliferation assays and confocal time-lapse imaging. These analyses showed that Mdka neither acts on proliferation of MFP or precursor cells, nor on ingression of mesodermal cells into the MFP. Instead, we suppose that Mdka controls the allocation of MFP cells within a population of midline precursor cells. This is consistent with the function of other known MFP inducing factors, which promote development of MFP and hypochord at the expense of the notochord or vise versa (Halpern et al., 1997; Appel et al., 1999; Amacher et al., 2002). However, while these MFP inducing factors are expressed in the embryonic shield and in the tailbud, *mdka* is excluded from these regions. We instead observed dynamic expression of *mdka* in the paraxial mesoderm and later in the neural tube. *Mdka* expression starts in the rostral paraxial mesoderm and moves as a wave from anterior to posterior. The caudal front of this wave progresses in parallel to the formation of a morphologically distinct MFP. This indicates that during zebrafish neurulation trunk derived factors from outside the tailbud are involved in MFP formation.

To test whether Mdka interacts with early inducing factors during MFP formation, we performed overexpression and knock-down of Mdka in different mutant lines. In most mutants, Mdka was able to rescue defects in MFP formation. This indicated that Mdka acts either downstream or independent of these factors. Expression analyses of *mdka* in these mutants, however, have shown that *mdka* is not regulated by these factors on a transcriptional level. Most notably, in all mutant lines *mdka* was not ectopically expressed in the tailbud region, where midline precursor cells are located. Therefore, we conclude that Mdka acts independent of MFP inducing factors during neurulation.

Based on these data, we have suggested a two-step model for MFP formation in zebrafish (Schäfer et al., 2005b). In the first phase during gastrulation, MFP cells are induced in a pool of midline precursor cells by shield-derived factors. After gastrulation, these midline precursor cells persist in the tailbud and continuously differentiate into MFP, notochord and hypochord. We propose a second phase of MFP formation during these later stages, in which Mdka secreted from the paraxial mesoderm controls the allocation of a subset of midline precursor cells into MFP.

Our functional analyses of Mdka support the second proposed model of an origin of MFP cells from a population of midline precursor cells. However, we show that during neurulation, signals from outside the shield or tailbud are involved in MFP formation. This is therefore in

line with the first model, which proposes a specification of MFP by trunk-derived signals. However, while in higher vertebrates signals from the notochord induce MFP formation, in zebrafish *mdka* is dynamically expressed in the paraxial mesoderm. An involvement of the paraxial mesoderm in MFP formation has also been shown in higher vertebrates by BMP antagonists, like e.g. Follistatin in chicken (Liem et al., 2000). However, this activity appears rather indirect via the functional interaction of BMP and Shh and is not involved in formation of the notochord and hypochord. Future analyses of the function of *mdk* genes in higher vertebrates will provide insights into the mechanisms of MFP formation in the different vertebrate species.

5.3. Anaplastic lymphoma kinase (Alk) – a putative receptor of zebrafish Midkine proteins?

Currently, it is not fully understood which signaling cascades are activated by Mdk. Although an activation of the PI3 kinase, MAPK, ERK pathway, as well as Jak-Stat pathway have been observed *in-vitro*, it is not clear how the Mdk signal is transduced into the target cells (reviewed in Muramatsu, 2002). This is due the fact that the Mdk receptor has not been found so far. In recent years, *in-vitro* binding assays have identified putative receptors of Mdk, like e.g. the receptor-type protein tyrosine phosphatase PTPζ (Maeda et al., 1999) or Anaplastic lymphoma kinase (Alk; Stoica et al., 2002). However, it has not been shown so far which of these receptors binds Mdk *in-vivo*.

We have started to investigate whether Alk is the endogenous receptor of Mdka during zebrafish embryogenesis. During this project, we are collaborating with Robert Kelsh and colleagues from the University of Bath, UK. *In-vitro* analyses have earlier shown a variety of Alk activities, which have also been reported for Mdk, like e.g. neurite outgrowth, proliferation and differentiation (Stoica et al., 2002). Consistent with a postulated function of Mdk during tumorigenesis, misexpression and constitutively active oncogenic activity of Alk has been demonstrated in non-Hodgekin lymphomas and glioblastomas (Morris et al., 1997; Powers et al., 2002). During mouse and human embryogenesis, *alk* is strongly expressed in brain and spinal cord and thus a function during neural development has been hypothesized (Iwahara et al., 1997). However, until now an *in-vivo* function of Alk in higher vertebrates has not been shown.

During the Tübingen large-scale mutagenesis screen, Kelsh and colleagues have isolated a zebrafish pigmentation mutant named *shady* (*shd*) (Kelsh et al., 1996). It has recently been shown by PAC-mediated rescue, knock-down phenocopy and positional cloning that *shd*

encodes an Alk ortholog (Lopes et al., in preparation). *Shd* mutants form an allelic series, in which iridophores are reduced to varying extends. Iridophores are silver or gold shining pigment cells with crystalline purine deposits, which together with melanophores (black cells containing melanin) and xantophores (yellow cells containing pteridine pigments) constitute the three major chromatophore types in zebrafish. Pigment cells develop from neural crest cells during embryogenesis in a stepwise manner. The mechanisms underlying this process are poorly understood, especially for iridophore development. Early pigment fate determination of neural crest is controlled by the HMG-domain transcription factor Sox10. It is predicted that the combinatorial activity of Sox10 and other cell fate determinants specifies the fate of different pigment cell lineages (Dutton et al., 2001; Kelsh and Eisen, 2000). Specification of melanophores fate, for example, occurs by Sox10 and Wnt signaling, which activates the transcription factor *mitf in-vivo* (Dorsky et al., 2000; Elworthy et al., 2003). The cofactor of Sox10 during iridophore specification, however, is currently not known.

Kelsh and colleagues have shown that in *shd* mutants very early steps of iridophore development are disturbed (Kelsh et al., 1996; Lopes et al., in preparation). Other pigment cells, as well as neural crest derived cartilage, neurons and glia cells develop normally. Endogenous *alk* is ubiquitously expressed during the first 24h of development, when iridoblast specification occurs. Later, it is restricted to iridophores and is turned off after differentiation at 48hpf. This has indicated a specific role of Alk during early linage specification of iridophores, possibly together with Sox10.

To test whether Alk is an endogenous receptor of Mdka, we analyzed if a *mdka* knock-down can phenocopy the iridophore defects of *shd* mutants. Injection of two different *mdka* morpholino oligonucleotides resulted in a severe reduction of iridophore number (n=67, p<0.0001 versus mock-injected control, two-tailed T-test; Lopes et al., in preparation), similar to the situation observed in strong *shd* alleles (Kelsh et al., 1996). A similar reduction of iridophores was observed when *mdkb* morpholinos were injected (Fig. 13). However, *mdkb* represses neural crest development already at early stages (Winkler and Moon, 2001). *Mdka* and *mdkb* are both strongly expressed in the neural tube during neurulation, when *alk* is supposed to specify iridophore fate in dorsal neural crest cells. These findings suggest that the receptor tyrosine kinase Alk is a possible receptor of both Mdk co-orthologes during iridophore formation.

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Fig. 13: Knock-down of *mdka* and *mdkb* reduces iridophore number. Lateral view of embryos at 4 days post fertilization (dpf). In *mdka* (B) and *mdkb* (C) morpholino injected embryos, iridophores are strongly reduced, when compared to the wild-type control (A).

To find out whether Alk, as receptor for Mdk is also involved in other Mdk mediated functions, we have started to investigate other developmental defects in *shd* mutants. The existence of such defects is indicated by an embryonic lethality of *shd* mutants, which cannot be merely explained by the lack of iridophores. We first analyzed floor plate, notochord and ventral neural tube development in *shd* mutants, because of the prominent function of Mdka during MFP development. However, so far no defects could be observed by in-situ hybridization with different molecular markers for these structures. This indicates that Alk is not the receptor of Mdka during MFP formation. Further characterization of defects in *shd* mutants will show whether Alk functions as Mdka receptor during other processes, like e.g. neurite outgrowth, axonal guidance and development of somites. In addition, biochemical binding studies are required to test whether Mdka and Mdkb directly bind to Alk.

