Analysis of the arachidonyl-CoA synthetase ACSL4a as a potential regulator of BMP expression and of the role of BMPs in timing of cell commitment along the dorsoventral axis of the gastrulating zebrafish embryo

I n a u g u r a l - D i s s e r t a t i o n

zur

Erlangung des Doktorgrades

der Mathematisch-Naturwissenschaftlichen Fakultät

der Universität zu Köln

vorgelegt von

Björn Renisch

aus Bad Soden a. Ts.

Köln, 2009

Berichterstatter:	Prof. Dr. Matthias Hammerschmidt
Berichterstatter:	Prof. Dr. Siegfried Roth
Tag der mündlichen Prüfung:	27. November 2009

1. Summary

Signaling by Bone Morphogenetic proteins (BMPs) plays a pivotal role during early dorsoventral (D-V) patterning of fish and frog embryos, determining both differential cell fates as well as the direction of cell movements along the D-V axis during gastrulation. Several forward genetics screens with ENU-mutagenised zebrafish have unravelled the indispensable function of several components of the BMP signaling pathway, including the BMP-ligands themselves, BMP receptors, the BMP-regulated transcription factor Smad5 and the extracellular BMP inhibitor Chordin. The BMP signaling pathway interacts with that of other signaling molecules, like Fibroblast Growth Factors or Wnts. However, there are still new genes to be discovered involved in early patterning processes; also the exact role of the BMP gradient in cell fate determination along the D-V axis of the gastrulating zebrafish embryo is not absolutely clarified.

As starting point of my thesis work I participated in a reverse genetics screen based on antisense morpholino oligonucleotides for specific gene knock-downs in zebrafish. As part of a consortium of several labs, I designed and screened a collection of morpholinos for phenotypes in early patterning, pituitary development and skin development. A morpholino targeting the gene *acyl-CoA Synthetase longchain family member 4a* (*acsl4a*), whose product is involved in the metabolism of the longchain fatty acid arachidonic acid, caused defects in D-V patterning.

In the first part of this thesis I characterise the phenotype of embryos after *acsl4a* knock-down, pointing to an essential role of Acsl4a in maintaining the BMP signaling gradient during gastrulation. However, the phenotype could only be obtained by one of several tested morpholinos. Also, I failed to rescue the defects by concomitant forced expression of *acsl4a* that is not targeted by the morpholino. Furthermore, I tested other components of the arachidonic acid metabolism, none of which interfered with early patterning of the zebrafish embryo. In conclusion, the role of *acsl4a*, and its connection to BMP signaling during D-V patterning of the zebrafish embryo remains elusive.

In the second part of the thesis I address the question of cell fate commitment, thus the timing of cell determination, along the D-V axis and its dependence on BMP signaling. According to the morphogen concept, the BMP gradient with from dorsal-to-ventral progressively increasing BMP signaling determines differential cell fates along the D-V axis. In the ectoderm, high BMP levels are thought to induce epidermal fates, while blocking neural development, so that ventral cells will give rise to skin, whereas neurons are formed from the

dorsal ectoderm, where BMP levels are lowest. In other instances, BMPs have been described as factors that maintain the pluripotency of stem cells, thus acting as a principle inhibitor of cell specification processes. Interestingly, the BMP gradient is highly dynamic over time, with a rather broad expression early, which becomes progressively restricted to the ventral side of the embryo during the course of gastrulation. Therefore, I wondered whether BMPs might keep the ventral ectoderm in a more pluripotent state, while dorsal cells become specified and committed to their final fate significantly earlier, consistent with the earlier loss of BMP expression. To test this notion, I carried out differential cell commitment studies by heterotopic and heterochronic transplantations of dorsal versus ventral ectodermal cells of the zebrafish embryo during different stages of gastrulation. Surprisingly, neither ventral nor dorsal cells were found to be committed to their initial fates, indicated by the loss of their respective marker genes expression upon transplantation into the ectopic environment. However, only ventral cells were able to adopt the fate of their new environment (dorsal), whereas dorsal cells in an ectopic ventral environment lacked both dorsal and ventral marker gene expression. This indicates that ventral ectodermal cells do indeed maintain their pluripotency longer than dorsal cells. Further experiments with mutant embryos have to show whether this effect requires BMP signaling.

1.2 Zusammenfassung

Signale durch Bone Morphogenetic Proteins (BMPs) spielen im Zebrafish und Frosch Embryo eine entscheidende Rolle bei der Musterbildung entlang der dorsoventralen Achse, dabei bestimmen sie sowohl unterschiedliche Zellschicksale als auch die gerichtete Zellbewegung entlang der D/V-Achse während der Gastrulation.

Mehrere soegannte „forward genetic" Screens mit ENU-mutagenisierten Zebrafischen haben die unentbehrliche Funktion von mehreren Komponenten des BMP-Signal-Wegs aufgezeigt, darunter die BMP-Liganden selbst, die Rezeptoren von BMPs, der durch BMPs regulierte Transkriptionsfaktor Smad5 und der extrazelluläre BMP-Inhibitor Chordin. Der BMP-Signalweg interagiert mit denen anderer Signalwege, wie Fibroblast Growth Factors oder Wnts. Jedoch gibt es immer noch neue Gene zu entdecken, die in Prozessen der frühen Musterbildung beteiligt sind; weiterhin ist die genaue Rolle des BMP Gradienten in der Festlegung von Zellschicksalen entlang der D-V Achse eines gastrulierenden Zebrafisch Embryos nicht vollkommen geklärt.

Als Ausgangspunkt meiner Doktorarbeit habe ich an einem rückwärts gerichteten genetischen Screen, basierend auf Antisense Morpholino Oligonukleotiden für spezifischen Knock-Down von Genen im Zebrafisch, teilgenommen. Als Teil eines Konsortiums mehrerer Arbeitsgruppen habe ich eine Kollektion von Morpholinos erstellt und auf Phänotypen in der Frühentwicklung, Entwicklung der Hypophyse und der Haut untersucht. Ein Morpholino, gerichtet gegen das Gen *acyl-CoA Synthetase longchain family member 4a* (*acsl4a*), dessen Produkt im Metabolismus der langkettigen Fettsäure Arachidonsäure beteiligt ist, verursachte Defekte in der D-V Musterbildung.

Im ersten Teil dieser Arbeit beschreibe ich den Phänotyp von Embryonen nach dem Knock-down von *acsl4a*, der auf eine essentielle Rolle von Acsl4a in der Aufrechterhaltung des Gradienten des BMP Signals während der Gastrulation hindeutet. Jedoch konnte der Phänotyp nur mit einem von mehreren untersuchten Morpholinos erreicht werden. Ebenfalls ist es mir missglückt die Defekte durch gleichzeitige Überexpression von *acsl4a,* welches nicht von dem Morpholino erkannt wird, zu retten. Ausserdem habe ich weitere Komponenten des Archidonsäure Metabolismus untersucht, jedoch keine der getesteten Komponenten interferierte mit der Frühentwicklung des Zebrafisch Embryos. Zusammengefasst bleibt die Rolle von *acsl4a* und seine Verbindung zum BMP-Signalweg während der D-V Musterbildung des Zebrafisch Embryos ungeklärt.

Im zweiten Teil dieser Arbeit befasse ich mit der Frage der Festlegung von Zellschicksalen, genauer der zeitlichen Folge der zellulären Bestimmung, entlang der D-V Achse und ihrer Abhängigkeit von BMP Signalen. Entsprechen dem Konzept von Morphogenen bestimmt der stufenweise von dorsal nach ventral ansteigende BMP Gradient die verschiedenen Zellschicksale entlang der D-V Achse. Im Ektoderm wird angenommen, dass ein hoher Grad von BMP Signal epidermale Schicksale induziert, bei gleichzeitigem blockieren der neuralen Entwicklung, so dass ventrale Zellen zu Haut werden, während Neuronen sich aus dem dorsalen Ektoderm, wo der Grad der BMP Signale am niedrigsten ist, entstehen. In anderen Zusammenhängen wurden BMPs als Faktoren beschrieben die die Pluripotenz von Stammzellen aufrechterhalten, dabei arbeiten sie als prinzipieller Inhibitor von Zellspezifikationsprozessen. Interessanterweise ist der Gradient von BMPs sehr dynamisch über die Zeit, mit einer sehr breiten Expression zu einem frühen Zeitpunkt, die während der Gastrulation schrittweise auf die ventrale Seite des Embryos beschränkt wird. Deswegen habe ich mich gefragt, ob BMPs das ventrale Ektoderm in einem mehr pluripotenten Zustand halten, während dorsale Zellen zu einem wesentlich früheren Zeitpunkt spezifiziert und auf ihr endgültiges Zellschicksal festgelegt werden, passend zu dem früheren Verlust der Expression von BMPs. Um diesen Gedanken zu überprüfen habe ich Studien zur zellulären Festlegung mit heterotopischen und heterochronischen Transplantationen von dorsalen gegen ventralen ektodermalen Zellen des Zebrafisch Embryos während verschiedener Zeitpunkte der Gastrulation durchgeführt. Überraschenderweise, konnte ich weder für ventrale noch dorsale Zellen zeigen, dass sie auf ihr ursprüngliches Schicksal festgelegt sind, da sie die Expression ihrer entsprechenden Markergene nach der Transplantation in eine ektopische Umgebung verlieren. Jedoch, nur ventrale Zellen waren fähig das Schicksal ihrer neuen Umgebung (dorsal) anzunehmen, wobei dorsalen Zellen in einer ektopischen ventralen Umgebung sowohl die Expression dorsaler als auch ventraler Markergene fehlt. Dies zeigt, dass ventrale ektodermale Zellen tatsächlich ihre Pluripotenz länger als dorsale Zellen behalten. Weitere Experimente mit mutanten Embryonen haben zu zeigen ob dieser Effekt Signale durch BMPs benötigt.

2. General Introduction

Higher animals develop from a fertilised egg to a three-dimensional, highly structured organismn consisting of multiple different cell types and tissues which - in the case of bilateria – are organized along at least two main body axes: the anterior-posterior (A-P) and the dorsal-ventral (D-V) axis. After fertilisation of the egg by a sperm cell, the first developmental process called cleavage is initiated, during which the zygote divides by several rounds of mitosis and (total or partial) cytokinesis into the so-called blastomeres. During these rapid cell divisions, the cell cycle just consists of an M- (mitosis) and an S- (DNA synthesis) phase, development is driven by maternally provided gene products, and cells become progressively smaller. However, once a certain ratio of DNA and cytoplasm is reached, G1 and G2 phases are established, cell divisions slow down, and zygotic transcription starts (mid-blastula-transition). After the blastula stages, the process of gastrulation starts, during which the three germlayers, ectoderm, mesoderm and endoderm, are formed. The ectoderm will later give rise to the epidermis, the nervous system and the neural crest, the mesoderm will form notochord, muscle, parts of the body skeleton, the blood, as well as vasculature and excretion tissues, and the endoderm will form the gastrointestinal system. In addition, the gastrulating embryo changes its overall shape and acquires a much more complex architecture, driven by the so-called morphogenetic cell movements during which mesoderm and endoderm become internalized. In addition, the body axes are set up and the general body plan is acquired. Gastrulation is followed by neurulation, during which the neural tube of the central nervous system is formed, and by organogenesis, during which the organs of the animal develop (Gilbert, 2003).