5.4. Regulation and expression of the novel homeobox gene *nkx2.2b* during zebrafish lateral floor plate formation

During early embryogenesis, distinct types of neurons and glia cells develop along the DV axis of the vertebrate neural tube. In the ventral neural tube, this process is mainly controlled by Shh secreted from MFP and notochord, which establishes a regionally restricted expression pattern of homeobox genes. This defines five distinct neuronal progenitor domains in the ventral neural tube and subsequently the differentiation of neuronal subtypes within each domain (Briscoe et al., 2000). *Nkx2.2* is a homeobox gene, which belongs to the class II of Shh activated genes. It requires high concentrations of Shh and thus is expressed in the p3 neuronal domain or LFP, neighboring the MFP (Ericson et al., 1997; Briscoe et al., 2000). It

is speculated that *nkx2.2* acts redundantly with the related *nkx2.9* gene during formation of the p3 domain, but is required for formation of V3 neurons (Briscoe et al., 1999).

In zebrafish, an *nkx2.2* ortholog has been previously characterized, named *nkx2.2a* (*nk2.2*; Barth and Wilson, 1995). Like the ortholog in higher vertebrates, *nkx2.2a* is expressed in the LFP and activated by Shh signaling (Barth and Wilson, 1995). However in contrast to higher vertebrates, *nkx2.2a* is expressed in a rostral to caudal gradient, with only faint expression in the developing spinal cord (Barth and Wilson, 1995; Shimamura et al., 1995). We have isolated a *nkx2.2a* co-ortholog in zebrafish, which we named *nkx2.2b* (Schäfer et al., 2005a). The zebrafish *nkx2.2* co-orthologes are highly divergent and share only 58% identity on the protein level. However, phylogenetic analyses have shown that both co-orthologs clearly belong to the group of vertebrate Nkx2.2 proteins. *Nkx2.2* genes were mapped on linkage group 20 and 17, on which other zebrafish duplicates are located including *bmp2a/b* and *sox11a/b* (Taylor et al., 2003). Furthermore, *nkx2.2* is closely linked to *nkx2.4* forming a co-orthologous segment. These phylogenetic data are consistent with a duplication of *nkx2.2* genes during an ancient fish-specific genome duplication event.

In-situ analyses have shown that *nkx2.2b* expression starts at the end of gastrulation in neuroectodermal cells and anterior mesoderm (Schäfer et al., 2005a). During neurulation, *nkx2.2b* expression extends caudally in the ventral neural tube, following *shh* expression in the MFP. At the 18s stage, *nkx2.2b* is expressed almost continuously in the ventral neural tube, extending from forebrain to tail. In hindbrain and trunk, *nkx2.2b* is expressed in two parallel rows of LFP cells. Thus, *nkx2.2b* is the first marker exclusively expressed in the entire LFP and therefore constitutes an important tool for the analysis of LFP formation.

To get insight into the evolution of duplicated *nkx2.2* genes in zebrafish, we compared expression and regulation of both co-orthologes. At the 18s stage, *nkx2.2a* and *nkx2.2b* have mostly overlapping expression patterns. However, exclusive expression was found in the mid-diencephalon and mid-hindbrain boundary, as well as in pancreatic progenitor cells. Most importantly in the trunk neural tube, *nkx2.2a* is only weakly expressed in a rostral to caudal gradient, while *nkx2.2b* is strongly expressed along the entire AP axis. Overexpression and mutant analysis have shown that the MFP inducing factor Cyclops (Cyc; Nodal-related 2) does not regulate *nkx2.2b* expression. *Nkx2.2a*, in contrast, is strongly reduced in *cyc* mutants (Barth and Wilson, 1995). Similarly, overexpression of Shh resulted in a differential expansion of *nkx2.2b* expression in different brain compartments, while *nkx2.2a* was only expanded in the mid-diencephalon boundary (Barth and Wilson, 1995; Schäfer et al., 2005a).

These data suggest that after gene duplication both *nkx2.2* genes have acquired different modes of transcriptional control resulting in different response to signaling factors.

To get further insight into the regulatory mechanisms of LFP formation, we have analyzed *nkx2.2* expression in different mutants of the Hedgehog (HH) signaling pathway. These were the mutants *detour* (*dtr;* Karlstrom et al., 2003) and *you-too* (*yot;* Karlstrom et al., 1999) with deficiencies in the downstream transcription factors Gli1 and Gli2, *sonic you* (*syu;* Schauerte et al., 1998) deficient for the ligand Shh and *slow muscle omitted* (*smu;* Varga et al., 2001) with a mutation in the signal transducer Smoothend. In all homozygous mutants, *nkx2.2b* expression was completely absent in the trunk LFP. This indicates that Shh is required for *nkx2.2b* expression and confirms that Shh is also required for LFP formation (Odenthal et al., 2000). In the embryonic brain, however, we observed a gradual reduction of *nkx2.2b*, ranging from mild reduction in *gli* mutants to a total loss in *smu* mutants. Thus, in the embryonic brain *nkx2.2b* is regulated in a complex manner, presumably due to the redundant functions of different Gli transcription factors and HH ligands.

Shh, secreted from the notochord and MFP establishes the regionally restricted expression of homeobox genes in the ventral neural tube by activation of class II and repression of class I genes (Briscoe et al., 2000). However, this does not explain how class I gene expression is initially activated. Recently, it has been shown that retinoids synthesized in the paraxial mesoderm are required for class I gene activation (Pierani et al., 1999; Swindell et al., 1999; Novitch et al., 2003). Co-electroporation of neural plate explants with Shh and an exogenous retinoic acid receptor (RAR) expands class I gene expression (Novitch et al., 2003). Due to the cross-repressive interaction of homoebox genes in these explants, class II genes including *nkx2.2* are repressed (Novitch et al., 2003). We have investigated, whether in zebrafish *nkx2.2b* in the LFP is similarly regulated by RA signaling. Incubation of embryos with increasing doses of all-trans RA resulted in a stepwise reduction of *nkx2.2b* expression in the developing brain. Most notably, high concentrations of all-trans RA blocked *nkx2.2b* expression completely, including in the LFP. On the other hand, incubation of embryos in DEAB, which inhibits RA synthesis (Perz-Edwards et al., 2001), led to a dorsal expansion of *nkx2.2b* expression in the LFP of hindbrain and trunk neural tube. Consistent with observations from higher vertebrates, the class I gene *olig2* was severely reduced in the neighboring pMN domain of these embryos. These data indicate that RA signaling is required to restrict *nkx2.2b* expression to the LFP, thus allowing correct formation of the pMN domain. Furthermore, this demonstrates for the first time that also in zebrafish retinoids are involved in DV patterning of the neural tube.

To investigate whether *nkx2.2b* expression is also regulated by motoneuron specific factors, we have overexpressed the LIM homeobox transcription factor *islet1* (*isl1*; Inoue et al., 1994) in zebrafish. This led to a loss of the entire floor plate including *nkx2.2* and a differentiation of ventral neural tube cells into motoneurons. As endogenous *isl1* is expressed earlier than *nkx2.2b,* we speculate that *isl1* prevents *nkx2.2b* expression in already determined motoneurons. Altogether, our analyses have shown that several interacting pathways are involved in the differential control of *nkx2.2b* expression. This indicates a tight regulation of homeobox gene patterning to ensure appropriate development of neurons and glias along the DV axis of the zebrafish ventral neural tube.

5.5. The zebrafish LFP contains two different cells populations that require different Hedgehog and Nkx2.2 activities

In most vertebrate species, floor plate marker genes have been identified that are not restricted to the MFP, but are also expressed in the LFP (Placzek et al., 1991; Placzek et al., 1993; Marti et al., 1995; Odenthal et al., 2000; Charrier et al., 2002). In chicken and mice, the LFP overlaps with the p3 neuronal domain, which is characterized by expression of the class II homeobox genes *nkx2.2* and *nkx2.9* (Briscoe et al., 1999; Briscoe et al., 2000; Charrier et al., 2002). Later, the p3 domain develops V3 interneurons that express *sim1*. As the LFP expresses established floor plate markers, it is not clear whether cells of this domain are non-neuronal floor plate cells or p3 neuronal cells. In zebrafish, LFP cells have been defined by *foxa2/HNF3ß* and *fkd4* expression, which extend more dorsally than *shh* and *netrin-1* expression in the MFP (Odenthal et al., 2000). So far, a p3 neuronal domain has not been described in zebrafish. However, GABAergic Kolmer-Agdhur neurons that project ipsilaterally into the ventral fasciculus are located in the LFP (Bernhardt et al., 1992). This indicates that also the zebrafish LFP consists of neuronal cells.