All of these developmental processes are under the control of a complex system of signaling pathways and transcription factors. These factors act in a spatially and temporally strictly regulated and coordinated fashion. While some of them have redundant roles, others are absolutely indispensable, so that a single loss of their function would cause major changes in the embryonic body plan.

2.1. Early zebrafish development

In 1981, George Steisinger introduced the zebrafish (*Danio rerio*) as a genetic model system for vertebrate development (Streisinger et al., 1981). The large number of progeny, the transparency of embryos and larvae, and the extracorporal development makes the zebrafish

highly suitable for large scale forward genetics screens and embryological methods like cell transplantations, treatments with chemical compounds and microinjection to overexpress and knock-down specific genes (reverse genetics). In the following, I will give an introduction into the early patterning processes during gastrulation of zebrafish, focussing on the establishment of the dorsal-ventral axis.

The anteriorposterior axis of the zebrafish embryo already become apparent shortly after fertilization, when the cytoplasm accumulates on one side of the zygote (the animal pole) and the yolk granules on the other (the vegetal pole). This animal-vegetal axis corresponds to the later anterior-posterior axis, with the head forming at the animal pole and the tailbud at the vegetal pole. The first cleavage of the zebrafish zygote occurs around 45 minutes after fertilisation, followed by meroblastic cleavages every 15 minutes until the 128-cell-stage. Between two hours and five hours post fertilisation (hpf), the blastula stages, the embryo, also called blastoderm, sits like a cap on the huge yolk cell, which does not cleave. At the 512-cell-stage (2.75 hpf), the embryo enters mid-blastula-transition (MBT; see above) and the first two cell lineages segregate from each other, the deep cells, which will form the proper embryo, and the Yolk Syncitial Layer (YSL), which does not contribute to the later animal. During cleavage, the cytoplasm of the most marginal blastomere has remained in contact with the yolk cell and the YSL is formed when these blastomeres fuse to the yolk cell, thereby releasing their nuclei into the cytoplasma of the yolk cell, where they form a syncytial layer (Kimmel and Law, 1985b). Shortly after the MBT a third cell lineage is formed, the enveloping layer (EVL). It derives from superficial blastomeres that flatten out to form an embryonic skin, also called the periderm (Kimmel and Law, 1985c). It is currently unclear whether the periderm is maintained throughout the entire life of the fish, or eventuall shed off and replaced by derivatives of basal keratinocytes from the ventral ectoderm (see below). Gastrulation starts at approximately 4.5 hpf with the morphogenetic movement of epiboly, which leads to a progressive covering of the yolk cell by the blastoderm (Kimmel et al., 1995; Kimmel and Law, 1985a). Accordingly, gastrula stages are named in percentage of yolk coverage. The 30% epiboly stage is reached shortly after the onset of gastrulation, the 50% epiboly stage at 5.5 hpf, the 70% epiboly stage (mid-gastrulation) and the 100% epiboly stage (end of gastrulation) at 10 hpf. In addition to epiboly, two other main morphogenetic movements of gastrulation can be distinguished: emboly (by some authors also called involution) and convergence and extension (C&E), both of which start at the 50% epiboly stage (Warga et al., 1990; Solnica-Krezel, 2005). During emboly, the mesoderm and the endoderm, which are induced in marginal cells facing the yolk cell and by signals emanating

from the YSL (Schier and Talbot, 2005), are brought into the interior of the embryo, whereas cells of the ectoderm remains outside. During convergence (Myers et al., 2002), lateral cells of both the ectoderm and the mesendoderm move towards the dorsal side to form the body axis. The dorsal side thickens accordingly. This thickening becomes first apparent at 6 hpf as a structure called the embryonic shield, which marks the dorsal side and which for the first time allows to morphologically recognize the dorsovental axis. Within this shield, the dorsal organizer, also called the Spemann-Mangold organizer, is located (see below). Driven by extension movements, the dorsal tissue further extends along the animal-vegetal / anterior-posterior axis of the embryo to form the body axis.

2.2 The establishment of the dorsal-ventral axis in zebrafish

Fate mapping experiments have shown that the future fate of an embryonic cell depends on its position within the late blastula and early blastula embryo (Kimmel et al., 1990). As outlined above, marginal cells facing the yolk will give rise to mesodermal and endodermal derivatives, while animal cells form to ectoderm. Of particular importance is also the position along the dorsoventral axis. Within the mesoderm, dorsal-most cells will form prechordal plate and notochord, dorsolateral cells somatic muscle, ventrolateral cells blood and ventral-most cells kidney and anus. Similarly, within the ectoderm, dorsal cells give rise to the neuroectoderm, intermediate cells to neural crest and placodes, and ventral-most cells to the epidermis. Initially organized within one plane along the dorsoventral circumference of the embryo, all of these cells types will move dorsally, so that eventually, the neuroectoderm will also be internalized and covered by skin. Over the past ten to fifteen years, enourmous progress has been made in eludidating the genetic and molecular network regulating dorso-ventral axis formation during early zebrafish development (Schier and Talbot, 2005). Two major steps can be distinguished; first, the formation of the Spemann-Mangold-organizer as the initial step of dorsoventral pattern formation (Nojima et al., 2004; Pelegri and Maischein, 1998), and second, the function of the organizer to further pattern the dorsoventral axis (Hammerschmidt and Mullins, 2002). Similar to the effects obtained by Mangold and Spemann with amphibian embryos, transplantation of the shield of zebrafish embryos into ventral regions leads to secondary body axis formation by an inducing ectopic muscle and neural development in adjacent tissue, while the organizer itself gives rise to prechordal plate, notochord and some fore- and midbrain tissues (Saúde et al., 2000). On the molecular level, organizer formation can be first recognized by the presence of ß-catenin protein in the nuclei

of the dorsal marginal cells and the YSL (Schneider et al., 1996). ß-catenin is an intracellular transducer of canonical Wnt (named because of *D.melanogaster* Wingless and vertebrate Integrated) signaling. In the absence of Wnt signaling, ß-catenin is phosphorylated by a complex of APC, Axin and the kinase GSK3, and thereby targeted for proteasomal degradation. However, upon binding of Wnt ligands to their Frizzled transmembrane receptors and the co-receptor LRP, this destruction complex is inactivated by Dishevelled and GBP, and ß-catenin can accumulate in the cytoplasm and translocate into the nucleus, where it participates as a partner of LEF/TCF transcription factors in transcriptional regulation (Logan and Nusse, 2004). While the nature of the Wnt ligand remains elusive, zebrafish mutants in ß-catenin-2 display compromised organizer formation and severe hyper-ventralization (Bellipanni et al., 2006). Among the first genes activated by ß-catenin is *bozozok*, a homeobox containing gene, which acts as repressor of ventralising genes like *bmp2b* , encoding a Bone morpogentic protein (BMP; see below) and *vox/vent*, encoding transcription factors mediating BMP signaling (Fekany et al., 1999; Imai et al., 2001; Leung et al., 2003; Shimizu et al., 2002). Other targets of ß-catenin are Nodal-like factors expressed in the YSL, mainly involved in the induction of the mesendoderm, but also required for the formation of the dorsal organizer (Dougan et al., 2003; Feldman et al., 1998). A second wave of ß-catenin dependent gene activation results in the expression of Fibroblast Growth Factors (FGFs) (Maegawa et al., 2006).

Fibroblast Growth Factors are involved in mesoderm formation, neural induction, dorsoventral and anterior-posterior patterning. FGFs signal via receptor-tyrosine kinases and the Ras/ MAPK pathway. During late blastula, *fgf3*, *fgf8* and *fgf24* expression is initiated in dorsal marginal cells, the precursors of the Spemann-Mangold organizer, where they promote expression of the BMP inhibitor Chordin (see also below), thereby restricting BMP activity and regulating dorsoventral patterning (Maegawa et al., 2006; Draper et al., 2003; Furthauer et al., 1999; Furthauer et al., 1997; Kiefer et al., 1996). Later in gastrulation, FGFs induce posterior neuroectodermal cell fates, but independent of the BMP signaling or organizer function (Rentzsch et al., 2004).

Another gene activated by ß-catenin and Nodal signals is *dickkopf* (*dkk*), constituting a negative feedback and temporal restriction of active Wnt signaling in the organizer domain. *dkk1* is expressed at the dorsal margin and later in the prechordal plate. Dkk protein binds the LRP subunit of the Wnt-receptor, thereby preventing the action of canonical Wnt signals and promoting the specification of anterior neural fates and axial mesendoderm, probably by blocking Wnt signals (Hashimoto et al., 2000; Shinya et al., 2000). Thus, while being

essential for early steps, canonical Wnt signaling needs to be actively blocked to allow later steps of dorsal development during mid- and late gastrulation. At the same time, active Wnt signaling, mainly via the ligands Wnt8 and Wnt3a, becomes indispensable for the specification of ventral and posterior cell fates (Erter et al., 2001; Lekven et al., 2001; Shimizu et al., 2005), This switch form an early pro-dorsal to a later pro-ventral and anti-dorsal effect, while still quite puzzling, has also been observed for canonicalWnt signaling during amphibian development (Moon et al., 1997). Thus, it seems to be a more common phenomenon, which might point to a primary role of Wnt signaling in the timing of cell specification, rather than or in addition to cell fate determination per se (see also below).

2.3. The role of Bone Morphogenetic Proteins during gastrulation

Like the aforementioned Nodals, Bone Morphogenetic Proteins are members of the large family of TGFß signaling molecules. Zygotic expression of *bmp2b* and *bmp7* in zebrafish starts right after mid-blastula transition. However, transcripts encoding Bmp4, Bmp7 and Radar, a homologue of the mammalian TGFß-family member Growth Differentiation Factor 6 (GDF6), are also maternally provided (Goutel et al., 2000; Kramer et al., 2002; Sidi et al., 2003). GDF6 is driving the expression of *bmp2b* and *bmp7*, thereby most likely acting via the BMP type I receptor Alk8 and the BMP-regulated transcription factor Smad5 (see also below) (Kramer et al., 2002; Sidi et al., 2003).. During late blastula and gastrula stages three *bmps* are prominently expressed, *bmp2b*, *bmp7* and *bmp4* , all of which are required for dorsoventral patterning and the establishment of ventrolateral fates (Dick et al., 2000; Kishimoto et al., 1997; Nguyen et al., 1998; Schmid et al., 2000; Stickney et al., 2007). Thus, *bmp2b* and *bmp7* mutants are severely hyper-dorsalised, with an extension of muscle and neuroectodermal precursors into ventral most regions of the embryo, whereas ventral cell fates like epidermal, kidney or blood precursors are completely or largely absent. Compared to *bmp2b* and *bmp7*, however, the role of *bmp4* during dorsoventral patterning is minor, whereas it is more important in specifing left-right asymmetry (Chen et al., 1997; Chocron et al., 2007; Hwang et al., 1997; Martinez-Barbera et al., 1997; Stickney et al., 2007)

The Bmp-ligands act as dimers. During dorsoventral patterning, Bmp2b and Bmp7 most likely work as heterodimer (Schmid et al., 2000; Little and Mullins, 2009). They bind to type a complex of type I and type II transmembrane receptor Ser/Thr kinases. To date five type I receptors are identified in zebrafish, *alk3a and alk3b* (Nikaido et al., 1999; Little and Mullins, 2009), *alk6a and alk6b* (Nikaido et al., 1999b; Little and Mullins, 2009) and *alk8*, which is

most similar to mammalian Alk2 (Bauer et al., 2001; Mintzer et al., 2001). The Bmp2b/7 heterodimers signal through heteromeric type I receptors consisting of Alk8, which is indispensable, and either of the four Alk3/6 molecules, which can replace each other in a redundant manner (Little and Mullins, 2009). Similar to Bmp4, the zebrafish orthologues of the BMP type II receptors have been found to be largely involved mediating BMP signaling during in left-right patterning (Monteiro et al., 2008), whereas during dorsoventral patterning, ActRII, a type II receptor shared with TGFß-like Nodal and Activin factors, seems to be used to mediate BMP signaling (Nagaso et al., 1999). Upon ligand binding the receptor cross-phosphorylate each other by their Ser/Thr kinase domain, followed by the phosphorylation of the receptor-regulated Smad (R-Smad) proteins (named after *C.elegans* Sma (Savage et al., 1996)and *D.melanogaster* Mad (Sekelsky et al., 1995) via the type I receptors. Upon phosphorylation, R-Smad disaggregate from the cytoplasmic part of the BMP receptors and associate with so-called Co-Smad proteins, with which they are translocated into the nucleus where they work as transcriptional regulators (Massague, 1996; Massague and Weis-Garcia, 1996; Raftery and Sutherland, 1999) (Fig.2-1). R-Smads specifically involved in BMP signal transduction are Smad1, Smad5 and Smad8, the most common Co-Smad shared by BMP and Nodal/Activin signaling is Smad4, while for zebrafish dorsoventral patterning, only Smad5 could thus far be shown to play an indispensable role (Hild et al., 1999; Kramer et al., 2002; McReynolds et al., 2007). Several direct or indirect transcriptional target of Bmp/Smad target genes have been described, some of which in turn encode for regulators or components of the BMP signal transduction pathway, thereby constituting positive or negative feedback loops involved in the fine-tuning of BMP signaling. Examples are the *bmp2b/4/7* genes themselves, constituting a positive feedback (Kishimoto et al., 1997), or the the *smad7* gene, which encodes an inhibitory Smad protein, thereby constituting a negative feedback (Pogoda and Meyer, 2002).

In blastula stages *bmp2b* and *bmp7* are broadly expressed throughout the embryo, sparing just the dorsal-most regions of the future Spemann-Mangold organizer, where their expression is repressed by Boz (Fekany et al., 1999; Leung et al., 2003). In addition, BMP inhibitors start to be expressed on the dorsal side, attenuating the positive feedback of BMP signaling on *bmp2b/7* gene expression, and thereby progressively restricting BMP activity and *bmp* expression to the ventral side of the embryo (Hammerschmidt and Mullins, 2002).

The most relevant BMP inhibitor is the protein Chordin, which is secreted from organizer cells and has long-range signaling effects to attenuate BMP signaling in dorsolateral cells of both the ectoderm and the mesenderm (Schulte-Merker et al., 1997; Hammerschmidt et al.,

1996). Accordingly, *chordin* mutants are hyper-ventralised, with a reduction in the number of neuroectodermal and muscle precursor cells, while derivatives of the ventral ectoderm and mesoderm are made in excess. The organizer and adjacent dorsal tissues also make generate other extracellular Bmp inhibitors such as Noggin-1 or Follistatin-like 2. Compared to Chordin, however, they are less important (Bauer et al., 1998; Dal-Pra et al., 2006; Fainsod et al., 1997; Furthauer et al., 1999; Zimmerman et al., 1996). The activity of Chordin itself is also under tight control. Thus, it is cleaved by metalloproteases Tolloid/Bmp1, which are most active on the ventral side of the embryo (Blader et al., 1997; Connors et al., 1999; Muraoka et al., 2006). Tolloid, in turn, is inactived upon binding to Sizzled, a secreted protein promoting Chordin activity, but in contrast to Chordin made on the ventral side in response to Bmp signaling (Muraoka et al., 2006; Yabe et al., 2003). Further secreted and BMP-binding factors like Twisted gastrulation (Little and Mullins, 2004; Ross et al., 2001; Xi and Fisher, 2005) and Crossveinless-2 (Rentzsch et al., 2006; Zhang et al., 2008) can regulate BMP signaling in a context-dependent positive or negative manner.

The BMP activity gradient is important for the establishment of the dorsal-ventral axis, as BMP play important roles in cell fate determination and differentiation. Among the target genes of BMP signaling is *deltaNp63* that is directly activated by BMP/Smad5 signaling in the ventral ectoderm and involved in the development of basal keratinocytes of the epidermis (Lee and Kimelman, 2002; Rentzsch et al., 2003). Similarly, although no direct target genes have been identified via DNA binding and transactivation as yet, BMP signaling is supposed to activate the expression of blood or kidney-specifying genes in the ventral mesoderm. Furthermore, according to the morphogen concept, it should with lower thresholds activate ectodermal genes involved in the specification of neural crest and placodal ectoderm.

In addition to cell fate specification along the dorsoventral axis, the BMP gradient has been shown to govern cell migrations along the dorsoventral axis during dorsal convergence movements, establishing a reverse gradient of cadherin-dependent cell-cell adhesivness (von der Hardt et al., 2007).

An additional aspect of BMP signaling similar to that alluded to above for canoncial Wnt signaling could be concerned with the timing of cell specification, rather than or in addition to determining the exact fate specification cells will require. Such a role would be consistent with the stem cell-maintaining effect of BMP signaling in other developmental contexts.

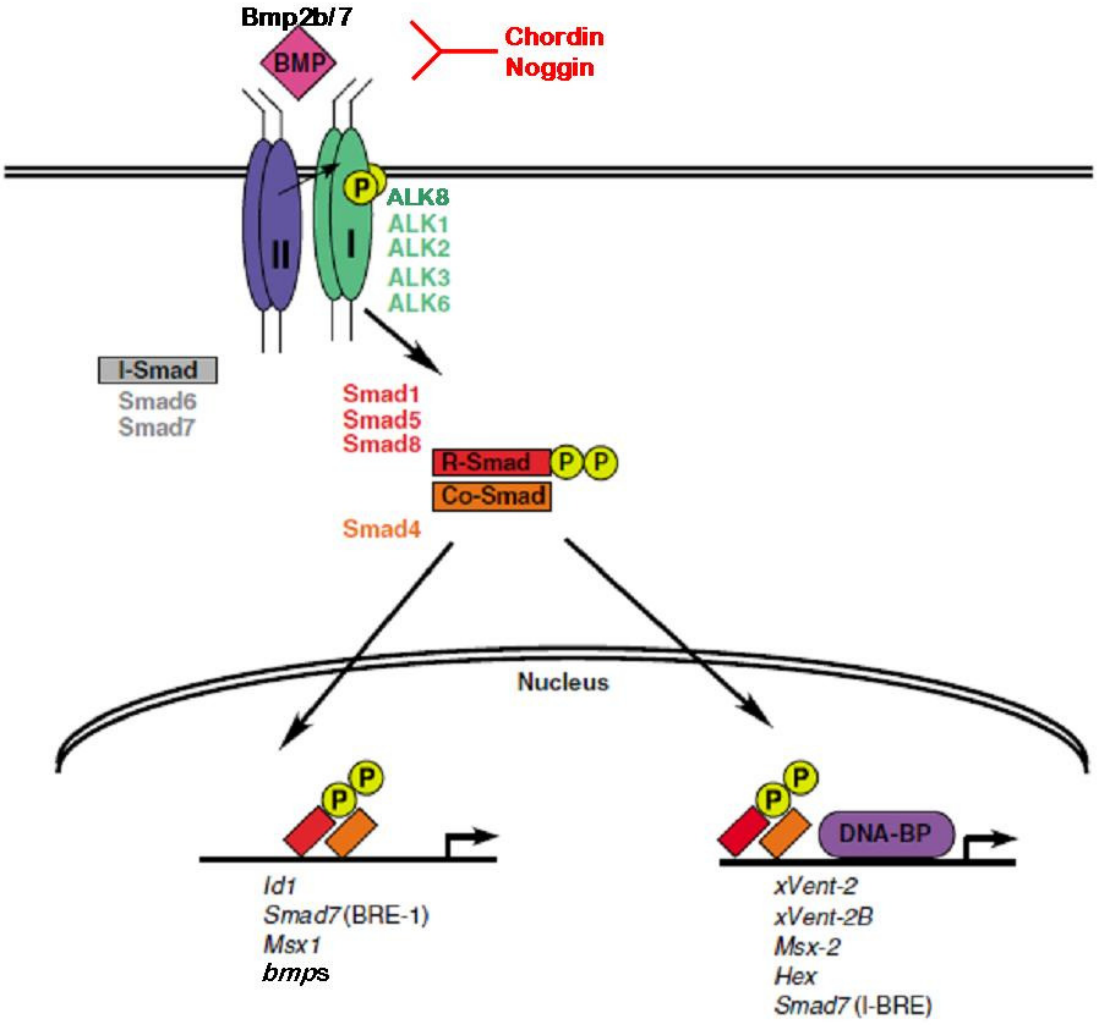


Figure 2-1: The signaling pathway of Bone Morphogenetic Proteins (modified from Varga and Wrana, 2005)
During dorsoventral patterning in zebrafish, the ligands Bmp2b and Bmp7 bind as heterodimer to a heteromeric complex of consisting of the type I receptors Alk8 and Alk3/6, and most likely the type II receptor ActRII. The intracellular signal transducers Smad1, 5, 8 become phosphorylated after receptor activation, associate with Smad4 and transloctate to the nucleus where they participate in transcriptional regulation. Inhibitors of BMP signaling like chordin and noggin bind to the ligands and prevent activation of the receptor. Among the target genes are the regulators and components of the BMP signal transducting pathway, thereby constituting positive and negative feedback loops.

2.4 The role of Bone Morphogenetic Proteins in stem cell biology

Stem cells are undifferentiated cells that have the ability of renewal while remaining capable of differentiating into a specialised cell (Chambers and Smith, 2004). The differentiation potentials can vary, depending on the stem cell type. A fertilized egg is totipotent, and can give rise to any cell type including extraembryonic tissues. Pluripotent stem cell is the term used for embryonic stem cells that can differentiate into any cell type of the embryo proper. Multipotent stem cells are usually stem cells that are restricted to a tissue or cell lineage, for example hematopoietic stem cell, the progenitors of all blood cells. Even more restricted cells are often called oligopotent. And cells that are still self-renewing but only differentiate into one cell type by asynchronous cell division are called unipotent stem cell, for example the satellite cell of adult muscles (Scholer, 2004).