We have analyzed the expression pattern of different floor plate markers to get insight into the organization of the LFP in zebrafish (Schäfer et al., submitted). These analyses led to the identification of two different cell populations in the LFP that alternate along the AP axis. One population expresses high levels of the floor plate marker *foxa2,* while the other expresses the homeobox gene *tal2*. Both populations simultaneously express *nkx2.2* genes. Proliferation analysis has shown that during neurulation *tal2* expressing cells are postmitotic, while *foxa2* expressing cells still proliferate. This indicates that at this stage *tal2* expressing cells start to differentiate. At 48hpf, *tal2* positive cells coexpresses the V3 neuronal marker *sim1*. A similar pattern was found for GABA positive Kolmer-Agdhur neurons, indicating

that *tal2* positive cells are neuronal cells. LFP cells with high levels of *foxa2*, in contrast, express no neuronal markers. This shows that the LFP in zebrafish consists of non-neuronal floor plate cells and neuronal cells that develop into V3 and most likely Kolmer-agdhur neurons (Fig. 14). Thus in zebrafish, floor plate cells and p3 neuronal cells are strictly separated and alternate along the AP axis.

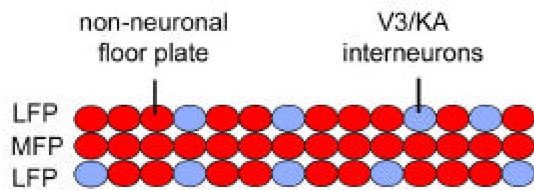

Fig. 14: The zebrafish LFP consists of non-neuronal floor plate cells (red color) and V3/KA (Kolmer-agdhur) neurons (blue color), which alternate along the AP axis.

In higher vertebrates it has been shown that neuronal progenitor cells are formed according to distinct threshold concentrations of Shh activity along the DV axis of the neural tube (Briscoe et al., 2000). Cells of the p3 domain or LFP require high levels of Shh activities and consequently are formed directly adjacent to the MFP. We have analyzed in zebrafish how the two distinct cell populations of the LFP, which are located at the same level of the DV axis, are formed by Shh signaling (Schäfer et al., submitted). For this, we analyzed formation of the two LFP populations in the Hedgehog (HH) signaling mutants *dtr* (*detour; gli1*) and *yot* (*you-too; gli2*). In homozygous mutants of both lines, where HH signaling is completely absent, the LFP is not formed along the entire AP axis. In heterozygous mutants, on the other hand, where the level of HH signaling is reduced, we found that only neuronal LFP cells are formed, while non-neuronal LFP cells are absent. This observation could be confirmed by incubation of embryos in varying doses of the alkaloid cyclopamine, which inhibits activity of the HH signal transducer Smoothend (Chen et al., 2002). Altogether, these experiments have shown that non-neuronal LFP cells require higher levels of HH activities than the neuronal LFP cells. This shows for the first time that HH dependent specification of neural tube cells occurs not only along the DV axis, but also in an alternating fashion along the AP axis. Future experiments will show, whether HH activities differ along the AP axis of the LFP or whether a so far unknown factor prespecifies the two LFP populations to respond differently to a given HH concentration.

The homeobox gene *nkx2.2* plays an important role during specification of the ventral neural tube of higher vertebrates. We have analyzed the function of the *nkx2.2a* and *nkx2.2b* genes in zebrafish, which are expressed in both LFP subpopulations (Schäfer et al., submitted; Schäfer et al., 2005a). Morpholino knock-down of *nkx2.2* genes resulted in a reduction of non-neuronal cells, while neuronal cells were formed normally. The efficiency of morpholino

knock-down was much higher for *nkx2.2a* than for *nkx2.2b*. This suggests a complex functional redundancy of these homoebox genes during patterning of the ventral neural tube. The specific function of the two *nkx2.2* genes only on non-neuronal LFP cells opens the possibility that *nkx2.2* genes act redundantly, for example with *nkx2.9* or *tal2* during specification of neuronal LFP cells. Functional characterization of these factors will show in the future, how these neuronal LFP cells are formed. Furthermore, it has to be analyzed whether *nkx2.2* genes are involved in specification of V3 neurons, like in higher vertebrates.

6. Conclusions

The floor plate is the most ventrally located structure in the developing spinal cord, which specifies neurons and glia cells along the DV axis and guides the trajectory of outgrowing axons. During my PhD thesis, I have obtained novel insights into the structure and formation of two domains of the floor plate, the medial (MFP) and the lateral floor plate (LFP). The origin and time of MFP specification is currently explained by two controversial models. One model suggests an early induction of MFP cells, within a pool of midline precursor cells in the organizer. The other model postulates a late induction of MFP in the trunk neural plate by signals of the underlying notochord. In zebrafish, the mechanisms and factors for MFP induction during gastrulation are well characterized. However, it is unclear how MFP forms during later stages. I have characterized the function of the secreted growth factor Mdka during formation of the MFP in zebrafish. These analyses have shown that during neurulation Mdka controls the allocation of MFP cells from a pool of initially induced midline precursor cells. However, *mdka* is not expressed in the organizer but dynamically in the trunk paraxial mesoderm. This, therefore, indicates a novel mechanism of MFP formation during zebrafish neurulation, which requires midline precursor cells and trunk-derived signals and thus combines two major aspects of both controversial models.

In higher vertebrates, it has been shown that LFP cells are located within the p3 neuronal domain, which develops V3 neurons. It is currently not clear how these different cell populations are organized during early embryogenesis. I have shown that in zebrafish the one cell wide LFP consists of floor plate cells and p3 neuronal cells, which develop V3/Kolmer-Agdhur neurons. The two different cell populations are clearly separated and alternate along the AP axis. This provides novel insights into the structure of the LFP in vertebrates. In the ventral neural tube of vertebrates, neurons and glia cells are specified by a ventral to dorsal activity gradient of Sonic hedgehog (Shh). I have shown that the two different cell populations of the LFP, which are positioned at the same DV level, but alternate along the AP axis require different levels of Hedgehog (HH) activities. Moreover, I have shown that the homeobox genes *nkx2.2a* and *nkx2.2b,* which are both expressed in the LFP are required for specification of only the non-neuronal LFP cells. This shows for the first time that directly adjacent LFP cells that alternate along the AP axis respond differently to HH signaling and activities of *nkx2.2* homeobox transcription factors. This indicates a novel mechanism of neuron specification in the ventral neural tube of vertebrates.

7. References

Albert, S., Muller, F., Fischer, N., Biellmann, D., Neumann, C., Blader, P. and Strahle, U. (2003). Cyclops-independent floor plate differentiation in zebrafish embryos. *Dev Dyn* **226**, 59-66.

Amacher, S. L., Draper, B. W., Summers, B. R. and Kimmel, C. B. (2002). The zebrafish T-box genes no tail and spadetail are required for development of trunk and tail mesoderm and medial floor plate. *Development* **129**, 3311-23.

Amet, L. E., Lauri, S. E., Hienola, A., Croll, S. D., Lu, Y., Levorse, J. M., Prabhakaran, B., Taira, T., Rauvala, H. and Vogt, T. F. (2001). Enhanced hippocampal long-term potentiation in mice lacking heparin-binding growth-associated molecule. *Mol Cell Neurosci* **17**, 1014-24.

Amores, A., Force, A., Yan, Y. L., Joly, L., Amemiya, C., Fritz, A., Ho, R. K., Langeland, J., Prince, V., Wang, Y. L. et al. (1998). Zebrafish hox clusters and vertebrate genome evolution. *Science* **282**, 1711-4.

Appel, B., Fritz, A., Westerfield, M., Grunwald, D. J., Eisen, J. S. and Riley, B. B. (1999). Delta-mediated specification of midline cell fates in zebrafish embryos. *Curr Biol* **9**, 247-56.