Several intrinsic and extrinsic factors are known to regulate and maintain the fate of a stem cell and to prevent it from differentiating, like the POU-domain transcription factor Oct4 (Nichols et al., 1998a), homeodomain protein Nanog (Chambers et al., 2003; Mitsui et al., 2003), the cytokine Leukemia Inhibitory Factor (LIF) (Matsuda et al., 1999), the prostaglandin PGE2 (North et al., 2007), Wnts (Nusse, 2008; Sato et al., 2004), FGFs (Dvorak et al., 2006) and BMPs (Varga and Wrana, 2005; Ying et al., 2003; Zhang and Li, 2005).

The effects of BMPs on stem cells can vary up to being seemingly contrary, depending on the cell types and the context. Embryonic stem cells of mouse (mES) cultured on a stromal feeder layer and treated with serum differentiate into neurons unless treated with BMPs, which will drive them towards epidermal differentiation (Kawasaki et al., 2000), consistent with the aforementioned positive effects of BMP signaling on the developmental of ventral (skin) versus dorsal (neural) cell types in the ectoderm of the zebrafish embryo. However, when mES cells are incubated in a less complex medium (supplemented with LIF and FGF2), BMP will also exhibit their a neural-blocking effect. However, rather than promoting their epidermal differentation, they will keep the mES in an undifferentiated state, as for instance indicated by the expression of the stem cell-specific marker Oct4 (Munoz-Sanjuan and Brivanlou, 2002; Tropepe et al., 2001; (Ying et al., 2003). This effect is most likely achieved by interfering with LIF function and suppressing the transcription of differentiation genes via blocking MAPK pathways (Qi et al., 2004). Along these lines, it is tempting to speculate that the primary function of BMP signaling might be concerned with blocking neural development, consistent with the neural default model (Stern, 2005), while for epidermal

specification, additional factors are necessary. Similarly, neural specification most likely requires other factors in addition to BMP inhibitors. Thus, Chordin and Noggin fail to induce neural fate in the absence of FGF signaling (Launay et al., 1996; Sasai et al., 1996). In addition, Wnts have been shown to be required for neural induction (Baker et al., 1999). However, it remains a matter of debate to which extent FGFs and Wnts act as direct neural inducers, and to which extent by interfering with and blocking BMP signaling (Furthauer et al., 1997; Furthauer et al., 2004; Rentzsch et al., 2004). FGF signaling for instance can block BMP signal transduction via its downstream effector MAPK, which phosphoralytes Smad1 at a specific site of the linker region., thereby preventing its translocation into the nucleus (Pera et al., 2003). Similarly, Wnts can crosstalk to BMPs via phosphorylation of Smads through GSK3 (Fuentealba et al., 2007). In conclusion, the role of BMP signaling in stem cell biology has to be seen in relation to other signaling pathways.

A specification-blocking effect as observed in mES cells would also be consistent with the reported roles of Bmp2, Bmp4 and Bmp8b and their Drosophila orthologue Decapentaplegic to maintain germ line stem cells in the mammalian testis and the fly ovary, respectively (Xie et al., 1998; (Funk et al., 2002). However, experiments with human embryonic stem cells (hES) indicate an opposite role for BMPs. Here, BMP-2 promotes the differentiation of hES, mainly into extraembryonic endoderm, accompanied by a loss of stem cell marker expression, whereas the BMP inhibitor Noggin blocks this differentiation (Pera et al., 2004).

For dorsovental patterning of the zebrafish embryo, there are different data sets in line with a possible differentiation -blocking and/or stem cell-maintaining role of BMP signaling. One comes form the analysis of mutants in Pou2, the zebrafish orthologue of the stem cell maintenance factor Oct4 (Belting et al., 2001; Reim and Brand, 2002). Mutants lacking both maternal and zygotic *pou2* gene products display loss of *bmp* expression and a hyper-dorsalization similar to that of *bmp* mutants. Futhermore, the dorsalised phenotype of *pou2* mutants can be rescued by re-introduction of *bmp* transcripts (Reim and Brand, 2006), suggesting that Pou2 acts via BMPs, and that the dorsalised phenotype might result from precocious specification of ventral cells. Consistent with this notion, experiments with transgene-driven temporally controlled BMP inhibition revealed that compared to dorsal cell types, deriviates of ventral cells require sustained BMP signaling (Pyati et al., 2005, 2006).

2.5. Aim of the work

The genetic network controlling dorsoventral patterning and axis formation of the vertebrate embryo, and the pivotal role of BMP signaling within this control system, are rather well understood. However, several questions remain open. To identify new genes involved in developmental processes I joined a consortium for a large-scale reverse genetic screen using antisense morpholino oligonucleotides (Pickart et al., 2006). During the screen I found *acyl-CoA synthetase longchain family member 4a* (*acsl4a*) to be required for dorsal-ventral patterning of the zebrafish gastrula. Acsl4a catalyzes the synthesis of a range of arachachidonic acid derivates, such as prostaglandins. Interestingly, the prostaglandin PGE2 has recently been shown to be required for the maintainence of hematopoietic stem cells of zebrafish larvae (North et al., 2007), raising the possibility that it might fulfil a similar function to allow proper ventral development during dorsoventral patterning of the early zebrafish embryo. This would be consistent with the stem cell maintaining roles of Pou2 and BMP signaling described above, and was subject of the first part of my thesis work.

If BMPs and their partners do indeed pattern the dorsoventral axis by attenuating cell specification, ventral cells, which receive high and longer BMP signaling, should become specified later than dorsal cells, in which BMP signaling is weaker and less persistent. In the second part of my thesis, I tested this notion, carrying out cell commitment studies via heterotopic and heterochronic transplantations of ectodermal cells.

3. Material and Methods

3.1. Zebrafish lines and husbandary

Wild-type embryos were obtained from crosses of TL/EK or AB zebrafish. gsc::GFP transgenic embryos were obtained from outcross of heterozygous fish with wildtype strains. Embryos were raised and staged according to Kimmel *et al.* (1995). Dechorionation was performed with Watchmakers forceps, and embryos older than 15 hpf, which were to be manipulated were anaesthetized by addition of 0.2% v/v Tricaine to embryo medium. Dechorionated embryos were kept in petridishes coated with 1% agarose.

3.2. Embryological methods

Microinjection of zebrafish embryos

Morpholino oligonucleotides (MOs) were either purchased from Gene Tools and a stock of 1mM was prepared, or the screen consortium sent aliquots of 25µg/µl stocks.

Synthetic mRNA, MOs, or linage tracers were diluted to working concentrations in Danieau-buffered solution (Nasevicius and Ekker, 2000) containing 10% phenol red (Sigma). A volume of 2 nl was injected into the yolk cell of 1-2 cell stage embryos with a fine glas needle connected to a pressure-driven P420 picopump (World Precision Instruments) and an M3310 manual micromanipulator (Science Products).

For the injection of MOs and dyes into the yolk cell, embryos were dechorionated and injected through a very narrow glass capillary with a M3310 Manual micromanipulator at high stage (3-3,5hpf).

The working concentrations are specified in the text; used RNA constructs and MO sequences are given below.

Cell transplantation experiments

Donor embryos were injected at 1-cell stage with fluorescent lineage tracers like fluorescein dextran (1mg/ml) or rhodamin dextran (1mg/ml). For the heterochronic transplantations the Host embryos were injected with *in vitro* synthesised RNA of *truncated BMP receptor Ia* or constitutive active *alk8*, three hours after the donor embryos were injected. Donor and Host embryos were manually dechorionated and placed into 1% methylcellulose on groove-slides. Depending on the experiments around 10 cells were transplanted with a glass capillary from the dorsal or ventral ectoderm embryo to the ventral or dorsal ectoderm, respectively, of the

host embryos in case of heterotopic transplantation; or into the animal pole of ventralised or dorsalised Host embryos in case of heterochronic transplantations; for a detailed describtion of the experiments performed see chapter 5. After the transplantation procedure the embryos were transferred into a coated petridish.

Treatment of embryos with chemical compounds

The chemical compounds were diluted in DMSO, MeOH or water to prepare stock solutions according to the instruction manuals. The working solutions were prepared just prior to use by the desired dilution of the stock solution in Embryo medium, in case of acidic solution an embryo medium with citrate-buffer was used. Embryos to be treated with agonist and antagonist of the arachidonic acid cascade were dechorionated and transferred into 20ml glass vials, maximum 20 embryos per vial and covered with 1-2ml Embryo medium, so that the embryos are just covered with water. The medium was replaced with working solutions; the control clutch was treated with embryo medium containing the appropriate solvent. Embryos were kept in the dark and slightly shaked during incubation. The working solutions were replaced by a fresh one every two hours until end of the treatment.

***in vitro* transcription of sense RNA (RNA synthesis for micro-injection)**

For *in vitro* transcription of the different sense RNAs 10µg of the respective plasmids were linearized with 2µl restriction enzyme and 10µl Buffermix in a total volume of 100µl for 2h at appropiate temperature; followed by a Phenol/ Chloroform extraction. Linearised plasmids were checked on a 0,8% agarose gel and quantified by photometry.

For *in vitro* transcription the mMessage mMachine Kit was used. 2 µg linearized plasmid DNA was mixed with 2 µL 10x transcription buffer, 2 µL NTP Mix, 2 µL enzyme mix in a 20 µL reaction and incubated at 37°C for 2 hours. 1 µL DNase was added and the mixture was incubated at 37°C for another 20 minutes to remove the template DNA. The reaction was stopped and purified by addition of 15µl ammonium acetate, 115 µL water and 150µl of Phenol/ Chloroform Mixture (Roth), followed by centrifugation at 14000 rpm for 3 minutes. The liquid phase was transferred into a new microreaction tube and mixed with 150µl Isopropanol, incubated for 20 minutes at -20°C and followed by centrifugation at 14000 rpm at 4°C for 15 minutes. The pellet was resuspend in 90µl ddH_2O, 10µl NH_4Ac and 100µl Isopropanol, incubated for 20 minutes at -20°C and followed by centrifugation at 14000 rpm at 4°C for 15 minutes again. The pellet was rinsed in 500 µL 70% ethanol and resuspended in 20 µL ddH_2O. The RNA was checked on a 1% agarose gel and stored at -20°C.

Whole-mount *in situ*-hybridizations

Synthesis of labeled anti-sense RNA (in situ probe synthesis)

Linearisation and purification of Plasmid were done as described for sense RNA synthesis.

For *in vitro* transcription 2 µg linearized plasmid DNA was mixed with 2 µL 10x transcription buffer, 2 µL Dig-RNA labeling Mix or FITC-RNA labeling Mix (Roche), 1 µL RNasin (Promega), 2 µL of the appropiate polymerase (T7, T3, Sp6) in a 20 µL reaction and incubated at 37°C for 2 hours. 2 µL of 10x transcription buffer, 17 µL ddH_2O and 1 µL DNase (RNase free, Roche) were added and the mixture was incubated at 37°C for another 30 minutes to remove the template DNA. Free nucleotides were removed by addition of 20 µL 7.8 M ammonium acetate and 120 µL ethanol and incubation at room temperature for 50 minutes, followed by centrifugation at 14000 rpm for 20 minutes. The pellet was rinsed in 300 µL 70% ethanol and resuspended in 20 µL ddH_2O. 20 µL formamide (Fluka) was added and RNA was stored at -20°C.

Whole-mount in situ hybridization

Embryos for *in situ*-hybridization were fixed in 4% PFA/PBS overnight at 4°C or 4 hours at room temperature, washed in PBST, dechorionated, transferred though a methanol series and stored in 100% methanol at -20°C.

For *in situ*-hybridization embryos were transferred from MeOH to PBST and depending on their developmental stage digested with 1 mg/ml proteinase K (24 hpf: 8 minutes; 36 hpf: 10 minutes; 48 hpf: 15 minutes; 72 hpf: 20 minutes) at room temperature and re-fixed in 4% PFA/PBS for 20 minutes. From PBST embryos were rinsed in Hyb- and incubated in Hyb+ at 70°C for a minimum of 4 hours. RNA-probes were diluted in Hyb+ to working concentration and incubated with embryos at 70°C overnight. On the next day embryos were washed at 70°C with the following buffers: 100% Hyb-, 25% 2xSSCT/ 75% Hyb-, 50% 2xSSCT/ 50% Hyb-, 75% 2xSSCT/25% Hyb-, 2x SSCT for 15 minutes each and 0.2x SSCT for 30 minutes two times. Then embryos were transferred into PBS buffered conditions at room temperature (75% 0.2xSSCT/ 25% PBST; 50% 0.2x SSCT/ 50% PBST; 25% 0.2x SSCT/ 75% PBST; PBST for 10 minutes each). Embryos were blocked against unspecific binding for at least 2 hours in 2% sheep serum/ 2% BSA/ PBST. For probe detection embryos were incubated with alkaline phosphatase-coupled antibody overnight at 4°C. Embryos were intensively washed with PBST, transferred into staining buffer (5 minutes for three times) followed by detection of alkaline phosphatase activity by addition of 1:50 NBT/BCIP (blue) in staining buffer. For fluorescent *in situ*s embryos were transferred into 0,1M Tris-HCl buffer adjusted to pH 8.0 (three times for 5 minutes) followed by addition of 1 tablet/ ml of FastRed and Tris buffer

(Sigma). The color reaction was stopped by rinsing the embryos with PBST and fixing in 4% PFA/PBS overnight. Stained embryos were either transferred though a methanol series into benzylbezoat/benzylalcohol (2:1) for microscopy or transferred into 80% glycerol in PBS.

PBST: PBS (D8662, Sigma), 0.1% Tween20

20xSSC: 175.3 g NaCl, 88.2 g Sodiumcitrate dehydrate ($C_6H_5Na_3O_7$ x $2H_2O$), pH 7.0; adjust to 1 L with distilled water.

Stain: 100 mM Tris (pH 9.5), 50 mM $MgCl_2$, 100 mM NaCl, 0.1% Tween20

Immunohistochemistry/ whole-mount antibody staining

After fixation and reconstitution as described in 7.2.2, embryos were incubated for 1-2 hours at room temperature in antibody blocking solution. This was replaced by primary antibody in blocking solution and incubated overnight at 4°C. The following embryos were washed six times in PBST for 15 minutes, blocked for 1 hour before appropriate secondary antibody in blocking solution was added for 2 hours at room temperature or overnight at 4°C. The secondary antibody was discarded and embryos were washed six times in PBST.

Fluorescently stained specimens were fixed in 4% PFA/PBS and transferred to 60% glycerol/40% PBS.

For DAB staining, the signal was amplified using the Vectastain Elite ABC Kit (Vector Laboratories). This system consists of avidine and biotin-labeled horseradish peroxidase, the two of which form multi-molecule complexes of avidine. The peroxidase is recruited to the secondary antibody by free biotin-binding sites in the avidine molecules. After incubation in AB solution (<24 hpf for 45 minutes; 24 hpf for 1 hour; 2-3 dpf 3-4 hours; >3 dpf for 4 hours) embryos were rinsed and washed six times for 30 minutes in PBST and incubated 30 minutes in DAB solution. The detection reaction was started by addition of 1 µL 0.3% H_2O_2 per 1 ml DAB solution. Staining reactions were stopped by rinsing in PBST, followed by fixation in 4% PFA/PBS. Stained embryos were transferred through a methanol series and stored and analyzed in benzylbenzoat/benzylalcohol (2:1).

Blocking solution: 10% fetal calf serum; 1% DMSO; 0.1% Tween 20; in PBS

DAB solution: 1 DAB tablet (3, 3-Diaminobenzidine tetrahydrochloride, Sigma, D5905) was dissolved in 15 mL PBST and filtered through a 0.4 µm micro filter prior to use.

Microscopy

Stained embryos were analysed in either benzylbenzoat/benzylalcohol (2:1) mixture or in 80%Glycerol/20%PBS; live embryos were mounted in 1% methylcellulose or 1% low melting agarose. Documentation was done using a Zeiss AxioImager with an AxioCam MRc for light microscopy and a Zeiss LSM 510/ 710 for confocal microscopy.

3.3. Molecular methods

Bacterial growth and plasmid preparation

Available bacterial clones were ordered from Imagenes.

Glycerol stocks or agar stabs of bacterial cell stocks were propagated by streaking on LB-agar plates containing 50 µg/mL ampicillin or 25 µg/mL kanamycin, and incubated overnight at 37°C. After inoculation with single colonies, bacterial cultures were grown in LB-medium supplemented with appropriate selective antibiotics in a shaking 37°C-incubator.

Small yield plasmid preparations from 3 mL bactertial cultures (Mini-preparations) were done with the QIAprepSpin Miniprep Kit (Qiagen 27106) as per manufacturer instructions, to obtain high quality DNA for subsequent sequencing and cloning procedures.

Medium yield plasmid preparations from 50 mL cultures (Midi-preparations) were made with the QIAfilter plasmid Midi Kit (Qiagen 12243), for cloning and preparing stocks of plasmid DNA.

Isolation of total RNA from embryos

50 embryos were mixed with 500 µL E3-medium and 1.5 mL Trizol LS Reagent (Gibco, BRL) and homogenized using a seringe. 400 µL chloroform was added after 5 minutes incubation, followed by vortexing and 10 minutes incubation at room temperature. Afterwards the cell debris was spun down at 13000 rpm and the supernatant, containing DNA and RNA, transferred into a fresh tube. For the precipitation 1mL isopropanol was added. After centrifugation at 14000 rpm at 4°C the pellet was washed with 70% ethanol, dried and taken up in RNAse-free ddH_2O.

Genomic DNA was removed by a DNase (Boehringer) treatment, followed by a phenol/chloroform (Roth) extraction. The concentration of the purified RNA was measured at 540nm using Standard photometer.

cDNA synthesis

In a 10.6 µL reaction, 2 µg of total RNA together with 1 µL oligo $(dT)_{15}$ (100pmol, Roche) was denatured at 70°C for 5 minutes then stored on ice. Reverse transcriptase buffer, 2 mM DTT, 10 mM dNTP and 200 U SuperScript II reverse transcriptase (Gibco, BRL) were added to give a final volume of 20 µL. After incubation at 42°C for 1.5 hours 20µl Tricine EDTA buffer (Clontech RACE) was added and the cDNA stored at -80°C.

Restriction digests

Digestion of DNA by restriction enzymes was performed using the conditions recommended by the manual (New England Biollabs).

Electrophoretic separation of DNA

Size fractionation of DNA fragments was performed by electrophoresis on 0.8-2% (w/v) agarose (Roth) gels in 1xTAE containing 100 ng/mL ethidium bromide, submerged in 1xTAE running buffer. Samples were mixed with loading buffer and run at 100-150 V. The DNA was visualized and photographed under UV light (λ=312 nm).

50x TAE (1L): 242 g Tris base, 57.1 mL glacial acetic acid, 100 mL 0.5 M EDTA (pH 8.0)

Purification of DNA fragments

For recovery of DNA from agarose gels, bands of specific sizes were cut from the gel on a transilluminator and purified with the QIAquick SpinColumn Gel Elution as per manufacturer instructions.

DNA concentration was determined by UV spectrophotometry at a wavelength of 260 nm.

Polymerase Chain Reaction (PCR)

PCR reactions were usually carried out at a final volume of 25 µL, containing 1xPCR-buffer, 0.2 mM dNTP (PeqLab), 1 µM forward and reverse primers (MWG or Sigma), 2.5 U *Taq* DNA polymerase (Genaxxon), or proof reading polymerase *Pfu* or advantage *Taq* (Roche), and template DNA.

Amplification was done in DNAengine Thermocycler (Applied Biosystems), using programs with 35-40 cycles of denaturation, annealing and elongation.

For temporal expression profiling, RT-PCR was performed using 1 µL cDNA (described in 7.1.5) in above described PCR mixtures. cDNA samples were adjusted to equivalent relative

mRNA amounts via control PCR reactions with primers specific for the house-keeping gene *ef1α* (Nordnes et al., 1994).

Sequencing

DNA sequencing was done using the chain termination method, which uses fluorophore labeled didesoxynucleotide phosphate to terminate strand elongation. Sequencing reactions were performed using 400-600 ng plasmid-DNA or 10-50 ng PCR product purified with the appropriate Qiagen Kits mentioned above. The DNA, 3 pmol primer and 1 µL BigDyeTerminator Mix (Applied Biosystems) were mixed to a 12 µL reaction. The amplification program for plasmid sequencing started with an initial template denaturation at 96°C for 2 minutes, followed by 25 cycles consisting of denaturation at 96°C for 10 seconds, primer annealing at 53°C (unless otherwise required with gene specific primers) for 5 seconds and extension at 60°C for 4 minutes. After addition of 8 µL ddH_20, samples were purified and column chromatography was performed at the in-house sequencing facility with an ABI DNA sequencer module.

Cloning of DNA fragments

For cloning of coding sequences from cDNA, PCR products were amplified using proofreading polymerases (Pfu, Fermentas). For further subcloning, primers with restrictions sites at the 5'end were designed; the restriction enzymes have be non-cutters in the cloned sequence. After purification, PCR products were cloned by non-directional cloning into pGem-T Easy vector (Promega) or pCRII Topo vector (Invitrogen) following manufators instructions.

To clone the DNA fragments into the destination vector, pGem-T easy or Topo vector containing the correct insert, proven by sequencing, were completely digested with the choosen restriction enzymes. After complete DNA restriction und purification of the insert DNA, ligations were performed using 50-100 ng vector DNA and a three molar excess of insert DNA, catalysed by 10 U T_4-DNA-ligase in a 10 µL reaction. When required, dephosphorylation of DNA ends prior to ligation was achieved by addition of 1 U Shrimp Alkaline Phosphatase in 10 µL, incubation at 37°C for 30 minutes and inactivation of the phosphatase at 65°C for 15 minutes. Ligations were incubated at 16°C overnight or at room temperature for 2 hours.