Bainter, J. J., Boos, A. and Kroll, K. L. (2001). Neural induction takes a transcriptional twist. *Dev Dyn* **222**, 315-27.

Barth, K. A. and Wilson, S. W. (1995). Expression of zebrafish nk2.2 is influenced by sonic hedgehog/vertebrate hedgehog-1 and demarcates a zone of neuronal differentiation in the embryonic forebrain. *Development* **121**, 1755-68.

Bernhardt, R. R., Patel, C. K., Wilson, S. W. and Kuwada, J. Y. (1992). Axonal trajectories and distribution of GABAergic spinal neurons in wildtype and mutant zebrafish lacking floor plate cells. *J Comp Neurol* **326**, 263-72.

Briscoe, J. and Ericson, J. (2001). Specification of neuronal fates in the ventral neural tube. *Curr Opin Neurobiol* **11**, 43-9.

Briscoe, J., Pierani, A., Jessell, T. M. and Ericson, J. (2000). A homeodomain protein code specifies progenitor cell identity and neuronal fate in the ventral neural tube. *Cell* **101**, 435-45.

Briscoe, J., Sussel, L., Serup, P., Hartigan-O'Connor, D., Jessell, T. M., Rubenstein, J. L. and Ericson, J. (1999). Homeobox gene Nkx2.2 and specification of neuronal identity by graded Sonic hedgehog signalling. *Nature* **398**, 622-7.

Catala, M., Teillet, M. A., De Robertis, E. M. and Le Douarin, M. L. (1996). A spinal cord fate map in the avian embryo: while regressing, Hensen's node lays down the notochord and floor plate thus joining the spinal cord lateral walls. *Development* **122**, 2599-610.

Chamoun, Z., Mann, R. K., Nellen, D., von Kessler, D. P., Bellotto, M., Beachy, P. A. and Basler, K. (2001). Skinny hedgehog, an acyltransferase required for palmitoylation and activity of the hedgehog signal. *Science* **293**, 2080-4.

Chang, C. and Hemmati-Brivanlou, A. (1998). Cell fate determination in embryonic ectoderm. *J Neurobiol* **36**, 128-51.

Charrier, J. B., Lapointe, F., Le Douarin, N. M. and Teillet, M. A. (2002). Dual origin of the floor plate in the avian embryo. *Development* **129**, 4785-96.

Charron, F., Stein, E., Jeong, J., McMahon, A. P. and Tessier-Lavigne, M. (2003). The morphogen sonic hedgehog is an axonal chemoattractant that collaborates with netrin-1 in midline axon guidance. *Cell* **113**, 11-23.

Chen, J. K., Taipale, J., Cooper, M. K. and Beachy, P. A. (2002). Inhibition of Hedgehog signaling by direct binding of cyclopamine to Smoothened. *Genes Dev* **16**, 2743-8.

Chiang, C., Litingtung, Y., Lee, E., Young, K. E., Corden, J. L., Westphal, H. and Beachy, P. A. (1996). Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function. *Nature* **383**, 407-13.

Chizhikov, V. V. and Millen, K. J. (2005). Roof plate-dependent patterning of the vertebrate dorsal central nervous system. *Dev Biol* **277**, 287-95.

Dai, L., Xu, D., Yao, X., Lu, Y. and Xu, Z. (2005). Conformational determinants of the intracellular localization of midkine. *Biochem Biophys Res Commun* **330**, 310-7.

Diez del Corral, R., Olivera-Martinez, I., Goriely, A., Gale, E., Made n, M. and Storey, K. (2003). Opposing FGF and retinoid pathways control ventral neural pattern, neuronal differentiation, and segmentation during body axis extension. *Neuron* **40**, 65-79.

Dorsky, R. I., Raible, D. W. and Moon, R. T. (2000). Direct regulation of nacre, a zebrafish MITF homolog required for pigment cell formation, by the Wnt pathway. *Genes Dev* **14**, 158-62.

Driever, W., Solnica-Krezel, L., Schier, A. F., Neuhauss, S. C., Malicki, J., Stemple, D. L., Stainier, D. Y., Zwartkruis, F., Abdelilah, S., Rangini, Z. et al. (1996). A genetic screen for mutations affecting embryogenesis in zebrafish. *Development* **123**, 37-46.

Dutton, K. A., Pauliny, A., Lopes, S. S., Elworthy, S., Carney, T. J., Rauch, J., Geisler, R., Haffter, P. and Kelsh, R. N. (2001). Zebrafish colourless encodes sox10 and specifies non-ectomesenchymal neural crest fates. *Development* **128**, 4113-25.

Elworthy, S., Lister, J. A., Carney, T. J., Raible, D. W. and Kelsh, R. N. (2003). Transcriptional regulation of mitfa accounts for the sox10 requirement in zebrafish melanophore development. *Development* **130**, 2809-18.

Ericson, J., Rashbass, P., Schedl, A., Brenner-Morton, S., Kawakami, A., van Heyningen, V., Jessell, T. M. and Briscoe, J. (1997). Pax6 controls progenitor cell identity and neuronal fate in response to graded Shh signaling. *Cell* **90**, 169-80.

Fan, Q. W., Muramatsu, T. and Kadomatsu, K. (2000). Distinct expression of midkine and pleiotrophin in the spinal cord and placental tissues during early mouse development. *Dev Growth Differ* **42**, 113-9.

Faure, S., de Santa Barbara, P., Roberts, D. J. and Whitman, M. (2002). Endogenous patterns of BMP signaling during early chick development. *Dev Biol* **244**, 44-65.

Furutani-Seiki, M. and Wittbrodt, J. (2004). Medaka and zebrafish, an evolutionary twin study. *Mech Dev* **121**, 629-37.

Gilbert, S. F. (2000). Developmental Biology. Sunderland: Sinauer Associates, Inc.

Haffter, P., Granato, M., Brand, M., Mullins, M. C., Hammerschmidt, M., Kane, D. A., Odenthal, J., van Eeden, F. J., Jiang, Y. J., Heisenberg, C. P. et al. (1996). The identification of genes with unique and essential functions in the development of the zebrafish, Danio rerio. *Development* **123**, 1-36.

Haffter, P. and Nusslein-Volhard, C. (1996). Large scale genetics in a small vertebrate, the zebrafish. *Int J Dev Biol* **40**, 221-7.

Halpern, M. E., Hatta, K., Amacher, S. L., Talbot, W. S., Yan, Y. L., Thisse, B., Thisse, C., Postlethwait, J. H. and Kimmel, C. B. (1997). Genetic interactions in zebrafish midline development. *Dev Biol* **187**, 154-70.

Hoegg, S., Brinkmann, H., Taylor, J. S. and Meyer, A. (2004). Phylogenetic timing of the fish-specific genome duplication correlates with the diversification of teleost fish. *J Mol Evol* **59**, 190-203.

Hongo, I., Kengaku, M. and Okamoto, H. (1999). FGF signaling and the anterior neural induction in Xenopus. *Dev Biol* **216**, 561-81.

Inoue, A., Takahashi, M., Hatta, K., Hotta, Y. and Okamoto, H. (1994). Developmental regulation of islet-1 mRNA expression during neuronal differentiation in embryonic zebrafish. *Dev Dyn* **199**, 1-11.

Iwahara, T., Fujimoto, J., Wen, D., Cupples, R., Bucay, N., Arakawa, T., Mori, S., Ratzkin, B. and Yamamoto, T. (1997). Molecular characterization of ALK, a receptor tyrosine kinase expressed specifically in the nervous system. *Oncogene* **14**, 439-49.

Jacob, J. and Briscoe, J. (2003). Gli proteins and the control of spinal-cord patterning. *EMBO Rep* **4**, 761-5.

Jeong, J. and McMahon, A. P. (2005). Growth and pattern of the mammalian neural tube are governed by partially overlapping feedback activities of the hedgehog antagonists patched 1 and Hhip1. *Development* **132**, 143-54.

Jeong, Y. and Epstein, D. J. (2003). Distinct regulators of Shh transcription in the floor plate and notochord indicate separate origins for these tissues in the mouse node. *Development* **130**, 3891-902.