Plasmids were transformed into chemo-competent E. coli strains (DH5α or TOP10) via heat-shock at 42°C for 45 seconds, followed by 2 minutes cooling on ice.

3.4. Materials

General laboratory chemicals were of analytical research grade and were purchased mainly from the following manufacturers: Aldrich; Baker Chemicals; Boehringer-Ingelheim; Bethesda Research Laboratories (BRL); Cayman Chemicals; Difco Laboratories; Fluka; Hoechst AG; Invitrogen; Merck; Pharmacia; Roth; Serva; Sigma.

Enzymes and kits were obtained from the following suppliers: Ambion; Boehringer-Ingelheim; Fermentas; Invitrogen; New England Biolabs; Promega; Gibco Life Technologies; Qiagen; Roche GmbH; Stratagene.

Morpholinos

MOs referenced with MODB were distributed via the screen consortium and are designed as transcriptional blocker; further informations can be found in the Morpholino Database (MODB; http://secretomes.biocompute.umn.edu/MODB/index.php) (Knowlton et al., 2008).

Morpholino	sequence	Reference
SP3005a	TCTGCTGAGAAGCTGGAGGGGACTG	MODB
SP3005b	GCACTGAGGTCCATGTTCAGACGCA	MODB
Acsl4_splice	GGTTTCTTAAATAAAAGCTCACCTT	this work
Acsl4C	GCCTTGGGTCAAAGTTCAAAAAGAT	this work
Acsl4D	CTCCAAAATGTGCAACCGGCTTTTA	this work
zcPLA2	GGCAGCCAGCAGTTTGGAGGTCAGG	MODB
bmp7	GCACTGGAAACATTTTTAGAGTCAT	(Lele et al., 2001)
Alox5a	TCACCGTGTAGCTGGGCATTTTCAA	MODB
Alox5b	TGCAGCTCTTGATGACGCCTCTGAT	MODB
5-LO	TGTACGTGAACATTCTGCTGAAATG	MODB
Alox12	CTCTGTGGTGTGGTGGTGAAAATCT	MODB
12-LO	GGCCACTGTCACTTTGTACTCCATC	MODB
BR0001	GAACTGTCTTCACAATCCGAGAGTG	MODB
BR0002	TTGAAGTCGAACCCATCTCTGACGT	MODB
BR0003	TTTGACGCCATCCTCCAGCTTATGT	MODB
BR0004	CTGTAACTCATCCATCATCAAGCCT	MODB
BR0005	CCAGTTGGACACCACAGATGCGTAA	MODB
BR0006	CCAATTTCCAGTCCCATCGACATTT	MODB
BR0007	CTCACGCATCAGCCACGAAAGAATC	MODB
BR0008	TGGGCTCCAGTGTTTATTTCTTCCG	MODB
BR0009	TGCCCAAATCCACAAGTGACATCTC	MODB
BR0010	CAGTCCTTTATGTGCCATCTTCTCA	MODB
BR0011	GGCCTGGACAGCCACGAGACATCTC	MODB
BR0012	TTTAGGTCCCACGCTGCCATTCTGC	MODB
BR0013	TCCAGATCAGTCCGTGCGCCATTAC	MODB
BR0014	AACAAGGTCTTTGGGCTGATGAACA	MODB
BR0015	CTGACCTCCATACTGCTGTAACAGA	MODB
BR0016	GCACCAAGCAAACCAACCTCATAAT	MODB

BR0017	TTTTTGAGCTTCGGATTGTCCTGCT	MODB
BR0018	AGAATACATTGATCTCCATTATGCA	MODB
BR0019	ACTCATCCATCTTTGTGTTGGTCTG	MODB
BR0020	TTCAGTGTCCGCCATCTTCACCACA	MODB
BR0021	TTTGTTGCTACTGTTGACTATCCCG	MODB
BR0022	GTTTGTCAGTCCTATCTGTTGTCAG	MODB
BR0023	CGCCTGTAAAAACAACTCGTAAGTC	MODB
BR0024	TCCACGAAGTCCCCGTAATAATATC	MODB
BR0025	CTTCCATAGTCTCGTATCCTCTGCG	MODB
BR0026	CCGCTCCCTTCTGTCTGCTAAATAA	MODB
BR0027	TTTCCGGTGAAAGACATGGTTTACG	MODB
SP3001	GGCATCTTCCCCTCTGCTGGGCGTC	MODB
SP3002	CTTCATCTTGAGAGCAGCGCTGGGT	MODB
SP3003	CATTCTGACCTGAGCGATTTCCAAA	MODB
SP3004	CAGAACAAAGGAATGAGGCAGGAAC	MODB
SP3006	AGTCTACTGCTCGCTTCCTCCTACG	MODB
SP3007	CACATCACCATACCTTCTCAGTGGA	MODB
SP3008	TCATTCTCACTGATGGATCTATTCA	MODB
SP3009	GAAGACGGTGGTCCTGGATGAAGTA	MODB
SP3010	TCACTCTTAAGTCTCACTTGGAAAG	MODB
SP3011	GCATTTCGACTCTCAATTTCTCGAG	MODB
SP3012	ATTTCCAATCCTCTGCTGCTCCTGT	MODB
SP3013	CATGGTGTTATCCAAAAAATTGTTA	MODB
SP3014	AAAGCAACTCGATCCATCATGACCA	MODB
SP3015	CATTCTGCTGTCCGACACAAACTAA	MODB
SP3017	ATGGTTTAAAACAAATCCTCTTGAA	MODB
SP3019	TAATATCTCCACAAAGAGTCGCCCA	MODB
SP3020	TGTCCGGCAGAATGAGGTTCTCTGT	MODB
SP3021	CCATGTTTGAGTCTGAGTGCTGACG	MODB
SP3022	GCTCCATTAGAAACGCAGTCGAACC	MODB
SP3023	TGAACGCCATAATGGGTCAACTATC	MODB
SP3024	ATGGTTTCTGCTCTTCTCCTTCTTC	MODB
SP3026	TTGATGGACAGACAAGAACACCTGA	MODB
SP3054	GAAAAATGTCTTTCGCCGGGAATCC	MODB
BB0002	GACCTGAGATGAAATGGCAAAGTAA	MODB
BB0006	ATCTCTGAAAAGACAATCTAGCCTA	MODB
boc	AACGTGCAGTAGTTTGGAGTTGAAT	MODB
cndp2	ACAGTGACAATCCCTCAGTGACAGA	MODB
elmo1	TTTCTTCACACCGTTTACTCTGCAA	MODB
mcts1	GTCGTATTCGGTCACTGTATTCCTC	MODB
npc2	CATGGTTCAAATGAATGTAGGTTCT	MODB
nxph1	TCCCTTCTCTCAAACTTCCACTAAT	MODB
ppt1	CAATCTCAATCCTTTAATTTTCTGT	MODB
rfng	GCATTCTTCCAGTGCTGAACGCCAG	MODB

Probes

Name	*Vector*	*Cut. enzyme*	*Polymerase*	*Reference*
otx2	pBSSKII	EcoRI	T7	(Li et al., 1994)
pax2a	pBSSK	XbaI	T7	(Schulte-Merker et al., 1994a)
eve1	pBSSK	HindIII	T3	(Joly et al., 1993)

tbx24	pBSSK	SalI	T3	(Nikaido et al., 2002)
ntl	pBSSK	XhoI	T7	(Schulte-Merker et al., 1992)
sox19a	pBSSK	NotI	T7	(Okuda et al., 2006)
bmp4	pCR2.1	BamHI	T7	(Nikaido et al., 1997)
bmp7	pCRII	SpeI	T7	(Hild et al., 1999)
bmp2b	pBSSK	NotI	T7	(Martinez-Barbera et al., 1997)
acsl4a short	pGemT	SacII	Sp6	this work
acsl4a	pME18S-Fl	PCR amplified	T3	this work

RNA constructs for microinjection

Name	*Vector*	*Restr. enzyme*	*Polymerase*	*Reference*
zfacsl4a full length	pCS2+	NotI	Sp6	this work
Mouse *acsl4*	pCS2+	NotI	Sp6	this work
zfAcsl4a-gfp full length	Xlt.GFPCS2+	NotI	Sp6	this work
zfacsl4a-5'UTR-*gfp*	Xlt.GFPCS2+	NotI	Sp6	this work
alk8CA	pCS2+	NotI	Sp6	(Bauer et al., 2001)
XtBRIa	pCS2+	NotI	Sp6	(Graff et al., 1994)

Primers

acsl4_as_XbaI	GTT TCT AGA GTC CTA CTT CTG GCC ATA CAT
acsl4_asmut_XbaI	GTT TCT AGA GTC CAA CTT CTG GCC ATA CAT
acsl4_forBamHI	TGC GGA TCC AAC ATG GAC CTC AGT GCC GTT
mmacsl4 rev XbaI	GCT CTA GAT TAT TTG CCC CCA TAC ATT C
mmacsl4 rev BamHI	AGG GAT CCA CCA TGA ACC TTA AGC TAA ATG T
acsl4_RTfor_1286	GCA GCG TAC TGA AAC CTT CC
acsl4 RTfor	TGC CTT CTG ATT GGT CAC TG
acsl4_RTrev_1627	TGA TAG TGC CTG CTC CAC AG
acsl4 RTrev	ACG CAA ACA CTT GAC TGC TG
acsl4_896	TGA ACA CAG CAG GAT TTC CA
acsl4 splice_for	TGG TCA CCT TTT ATG CGA CTC
acsl4 splice_rev	CAC AGC ATG CAC TCA GTT GA

primary antibodies

anti-fluorescein	rabbit IgG	Molecular Probes
anti-p63 (4A4)	mouse IgG	Santa Cruz
anti-phospho Smad 1/5/8	rabbit IgG	Cell Signaling

secondary antibodies

Anti-mouse AlexaFluor647	goat IgG	Invitrogen
Anti-rabbit AlexaFluor488	goat IgG	Molecular Probes
Anti-rabbit biotinylated	goat IgG	Vector Labs

4. The role of *acyl-coA-synthetase longchain family member 4* in dorsoventral patterning during zebrafish gastrulation

4.1. Introduction

4.1.1 The arachidonic acid pathway

The first project of my PhD thesis is concerned with the characterisation of *acyl-CoA synthetase long-chain family member 4a*, an enzyme-encoding gene that I found essential for dorsoventral patterning of the zebrafish embryo in an antisense morpholino oligonucleotide (MO)-based screen conducted as part of a research consortium funded by the National Insitute of Heatlh (USA). ACSL4a's major catalytic substrate is arachidonic acid (AA) (Cao et al., 1998), a polyunsaturated omega-6-fatty acid that is also named 5,8,11,14-eicosatetraenoic acid. It consists of a chain of 20 carbon atoms with four cis-double bonds (C20:4:n-6). The common name arachidonic acid is deduced from the saturated arachidic acid initially described from peanuts (*Arachis hypogaea*). AA is one of the essential fatty acids of animal diet. It is either taken up directly in the intestine, or it is synthesised from linoelic acid, a C18 acid with 2 double bonds, in the liver. Within the body, it is transported via the blood stream by lipidproteins or albumin (Zhou and Nilsson, 2001). The uptake by the cell most likely occurs via transport proteins like fatty-acid binding proteins on the plasmamembrane (Brash, 2001). Upon uptake esterified arachidonic acid is incorporated into and stored within plasmamembranes, in particular the outer nuclear membrane, the endoplasmatic reticulum and the Golgi apparatus. Accordingly, most of the enzyme machinery involved in AA metabolism is thought to be located close to or within the nucleus (Capriotti et al., 1988; Luo et al., 2006). In the first step the carboxy-group of arachidonic acid has to be activated by forming an energy-rich thio-ester bond with the thiol group of coenzyme A (Fatty acid + CoA + ATP <=> Acyl-CoA + AMP + PP_i). This step is facilitated through Acyl-CoA Synthetase Longchain family member 4 (Cao et al., 1998), the enzyme that is subject of the studies presented here. In the second step Arachidonyl-CoA is integrated into the sn2-position of phospholipids by the action of acyltransferases, thus via phospholipid remodelling rather than phospholipid *de novo* synthesis. This step requires a deacylation of phospholipids by a Phospholipase, followed by reacylation of membrane phospholipids with arachidonyl-CoA by a long-chain fatty acyl-CoA acyl transferase. These steps are followed by further remodelling steps mediated by CoA-independent transacylases (Balsinde et al., 1997; Balsinde et al., 1995; Leslie, 2004a; Yamashita et al., 1997). Intracellularly, AA is stored conjugated to such

phospholipids, usually phosphatidylethanolamine, until it is released from them by the catalytic action of Phospholipase A2s (PLA2). Up to now 15 genes encoding PLA2s have been identified in mammals and they are subgrouped in three types, the intracellular calcium-independent PLA2s (iPLA2s), the secrected PLA2s (sPLA2s) and the cytosolic PLA2s (cPLA2s) (Leslie, 2004b). Among the Phospholipase A2s, cPLA2-alpha has shown specificity for arachidonic acid (de Carvalho et al., 1995; Leslie et al., 1988). It is regulated by intracellular Ca^{2+} and phosphorylation through MAPKs (Evans et al., 2001; Lin et al., 1993), which is a critical step in the regulation the avaibility of free intracellular AA. In an alternative pathway arachidonyl-CoA can be directly hydrolysed to free AA by an acyl-CoA hydrolase (Farooqui et al., 2000; Sakuma et al., 1999). Free AA in turn serves as substrate for eicosanoid synthesis (see below) (Brock and Peters-Golden, 2007). Thus, free intracellular AA for eicosanoid production can be either supplied directly, via the hydrolysation of arachidonyl-CoA that most likely derives from freshly uptaken AA, or in a more indirect fashion from stored AA that is conjugated to phospolipids, integrated into membrane integration and released from them by Phospholipase A2.

4.1.2 The cyclooxygenase pathway of prostanoid production

Arachidonic acid serves as precursor molecule for eicosanoid synthesis (see Figure 4-1 for an overview). Eicosanoid (from the Greek word for twenty, *eicosa*) are a very large family of lipid mediators that fall into two major groups, the leukotrienes and the prostanoids, which can be further subdivided into prostaglandins, prostacyclins and thromboxanes. They extert a range of diverse, hormone-like biological functions (Brock and Peters-Golden, 2007). However in contrast to the endocrine nature of classical hormones, eicosanoids are formed in almost all tissues, rather than in specialized glands, are not stored in tissues to any appreciable extent, and act locally in an autocrine or paracrine fashion on the secreting cells themselves or cells in their neighborhood, rather than circulating to distant sites of actions. All of them are synthesised from free AA in a multistep reaction (Evans et al., 2001). In the first step of prostanoid synthesis, the intermediate Prostaglandin H_2 (PGH_2) is formed from AA by Prostaglandin H_2 -Synthase (PGHS). The two isoforms of PGHS are also called Cyclooxygenase 1 and 2 (COX-1, COX-2). COX-1 is usually responsible for the constitutive prostaglandin synthesis, whereas COX-2 is important for induced synthesis of prostaglandins (Smith et al., 2000). In a tissue-specific manner, PGH_2 is further metabolised to thromboxanes, prostacyclins or prostaglandins. Thromboxanes are found in blood platelets

and promote platelet aggregation, whereas prostacyclins seem to inhibit blood clotting. Among the large group of Prostaglandins (PGs), PGE_2, PGF_2 and PGD_2 are the most active ones; PGE_2 is for example responsible for the induction or enhancement of inflammation and fever, vasodilatation and bone formation. By interfering with the canonical Wnt-signaling pathway and via PPARgamma signaling, PGE_2 can also promote colon cancer (Castellone et al., 2005; Wang and DuBois, 2007). PGF_2 can for instance induce contractions of the uterus, while PGD_2 is thought to be a bronchoconstrictor (Karlson et al., 2005). Prostanoids are released from cells by facilitated transport (Schuster, 2002). Signaling in target cells occurs via specific receptors that belong to the G-Protein-coupled receptor (GPCR) superfamily of seven transmembrane spanning proteins (Narumiya and FitzGerald, 2001). For PGE_2, four receptors are known (called EP1-4). Various downstream signaling pathways can be activated upon ligand binding, including G-Protein signaling via Phosphoinositide-3-Kinase (PI3K), Protein Kinase B (Akt), and / or cAMP/ Protein Kinase A (PKA) signaling, as well as signaling via Glycogen Synthase Kinase 3 and ß-catenin, thereby interfereing with the canonical Wnt transduction pathway (Dey et al., 2006; Sugimoto and Narumiya, 2007).

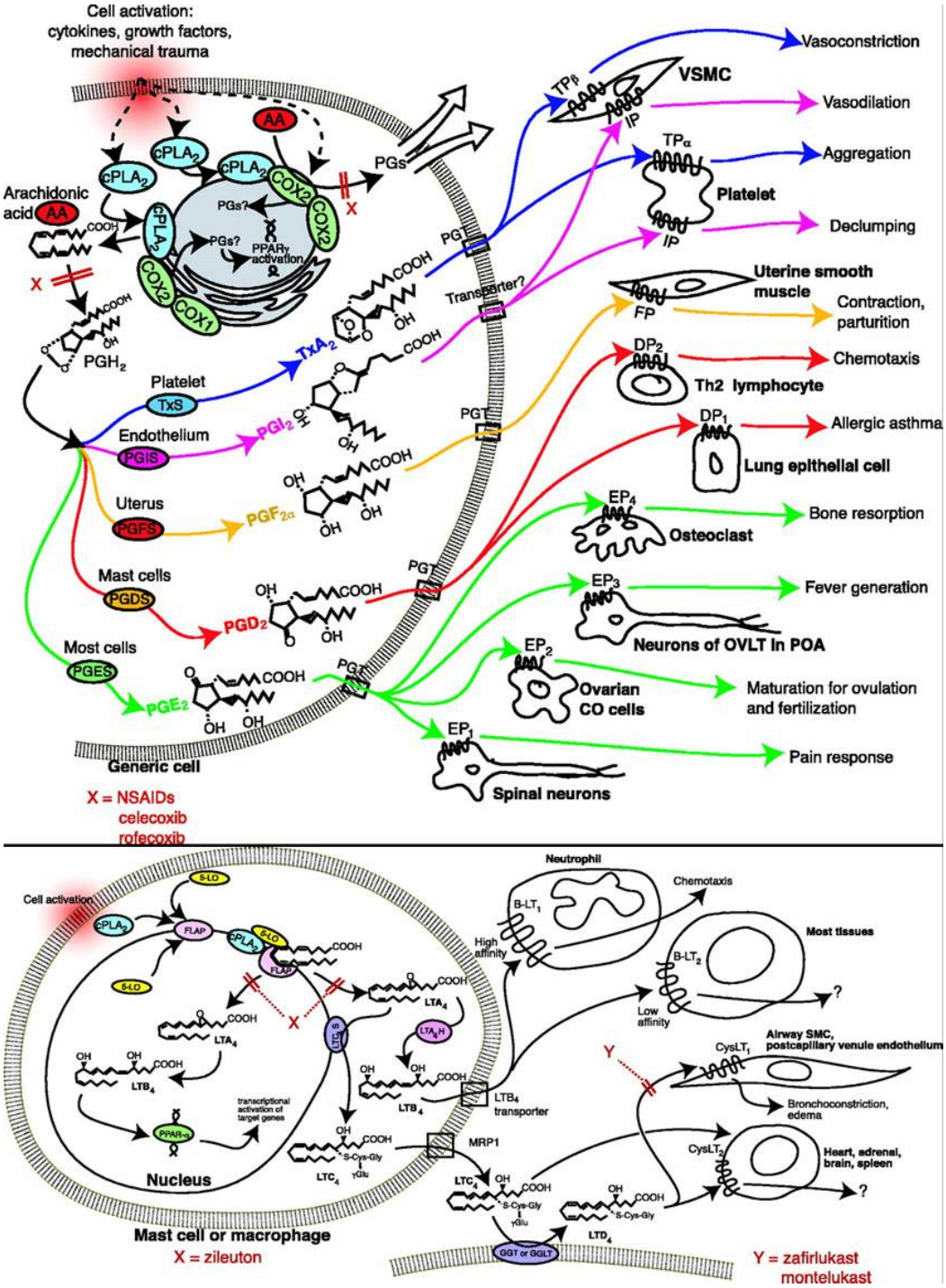


Figure 4-1: Overview of the most prominent pathways of eicosanoids
Arachidonic acid is released from phospholipids by the action of cPLA2, which is activated by extracellular or intracellular signals (cell activation). (Upper panel) Prostanoids are synthesised via Cyclooxygenase 1 and 2 (COX-1, -2), which form Prostaglandin H_2 (PGH_2). NSAIDs are blocking the cyclooxygenases. PGH_2 is the precursor for further prostaglandins, thromboxanes and prostacyclins synthesis. Upon release they act as autocrine or paracrine signaling molecules, via binding to receptors of the GPCR class or to PPAR nuclear receptors, in a huge variety of tissues and contexts, as indicated in the panel.
(Lower panel) after cell activation and release of AA the second major class of eicosanoids, the leukotrienes, are synthesised by the action of lipoxygensaes and FLAP. They act as autocrine or paracrine factors via PPAR or GPCR receptors, too, in a variety of tissues. Schemes from Funk, 2001.