Kadomatsu, K., Huang, R. P., Suganuma, T., Murata, F. and Muramatsu, T. (1990). A retinoic acid responsive gene MK found in the teratocarcinoma system is expressed in spatially and temporally controlled manner during mouse embryogenesis. *J Cell Biol* **110**, 607-16.

Kadomatsu, K. and Muramatsu, T. (2004). Midkine and pleiotrophin in neural development and cancer. *Cancer Lett* **204**, 127-43.

Kandel, E. R., Schwarz, J. H. and Jessell, T. M. (2000). Principles of Neural Science: McGraw-Hill.

Karlstrom, R. O., Talbot, W. S. and Schier, A. F. (1999). Comparative synteny cloning of zebrafish you-too: mutations in the Hedgehog target gli2 affect ventral forebrain patterning. *Genes Dev* **13**, 388-93.

Karlstrom, R. O., Tyurina, O. V., Kawakami, A., Nishioka, N., Talbot, W. S., Sasaki, H. and Schier, A. F. (2003). Genetic analysis of zebrafish gli1 and gli2 reveals divergent requirements for gli genes in vertebrate development. *Development* **130**, 1549-64.

Kelsh, R. N., Brand, M., Jiang, Y. J., Heisenberg, C. P., Lin, S., Haffter, P., Odenthal, J., Mullins, M. C., van Eeden, F. J., Furutani-Seiki, M. et al. (1996). Zebrafish pigmentation mutations and the processes of neural crest development. *Development* **123**, 369-89.

Kelsh, R. N. and Eisen, J. S. (2000). The zebrafish colourless gene regulates development of non-ectomesenchymal neural crest derivatives. *Development* **127**, 515-25.

Kennedy, T. E., Serafini, T., de la Torre, J. R. and Tessier-Lavigne, M. (1994). Netrins are diffusible chemotropic factors for commissural axons in the embryonic spinal cord. *Cell* **78**, 425-35.

Key, B. and Devine, C. A. (2003). Zebrafish as an experimental model: strategies for developmental and molecular neurobiology studies. *Methods Cell Sci* **25**, 1-6.

Lamb, T. M. and Harland, R. M. (1995). Fibroblast growth factor is a direct neural inducer, which combined with noggin generates anterior-posterior neural pattern. *Development* **121**, 3627-36.

Launay, C., Fromentoux, V., Shi, D. L. and Boucaut, J. C. (1996). A truncated FGF receptor blocks neural induction by endogenous Xenopus inducers. *Development* **122**, 869-80.

Le Douarin, N. M. and Halpern, M. E. (2000). Discussion point. Origin and specification of the neural tube floor plate: insights from the chick and zebrafish. *Curr Opin Neurobiol* **10**, 23-30.

Le Douarin, N. M., Teillet, M. A. and Catala, M. (1998). Neurulation in amniote vertebrates: a novel view deduced from the use of quail-chick chimeras. *Int J Dev Biol* **42**, 909-16.

Liem, K. F., Jr., Jessell, T. M. and Briscoe, J. (2000). Regulation of the neural patterning activity of sonic hedgehog by secreted BMP inhibitors expressed by notochord and somites. *Development* **127**, 4855-66.

Lopes, S. S., Müller, J., Carney, T., McAdow, R. A., Rauch, J., Schäfer, M., Jacoby, A. S., Hurst, L. D., Haffter, P., Winkler, C. et al. Endogenous role for Anaplastic Lymphoma Kinase signalling in neural crest development. *submitted.*

Maeda, N., Ichihara-Tanaka, K., Kimura, T., Kadomatsu, K., Muramatsu, T. and Noda, M. (1999). A receptor-like protein-tyrosine phosphatase PTPzeta/RPTPbeta binds a heparin-binding growth factor midkine. Involvement of arginine 78 of midkine in the high affinity binding to PTPzeta. *J Biol Chem* **274**, 12474-9.

Maeda, N., Nishiwaki, T., Shintani, T., Hamanaka, H. and Noda, M. (1996). 6B4 proteoglycan/phosphacan, an extracellular variant of receptor-like protein-tyrosine phosphatase zeta/RPTPbeta, binds pleiotrophin/heparin-binding growth-associated molecule (HB-GAM). *J Biol Chem* **271**, 21446-52.

Marti, E. and Bovolenta, P. (2002). Sonic hedgehog in CNS development: one signal, multiple outputs. *Trends Neurosci* **25**, 89-96.

Marti, E., Takada, R., Bumcrot, D. A., Sasaki, H. and McMahon, A. P. (1995). Distribution of Sonic hedgehog peptides in the developing chick and mouse embryo. *Development* **121**, 2537-47.

Megason, S. G. and McMahon, A. P. (2002). A mitogen gradient of dorsal midline Wnts organizes growth in the CNS. *Development* **129**, 2087-98.

Meyer, A. and Schartl, M. (1999). Gene and genome duplications in vertebrates: the one-to-four (-to-eight in fish) rule and the evolution of novel gene functions. *Curr Opin Cell Biol* **11**, 699-704.

Meyer, N. P. and Roelink, H. (2003). The amino-terminal region of Gli3 antagonizes the Shh response and acts in dorsoventral fate specification in the developing spinal cord. *Dev Biol* **257**, 343-55.

Mitsiadis, T. A., Salmivirta, M., Muramatsu, T., Muramatsu, H., Rauvala, H., Lehtonen, E., Jalkanen, M. and Thesleff, I. (1995). Expression of the heparin-binding cytokines, midkine (MK) and HB-GAM (pleiotrophin) is associated with epithelial-mesenchymal interactions during fetal development and organogenesis. *Development* **121**, 37-51.

Moreau, M. and Leclerc, C. (2004). The choice between epidermal and neural fate: a matter of calcium. *Int J Dev Biol* **48**, 75-84.

Morris, S. W., Naeve, C., Mathew, P., James, P. L., Kirstein, M. N., Cui, X. and Witte, D. P. (1997). ALK, the chromosome 2 gene locus altered by the t(2;5) in non-Hodgkin's lymphoma, encodes a novel neural receptor tyrosine kinase that is highly related to leukocyte tyrosine kinase (LTK). *Oncogene* **14**, 2175-88.

Munoz-Sanjuan, I. and Brivanlou, A. H. (2002). Neural induction, the default model and embryonic stem cells. *Nat Rev Neurosci* **3**, 271-80.

Muramatsu, H., Zou, K., Sakaguchi, N., Ikematsu, S., Sakuma, S. and Muramatsu, T. (2000). LDL receptor-related protein as a component of the midkine receptor. *Biochem Biophys Res Commun* **270**, 936-41.

Muramatsu, T. (2002). Midkine and pleiotrophin: two related proteins involved in development, survival, inflammation and tumorigenesis. *J Biochem (Tokyo)* **132**, 359-71.

Nakamura, E., Kadomatsu, K., Yuasa, S., Muramatsu, H., Mamiya, T., Nabeshima, T., Fan, Q. W., Ishiguro, K., Igakura, T., Matsubara, S. et al. (1998). Disruption of the midkine gene (Mdk) resulted in altered expression of a calcium binding protein in the hippocampus of infant mice and their abnormal behaviour. *Genes Cells* **3**, 811-22.

Nasevicius, A. and Ekker, S. C. (2000). Effective targeted gene 'knockdown' in zebrafish. *Nat Genet* **26**, 216-20.

Novitch, B. G., Wichterle, H., Jessell, T. M. and Sockanathan, S. (2003). A requirement for retinoic acid-mediated transcriptional activation in ventral neural patterning and motor neuron specification. *Neuron* **40**, 81-95.

Odenthal, J., van Eeden, F. J., Haffter, P., Ingham, P. W. and Nusslein-Volhard, C. (2000). Two distinct cell populations in the floor plate of the zebrafish are induced by different pathways. *Dev Biol* **219**, 350-63.

Owada, K., Sanjo, N., Kobayashi, T., Mizusawa, H., Muramatsu, H., Muramatsu, T. and Michikawa, M. (1999). Midkine inhibits caspase-dependent apoptosis via the activation of mitogen-activated protein kinase and phosphatidylinositol 3-kinase in cultured neurons. *J Neurochem* **73**, 2084-92.

Patten, I., Kulesa, P., Shen, M. M., Fraser, S. and Placzek, M. (2003). Distinct modes of floor plate induction in the chick embryo. *Development* **130**, 4809-21.

Pera, E. M., Ikeda, A., Eivers, E. and De Robertis, E. M. (2003). Integration of IGF, FGF, and anti-BMP signals via Smad1 phosphorylation in neural induction. *Genes Dev* **17**, 3023-8.

Perz-Edwards, A., Hardison, N. L. and Linney, E. (2001). Retinoic acid-mediated gene expression in transgenic reporter zebrafish. *Dev Biol* **229**, 89-101.

Pierani, A., Brenner-Morton, S., Chiang, C. and Jessell, T. M. (1999). A sonic hedgehog-independent, retinoid-activated pathway of neurogenesis in the ventral spinal cord. *Cell* **97**, 903-15.

Pierani, A., Moran-Rivard, L., Sunshine, M. J., Littman, D. R., Goulding, M. and Jessell, T. M. (2001). Control of interneuron fate in the developing spinal cord by the progenitor homeodomain protein Dbx1. *Neuron* **29**, 367-84.

Placzek, M. and Briscoe, J. (2005). The floor plate: multiple cells, multiple signals. *Nat Rev Neurosci* **6**, 230-40.

Placzek, M., Dodd, J. and Jessell, T. M. (2000). Discussion point. The case for floor plate induction by the notochord. *Curr Opin Neurobiol* **10**, 15-22.

Placzek, M., Jessell, T. M. and Dodd, J. (1993). Induction of floor plate differentiation by contact-dependent, homeogenetic signals. *Development* **117**, 205-18.

Placzek, M., Tessier-Lavigne, M., Yamada, T., Jessell, T. and Dodd, J. (1990). Mesodermal control of neural cell identity: floor plate induction by the notochord. *Science* **250**, 985-8.

Placzek, M., Yamada, T., Tessier-Lavigne, M., Jessell, T. and Dodd, J. (1991). Control of dorsoventral pattern in vertebrate neural development: induction and polarizing properties of the floor plate. *Development* **Suppl 2**, 105-22.

Porter, J. A., Young, K. E. and Beachy, P. A. (1996). Cholesterol modification of hedgehog signaling proteins in animal development. *Science* **274**, 255-9.

Postlethwait, J. H., Woods, I. G., Ngo-Hazelett, P., Yan, Y. L., Kelly, P. D., Chu, F., Huang, H., Hill-Force, A. and Talbot, W. S. (2000). Zebrafish comparative genomics and the origins of vertebrate chromosomes. *Genome Res* **10**, 1890-902.

Powers, C., Aigner, A., Stoica, G. E., McDonnell, K. and Wellstein, A. (2002). Pleiotrophin signaling through anaplastic lymphoma kinase is rate-limiting for glioblastoma growth. *J Biol Chem* **277**, 14153-8.

Qi, M., Ikematsu, S., Maeda, N., Ichihara-Tanaka, K., Sakuma, S., Noda, M., Muramatsu, T. and Kadomatsu, K. (2001). Haptotactic migration induced by midkine. Involvement of protein-tyrosine phosphatase zeta. Mitogen-activated protein kinase, and phosphatidylinositol 3-kinase. *J Biol Chem* **276**, 15868-75.

Ratovitski, E. A., Kotzbauer, P. T., Milbrandt, J., Lowenstein, C. J. and Burrow, C. R. (1998). Midkine induces tumor cell proliferation and binds to a high affinity signaling receptor associated with JAK tyrosine kinases. *J Biol Chem* **273**, 3654-60.

Robertson, C. P., Braun, M. M. and Roelink, H. (2004). Sonic hedgehog patterning in chick neural plate is antagonized by a Wnt3-like signal. *Dev Dyn* **229**, 510-9.

Robinson-Rechavi, M., Marchand, O., Escriva, H., Bardet, P. L., Zelus, D., Hughes, S. and Laudet, V. (2001a). Euteleost fish genomes are characterized by expansion of gene families. *Genome Res* **11**, 781-8.

Robinson-Rechavi, M., Marchand, O., Escriva, H. and Laudet, V. (2001b). An ancestral whole-genome duplication may not have been responsible for the abundance of duplicated fish genes. *Curr Biol* **11**, R458-9.

Rowitch, D. H. (2004). Glial specification in the vertebrate neural tube. *Nat Rev Neurosci* **5**, 409-19.

Sampath, K., Rubinstein, A. L., Cheng, A. M., Liang, J. O., Fekany, K., Solnica-Krezel, L., Korzh, V., Halpern, M. E. and Wright, C. V. (1998). Induction of the zebrafish ventral brain and floorplate requires cyclops/nodal signalling. *Nature* **395**, 185-9.

Sander, M., Paydar, S., Ericson, J., Briscoe, J., Berber, E., German, M., Jessell, T. M. and Rubenstein, J. L. (2000). Ventral neural patterning by Nkx homeobox genes: Nkx6.1 controls somatic motor neuron and ventral interneuron fates. *Genes Dev* **14**, 2134-9.

Sasai, Y., Lu, B., Piccolo, S. and De Robertis, E. M. (1996). Endoderm induction by the organizer-secreted factors chordin and noggin in Xenopus animal caps. *Embo J* **15**, 4547-55.

Schäfer, M., Kinzel, D., Neuner, C., Schartl, M., Volff, J. N. and Winkler, C. (2005a). Hedgehog and retinoid signalling confines nkx2.2b expression to the lateral floor plate of the zebrafish trunk. *Mech Dev* **122**, 43-56.

Schäfer, M., Kinzel, D. and Winkler, C. The lateral floor plate in zebrafish is composed of distinct cell populations that require different Hedgehog and Nkx2.2 activities. *in preparation.*

Schäfer, M., Rembold, M., Wittbrodt, J., Schartl, M. and Winkler, C. (2005b). Medial floor plate formation in zebrafish consists of two phases and requires trunk-derived Midkine-a. *Genes Dev* **19**, 897-902.

Schauerte, H. E., van Eeden, F. J., Fricke, C., Odenthal, J., Strahle, U. and Haffter, P. (1998). Sonic hedgehog is not required for the induction of medial floor plate cells in the zebrafish. *Development* **125**, 2983-93.

Schmidt, C. and Patel, K. (2005). Wnts and the neural crest. *Anat Embryol (Berl).*

Schulte-Merker, S., Ho, R. K., Herrmann, B. G. and Nusslein-Volhard, C. (1992). The protein product of the zebrafish homologue of the mouse T gene is expressed in nuclei of the germ ring and the notochord of the early embryo. *Development* **116**, 1021-32.

Shibata, Y., Muramatsu, T., Hirai, M., Inui, T., Kimura, T., Saito, H., McCormick, L. M., Bu, G. and Kadomatsu, K. (2002). Nuclear targeting by the growth factor midkine. *Mol Cell Biol* **22**, 6788-96.

Shih, J. and Fraser, S. E. (1995). Distribution of tissue progenitors within the shield region of the zebrafish gastrula. *Development* **121**, 2755-65.

Shimamura, K., Hartigan, D. J., Martinez, S., Puelles, L. and Rubenstein, J. L. (1995). Longitudinal organization of the anterior neural plate and neural tube. *Development* **121**, 3923-33.

Souttou, B., Ahmad, S., Riegel, A. T. and Wellstein, A. (1997). Signal transduction pathways involved in the mitogenic activity of pleiotrophin. Implication of mitogen-activated protein kinase and phosphoinositide 3-kinase pathways. *J Biol Chem* **272**, 19588-93.

Stamataki, D., Ulloa, F., Tsoni, S. V., Mynett, A. and Briscoe, J. (2005). A gradient of Gli activity mediates graded Sonic Hedgehog signaling in the neural tube. *Genes Dev* **19**, 626-41.

Stein, E. and Tessier-Lavigne, M. (2001). Hierarchical organization of guidance receptors: silencing of netrin attraction by slit through a Robo/DCC receptor complex. *Science* **291**, 1928-38.

Stern, C. D. (2005). Neural induction: old problem, new findings, yet more questions. *Development* **132**, 2007-21.

Stoica, G. E., Kuo, A., Aigner, A., Sunitha, I., Souttou, B., Malerczyk, C., Caughey, D. J., Wen, D., Karavanov, A., Riegel, A. T. et al. (2001). Identification of anaplastic lymphoma kinase as a receptor for the growth factor pleiotrophin. *J Biol Chem* **276**, 16772-9.

Stoica, G. E., Kuo, A., Powers, C., Bowden, E. T., Sale, E. B., Riegel, A. T. and Wellstein, A. (2002). Midkine binds to anaplastic lymphoma kinase (ALK) and acts as a growth factor for different cell types. *J Biol Chem* **277**, 35990-8.

Strähle, U., Lam, C. S., Ertzer, R. and Rastegar, S. (2004). Vertebrate floor-plate specification: variations on common themes. *Trends Genet* **20**, 155-62.

Streit, A. and Stern, C. D. (1999). Establishment and maintenance of the border of the neural plate in the chick: involvement of FGF and BMP activity. *Mech Dev* **82**, 51-66.

Swindell, E. C., Thaller, C., Sockanathan, S., Petkovich, M., Jessell, T. M. and Eichele, G. (1999). Complementary domains of retinoic acid production and degradation in the early chick embryo. *Dev Biol* **216**, 282-96.

Taylor, J. S., Braasch, I., Frickey, T., Meyer, A. and Van de Peer, Y. (2003). Genome duplication, a trait shared by 22000 species of ray-finned fish. *Genome Res* **13**, 382-90.

Taylor, J. S., Van de Peer, Y., Braasch, I. and Meyer, A. (2001). Comparative genomics provides evidence for an ancient genome duplication event in fish. *Philos Trans R Soc Lond B Biol Sci* **356**, 1661-79.

Teillet, M. A., Lapointe, F. and Le Douarin, N. M. (1998). The relationships between notochord and floor plate in vertebrate development revisited. *Proc Natl Acad Sci U S A* **95**, 11733-8.

Tian, J., Yam, C., Balasundaram, G., Wang, H., Gore, A. and Sampath, K. (2003). A temperature-sensitive mutation in the nodal-related gene cyclops reveals that the floor plate is induced during gastrulation in zebrafish. *Development* **130**, 3331-42.

Tomomura, M., Kadomatsu, K., Matsubara, S. and Muramatsu, T. (1990). A retinoic acid-responsive gene, MK, found in the teratocarcinoma system. Heterogeneity of the transcript and the nature of the translation product. *J Biol Chem* **265**, 10765-70.

Van de Peer, Y., Taylor, J. S. and Meyer, A. (2003). Are all fishes ancient polyploids? *J Struct Funct Genomics* **3**, 65-73.

van Straaten, H. W. and Hekking, J. W. (1991). Development of floor plate, neurons and axonal outgrowth pattern in the early spinal cord of the notochord-deficient chick embryo. *Anat Embryol (Berl)* **184**, 55-63.

Varga, Z. M., Amores, A., Lewis, K. E., Yan, Y. L., Postlethwait, J. H., Eisen, J. S. and Westerfield, M. (2001). Zebrafish smoothened functions in ventral neural tube specification and axon tract formation. *Development* **128**, 3497-509.

Vincent, S. D., Dunn, N. R., Hayashi, S., Norris, D. P. and Robertson, E. J. (2003). Cell fate decisions within the mouse organizer are governed by graded Nodal signals. *Genes Dev* **17**, 1646-62.

Volff, J. N. (2005). Genome evolution and biodiversity in teleost fish. *Heredity* **94**, 280-94.

Westerfield, M. (2000). The zebrafish book. A guide for the laboratory use of the zebrafish Danio (Brachydanio) rerio. Eugene: Univ. of Oregon Press.

Wijgerde, M., McMahon, J. A., Rule, M. and McMahon, A. P. (2002). A direct requirement for Hedgehog signaling for normal specification of all ventral progenitor domains in the presumptive mammalian spinal cord. *Genes Dev* **16**, 2849-64.

Wilson, L. and Maden, M. (2005). The mechanisms of dorsoventral patterning in the vertebrate neural tube. *Dev Biol* **282**, 1-13.

Winkler, C. and Moon, R. T. (2001). Zebrafish mdk2, a novel secreted midkine, participates in posterior neurogenesis. *Dev Biol* **229**, 102-18.

Winkler, C., Schäfer, M., Duschl, J., Schartl, M. and Volff, J. N. (2003). Functional divergence of two zebrafish midkine growth factors following fish-specific gene duplication. *Genome Res* **13**, 1067-81.

Wittbrodt, J., Meyer, A. and Schartl, M. (1998). More genes in fish? *Bio-Essays* **20**, 511-515.

Woods, I. G., Kelly, P. D., Chu, F., Ngo-Hazelett, P., Yan, Y. L., Huang, H., Postlethwait, J. H. and Talbot, W. S. (2000). A comparative map of the zebrafish genome. *Genome Res* **10**, 1903-14.

Yamada, T., Placzek, M., Tanaka, H., Dodd, J. and Jessell, T. M. (1991). Control of cell pattern in the developing nervous system: polarizing activity of the floor plate and notochord. *Cell* **64**, 635-47.

Yamaguchi, T. P. (2001). Heads or tails: Wnts and anterior-posterior patterning. *Curr Biol* **11**, R713-24.

Zardoya, R., Abouheif, E. and Meyer, A. (1996). Evolutionary analyses of hedgehog and Hoxd-10 genes in fish species closely related to the zebrafish. *Proc Natl Acad Sci U S A* **93**, 13036-41.

8. Original publications

8.1. Functional Divergence of two Zebrafish Midkine Growth Factors Following Fish-Specific Gene Duplication

8.2. Medial floor plate formation in zebrafish consists of two phases and requires trunk-derived Midkine-a

8.3. Hedgehog and Retinoid signaling confines *nkx2.2b* expression to the lateral floor plate of the zebrafish trunk

8.4. The lateral floor plate in zebrafish is composed of distinct cell populations that require different Hedgehog and Nkx2.2 activities

The following papers are available online:

Winkler, C., <u>Schäfer, M.,</u> Duschl, J., Schartl, M. and Volff, J. N. *(2003). Functional Divergence of Two Zebrafish Midkine Growth Factors Following Fish-Specific Gene Duplication. Genome Res.* ***13****, 1067-81.*

<u>Schäfer, M.,</u> Kinzel, D., Neuner, C., Schartl, M., Volff, J. N. and Winkler, C. *(2005). Hedgehog and retinoid signalling confines nkx2.2b expression to the lateral floor plate of the zebrafish trunk. Mech Dev.* ***122****, 43-56.*

<u>Schäfer, M.,</u> Rembold, M., Wittbrodt, J., Schartl, M. and Winkler, C. *(2005). Medial floor plate formation in zebrafish consists of two phases and requires trunk-derived Midkine-a. Genes Dev.* ***19****, 897–902.*

Due to copy right restrictions this paper draft is only available on request:

<u>Schäfer, M.,</u> Kinzel, D. and Winkler, C. *The lateral floor plate in zebrafish is composed of distinct cell populations that require different Hedgehog and Nkx2.2 activities. In preparation.*

(M.Schäfer)

9. Curriculum vitae (professional)

Name:	**Matthias Schäfer**
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University studies

1997-1999	**Basic study** Julius-Maximilians-University Würzburg Intermediate examination (Vordiplom), sehr gut
1999-2002	**Main study** Julius-Maximilians-University Würzburg Final examination (Diplom), sehr gut
06/2001-03/2002	**Diploma thesis** supervisors: Prof. Dr. Dr. Manfred Schartl, Prof. Dr. Ulrich Scheer group: Dr. Christoph Winkler, Physiological Chemistry I, Julius-Maximilians-University Würzburg Title: 'Patterning of the spinal cord in zebrafish: the role of Midkine1 (Mdk1) during specification of floor plate cells' mark: sehr gut
since 04/2002	**PhD thesis** at the 'International Graduate School - University of Würzburg' supervisors: Prof. Dr. Dr. Manfred Schartl, Prof. Dr. Thomas Brand, Dr. Christoph Winkler group: Dr. Christoph Winkler, Physiological Chemistry I, Julius-Maximilians-University Würzburg Title: 'Mechanisms of floor plate formation and neural patterning in zebrafish'

Teaching activities

since 2000	Instructor at the practical course 'Cell and Developmental Biology' for Biology students (main study)
since 2001	Instructor at the practical course 'Biochemistry' for Medical students (basic study)
07/2002	Student helper at the 'EMBO Course on Molecular and Genetic Tools for the Analysis of Medaka and Zebrafish Development', EMBL Heidelberg
05/2003-06/2003	Advisor F2 internship Doris Kinzel, title of project: 'Characterization of the *nkx2.2b* gene during zebrafish ventral neural tube patterning'
since 2004	Instructor at the practical course 'Model Organisms' for Biomedical students (Bachelor study)
04/2004-12/2004	Advisor Diploma thesis Doris Kinzel, title of thesis: 'Functional analysis of Nkx2.2b – a novel homebox gene expressed in the ventral neural tube in zebrafish'

Research visits

08/2000-10/2000	Prof. David Kimelman, Department of Biochemistry, University of Washington, Seattle, USA Establishment of a maternal loss of function approach in zebrafish by injection of morpholino antisense oligonucleotides and establishment of ET recombination in E. coli with the aim of application in a zebrafish BAC library
07/2002, 09/2002	Dr. Jochen Wittbrodt, EMBL, Heidelberg Confocal microscopy and real time imaging of the neurulating zebrafish embryo
01/2004	Prof. Stephen Wilson, University College London, UK Misexpression and knockdown of Midkine-a in *floating head* and *delta-notch* zebrafish embryos.

Ad hoc Reviewer

2004/2005	Gene (ISSN: 0378-1119)

Awards and Distinctions

7/2002	The 'George Streisinger Prize': Poster prize at the '5 th International Conference on Zebrafish Development and Genetics', Madison, Wisconsin, USA
07/2004	Opening talk/Inauguration of the International Graduate School-University of Würzburg
04/2005	Poster prize at the 'GfE (German Society of Developmental Biology) Meeting Developmental Biology', Münster, Germany
07/2005	Poster prize at the '4th European Zebrafish Genetics and Development Meeting', Dresden, Germany

Fellowships

8/2001-10/2001	Fonds Hochschule International: Travel grant for a 3 month internship in the lab of Prof. Dr. David Kimelman (University of Washington, Seattle, USA)
6/2001	Carl-August-Forster Stiftung: Literature grant
6/2002	GlaxoSmithKline Stiftung: Travel grant to participate at the '5th International Conference on Zebrafish Development and Genetics', Madison, Wisconsin, USA
01/2003-03/2005	Boehringer Ingelheim Fonds: PhD fellowship
since 07/2004	Graduate College, Molecular Basis of Organ Development in Vertebrates' (GK 1048): Associated member

Würzburg, 08 August 2005

10. Lebenslauf (beruflicher Werdegang)

Name:	**Matthias Schäfer**
Titel:	Diplom Biologe
Geburtstag:	05. Oktober 1975
Familienstand:	nicht verheiratet, keine Kinder

Studium

1997-1999	**Grundstudium** Julius-Maximilians-Universität Würzburg Vordiplom, sehr gut
1999-2002	**Hauptstudium** Julius-Maximilians-Universität Würzburg Diplom, sehr gut
06/2001-03/2002	**Diplomarbeit** Betreuer: Prof. Dr. Dr. Manfred Schartl, Prof. Dr. Ulrich Scheer Arbeitsgruppe: Dr. Christoph Winkler, Physiologische Chemie I, Julius-Maximilians-Universität Würzburg Titel: 'Patterning of the spinal cord in zebrafish: the role of Midkine1 (Mdk1) during specification of floor plate cells' Note: sehr gut
seit 04/2002	**Doktorarbeit** An der 'International Graduate School - University of Würzburg' Betreuer: Prof. Dr. Dr. Manfred Schartl, Prof. Dr. Thomas Brand, Dr. Christoph Winkler Arbeitsgruppe: Dr. Christoph Winkler, Physiologische Chemie I, Julius-Maximilians-Universität Würzburg Titel: 'Mechanisms of floor plate formation and neural patterning in zebrafish'

Lehre

seit 2000	Assistent Fortgeschrittenen (F1) Praktikum 'Zell- und Entwicklungsbiologie' Hauptstudium Biologie
seit 2001	Assistent Praktikum 'Biochemie' Grundstudium Medizin
07/2002	Assistent beim 'EMBO Course on Molecular and Genetic Tools for the Analysis of Medaka and Zebrafish Development', EMBL, Heidelberg
05/2003-06/2003	Betreuung F2 Laborpraktikum Doris Kinzel, Titel des Projektes: 'Charakterisierung des *nkx2.2r* Gens während der Musterbildung im ventralen Neuralrohr des Zebrafisch'
seit 2004	Assistent Praktikum 'Model Modelorganismen' Bachelor-Studium Biomedizin
04/2004-12/2004	Betreuer Diplomarbeit Doris Kinzel, Titel der Arbeit: 'Die Funktion des *nkx2.2b* Homeobox-Gens für die Zelldifferenzierung im ventralen Neuralrohr im Zebrafisch'

Forschungs-aufenthalte	
08/2000-10/2000	Prof. David Kimelman, Department of Biochemistry, University of Washington, Seattle, USA Establishment of a maternal loss of function approach in zebrafish by injection of morpholino antisense oligonucleotides and establishment of ET recombination in E. coli with the aim of application in a zebrafish BAC library
07/2002, 09/2002	Dr. Jochen Wittbrodt, EMBL, Heidelberg Confocal microscopy and real time imaging of the neurulating zebrafish embryo
01/2004	Prof. Stephen Wilson, University College London, UK Misexpression and knock-down of Midkine-a in *floating head* and *delta-notch* zebrafish embryos.
Ad hoc Gutachter	
2004/2005	Gene (ISSN: 0378-1119)
Preise und Auszeichnungen	
7/2002	’George Streisinger Prize’: Posterpreis beim ‘5 th International Conference on Zebrafish Development and Genetics’, Madison, Wisconsin, USA
07/2004	Vortrag bei der Eröffnung der ‘International Graduate School-University of Würzburg’
04/2005	Posterpreis bei der ‘Jahrestagung der GfE (Deutsche Gesellschaft für Entwicklungsbiologie)’, Münster
07/2005	Posterpreis beim ‘4th European Zebrafish Genetics and Development Meeting’, Dresden
Stipendien	
8/2001-10/2001	Fonds Hochschule International: Reisestipendium für einen dreimonatigen Forschungsaufenthalt im Labor Prof. Dr. David Kimelman (University of Washington, Seattle, USA)
6/2001	Carl-August-Forster Stiftung: Büchergeld
6/2002	GlaxoSmithKline Stiftung: Reisestipendium für die Teilnahme an der ‘5th International Conference on Zebrafish Development and Genetics’, Madison, Wisconsin, USA
01/2003-03/2005	Boehringer Ingelheim Fonds: Doktoranden-Stipendium
seit 07/2004	Graduiertenkolleg ‘Molecular Basis of Organ Development in Vertebrates’ (GK 1048): assoziiertes Mitglied

Würzburg, den 08. August 2005

11. Appendix

11.1. Erklärung

Ich erkläre hiermit ehrenwörtlich, dass ich die vorliegende Dissertation ‚Molecular mechanisms of floor plate formation and neural patterning in zebrafish' selbständig angefertigt und dabei keine andere als die von mir angegebenen Quellen und Hilfsmittel benutzt habe.

Ich erkläre außerdem, dass die vorliegende Dissertation weder in gleicher, noch in ähnlicher Form bereits in einem anderen Prüfungsverfahren vorgelegen hat.

Ich habe früher außer den mit dem Zulassungsantrag urkundlich vorgelegten Graden keine weiteren akademischen Grade erworben oder zu erwerben versucht.

Würzburg, den 08. August 2005

Matthias Schäfer

11.2. Danksagung

An dieser Stelle möchte ich mich bei allen bedanken, die mich bei der Durchführung und Fertigstellung dieser Arbeit unterstützt haben. Mein besonderer Dank gilt:

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- Den Mitgliedern der Arbeitsgruppe Winkler und des Lehrstuhls für Physiologische Chemie I für die freundliche Arbeitsatmosphäre und Unterstützung, sowie Frau Dr. Andrea Wizenmann für zahlreiche Diskussionen und Ideen.

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