4.1.3 The leukotriene and other pathway

A second major pathway of AA oxygenation is the leukotriene pathway, which leads to the formation of leukotrienes and the hydroperoxy and hydroxy eicosatetraenoic acids (abbreviated HPETEs and HETEs, respectively) (for leukotrienes, see lower panel of Figure 4-1). As for prostanoids the release of AA through cPLA2 is required, followed by the enzymatic reaction of 5-lipoxygenase (5-LO) and 5-lipoxygenase-activating protein (FLAP) (Peters-Golden and Brock, 2001; Peters-Golden and Brock, 2003). Leukotrienes signal through GPCRs, too, and play an important in the immune system, especially in leukocytes and mast cells, but also promote bronchoconstriction. Further HETEs as well as Lipoxins are produced by other Lipoxygenase (12-LO, 15-LO) (Karlson et al., 2005). In *Hydra spec*. HETEs have shown to be involved in head development (Di Marzo et al., 1993; Hassel et al., 1996; Muller et al., 1998). More common roles of them are in pulmonary function regulation and in vascular tone and inflammatory responses (Moreno, 2009). Lipoxins are anti-inflammatory mediators found in leukocytes (Chiang et al., 2005; Serhan et al., 1984).

In a further pathway, enzymes of the family of Cytochrome P450 oxidases can catalyse a reaction of arachidonic acid with O_2 to form epoxytrienoic acids (EET) that are described as vasodilators (Spector et al., 2004).

Beside its conversion by oxygenases, free AA has bioactive functions itself. It can stimulate Peroxisome Proliferator-Activated Receptors (PPARs), a group of nuclear hormone receptors that require binding to fatty acids for nuclear translocation and transcriptional regulation. Furthermore, AA regulates certain ion channels (Brash, 2001), induces apoptosis (Cao et al., 2000; Jantke et al., 2004) and is involved in hormone-induced steroidogenesis (Wang et al., 2000).

4.1.4 The enzyme acyl-coA-synthetase longchain family member 4 (ACSL4)

Acyl-CoA synthetases in general are required to form the thioester bond between CoenzymA and the carboxy group of fatty acids. The enzymes are subdivided into several subgroups, based on their specificity for particular fatty acids. The longchain family members are specific for C12-C20 fatty acids like palmitic acid, linoelic acid, oleic acid and arachidonic acid. Five ACSLs have been identified in mammals (Soupene and Kuypers, 2008); in zebrafish three members have been annotated (ZFIN Data). Mammalian *ACSL4* is expressed in a wide range of tissues, including heart, brain, liver, adipose tissue, adrenal gland, epididymis and ovaries

(de Jong et al., 2007; Kang et al., 1997). Immunohistochemistry has further localisized mammalian ACSL4 protein at the membrane of peroxisomes and the ER of liver cells (Lewin et al., 2001; Lewin et al., 2002).

Patients sufferings from Alport-Syndrome and non-specific X-linked mental retardation (MRX) showed chromosomal deletion including the human *ASCL4* (Meloni et al., 2002; Piccini et al., 1998). However, it remains contentious whether it is the loss of *ASCL4* or neighboring genes that is responsible for MRX (Verot et al., 2003).

In a mice model with disrupted gene *acsl4*, female heterozygous mice became pregnant less frequently and produced smaller litters, due to increased *in utero* death rates of heterozygous embryos from stage E12 onwards. This might be due to combined zygotic and maternal effects. Mothers suffered from enlarged uteri containing cysts and higher levels of prostaglandin. In was speculated that the abnormal accumulation of uterine prostaglandins might impair female fertility. Male hemizygous mice did not show a phenotype, homozygous animals are not described, so far (Cho et al., 2001). *In vitro* studies of steroidogenesis have further revealed that ACSL4 is involved in a pathway of trophic hormone-stimulated steroidogenesis. The release of AA is required for the expression of StAR protein (steroidogenic acute regulatory protein), a critical regulator of steroid biosynthesis. Thereby an isoform of ACSL4 is involved in a PLA2-independent pathway to release free AA (Cho et al., 2000; Maloberti et al., 2005; Stocco et al., 2001). Further *in vitro* studies have shown that the expression of *ACSL4* isoforms is upregulated in human hepatocellular carcinoma cells. Here, ACSL4 regulates AA-dependent cell growth and AA-induced apoptosis (Hu et al., 2008; Liang et al., 2005).

4.1.5 Aim of the project

As a starting point of this part of my PhD work I screened a collection of morpholino antisense oligonucleotides in collaboration with the laboratories of Stephen C. Ekker (University of Minnesota) and Steve A. Farber (Carnegie Institution, Baltimore). One of the first morpholinos I screened, named SP3005, caused dorsalisation of zebrafish embryos. This morpholino targets the 5'UTR of zebrafish *acyl-coA-synthetase longchain family member 4a* (*acsl4a*). In zebrafish, the arachidonic acid and eicosanoids networks have been little investigated thus far. Only the prostaglandin PGE_2 and the cyclooxygenases Cox1 and Cox2, the crucial enzymes of prostaglandin synthesis, have been described to be important for zebrafish development. Thus, PGE_2 is required for proper cell migration during gastrulation

(Cha et al., 2006a) as well as for vascular tube formation and maintenance of haematopoietic stem cell (Cha et al., 2005; North et al., 2007). However, there were no reports on a function of arachidonic acid metabolism and eicosanoids during embryonic patterning processes. Because of the long-standing interest of the Hammerschmidt laboratory in dorsal-ventral patterning, I decided to further characterise the phenotype caused by the morpholino targeting zebrafish *acsl4a*, and the correlation between Ascl4a and BMP signaling, a central pathway regulating dorsal-ventral patterning of the zebrafish embryo (see General Introduction). In addition, I investigated whether *ascl4a* is required for gastrulation movements, comparable to reported roles of Cox enzymes their product and AA derivative PGE_2. Finally, in collaboration with the laboratory of Steven A. Farber, an expert in lipid metabolim and its role during zebrafish development (Ghiselli and Farber, 2005; Ho et al., 2004), I knocked down other putative components of AA metabolism, such as a cPL2A phospholipase and different lipoxygenases, and treated embryos with chemical AA antagonists and agonists.

4.2. Results

4.2.1. Knock-down with morpholino SP3005a causes dorsalisation of the zebrafish embryo

The zebrafish *acsl4a* gene is located on chromosome 14 and encodes a protein of 706 amino acid residues with 74% identify to the human and mouse ACSL4 proteins. As part of the aforementioned large-scale antisense morpholino oligonucleotide (MO) screen, MO SP3005a was designed that targets the 5'UTR at position -38 of *acsl4a* (see Materials and Methods).

For a first characterisation of the morphant phenotype, I examined the morphology of embryos injected with different amounts of the MO (Fig. 4-2). Dorsoventral patterning and the establishment of the dorsal body axis take place during gastrulation. Defects during these patterning processes lead to a gain of dorsal tissues, like neuroectoderm and presomitic mesoderm, and concomitant loss of ventral tissues like epidermis and somatic mesoderm in the case of dorsalisation, and contrary shifts in the case of ventralisation. During gastrula and early segmentation stages (5-12 hours post fertilisation; hpf), these shifts can be best visualised with appropriate dorsal and ventral markers, such as genes that display a specific dorsoventral expression pattern. However, morphologically, the strength of dorsalisation or ventralisation of embryos can be best determined much later (24 hpf), when the respective derivatives of dorsal and ventral tissues have been formed and cell types have fully differentiated. For classification of dorsalisation strenghts into C1 (weakest) to C5 (strongest) (Fig. 4-2 K), I used criteria as previously described (Kishimoto et al., 1997).

After the injection of MO at a concentration of 0,125 mM, 74% (n=52/70) of the embryos had died before 24 hpf, most likely as a result of strongest dorsalization, which leads to an "explosion" of embryos due to somite constriction during early segmentation stages (Mullins et al., 1996), while 25% (n=17/70) showed strong dorsalisation (C4 or C5). Injection of 0,1 mM to 0,05 mM caused the whole spectrum of dorsalisation classes. Injection of 0,1 mM MO, caused 4% (n=4/100) lethality, 49% (n=49/100) C4/5 dorsalisation and 47% (n=47/100) moderate and mild dorsalisation (C3-C1), while 4% (n=4/100) had wild-type morphology. Embryos injected with 0.075 mM and 0.05 mM MO showed no lethality, reduced numbers of strong dorsalization, and correspondingly higher proportions of moderate and mild dorsalisation classes (C1-C3; 68% (n=35/51) for 0.075 mM; 72% (n=64/87) for 0.05 mM). Injection of 0.25 mM caused only mild phenotypes (0% C4/C5; 61% C1-C3, n=33/54), while upon injection of 0.0125 mM, embryos predominately appeared unaffected (91%, n=90/99) (Fig 4-1 I, K). In conclusion, injection of SP3005a MO caused dorsalization in a dose-

dependent fashion, possible reflecting the range of phenotypes obtained upon partial to complete knock-down of gene function. Importantly, complete knock-down appears to cause strongest dorsalization, as also obtained upon complete loss of BMP signaling (see also General Introduction and below).

I performed an analysis using whole mount *in situ*-hybridisations to confirm the observed dorsalisation. Injected and uninjected embryos were stained at mid gastrula stages (80% epiboly; 8 hpf) with previously described markers for dorsal-ventral patterning (Fig. 4-1). The gene *orthodenticle homolog 2* (*otx2*) encodes a homeodomain-containing transcription factor and is expressed in fore- and midbrain precursor cells of the anterior neuroecoderm, thereby serving as a marker for dorsal ectoderm (Li et al., 1994). In SP3005a morphant embryos (0,1 mM MO) the expression domain of this dorsal ectodermal marker was expanded into ventrolateral regions of the embryo (Fig. 4-1 A, B). Respectively, expression of *tbx24,* which encodes a T-Box transcription factor made in the paraxial mesoderm (Nikaido et al., 2002), was also ventrally expanded in *acls4* morphants (Fig. 4-1 E, F). In reverse, as expected for a dorsalised embryo, the expression of *gata 2*, a marker for ventral ectoderm (Detrich et al., 1995), was reduced and restricted to the most-ventral region of the embryo (Fig. 4-1 C, D). Similarly, the marker for ventral mesoderm, *eve1* (Joly et al., 1993), was expressed in a much smaller domain in ventral-most regions only (Fig. 4-1 G, H). Based on these results, I characterise the phenotype of SP3005a morphants as dorsalisation, pointing to an indispensable role of Acsl4a for the specification of ventral cell fates during dorsoventral patterning of the zebrafish embryo. However, I should also point out that according to the marker gene expression data, the dorsalization of the SP3005a morphants was weaker than that obtained upon complete inactivation of BMP signaling, which for instance leads to a complete loss of *gata2* and *eve1* expression (Mullins et al., 1996).

To determine the efficacy of acsl4a knock-down by the SP3005a MO, I generated an N-terminal fusion construct of Green Fluorescent Protein (GFP) and the morpholino binding site. For this purpose, I cloned parts of the *acsl4a* cDNA containing the 5'UTR target site of the morpholino and the translation initiation site, in front of and in frame with the coding region of *gfp*. The plasmid was used for *in vitro* synthesis of mRNA, which was injected into zebrafish embryos. Injection of the mRNA alone led to ubiquitous GFP expression, whereas GFP was completely suppressed when I coinjected the mRNA with SP3005a MO (Fig. 4-1 L, M). Thus, SP3005a MO efficiently blocks the translation of mRNAs containing the *acsl4a* target sequence.

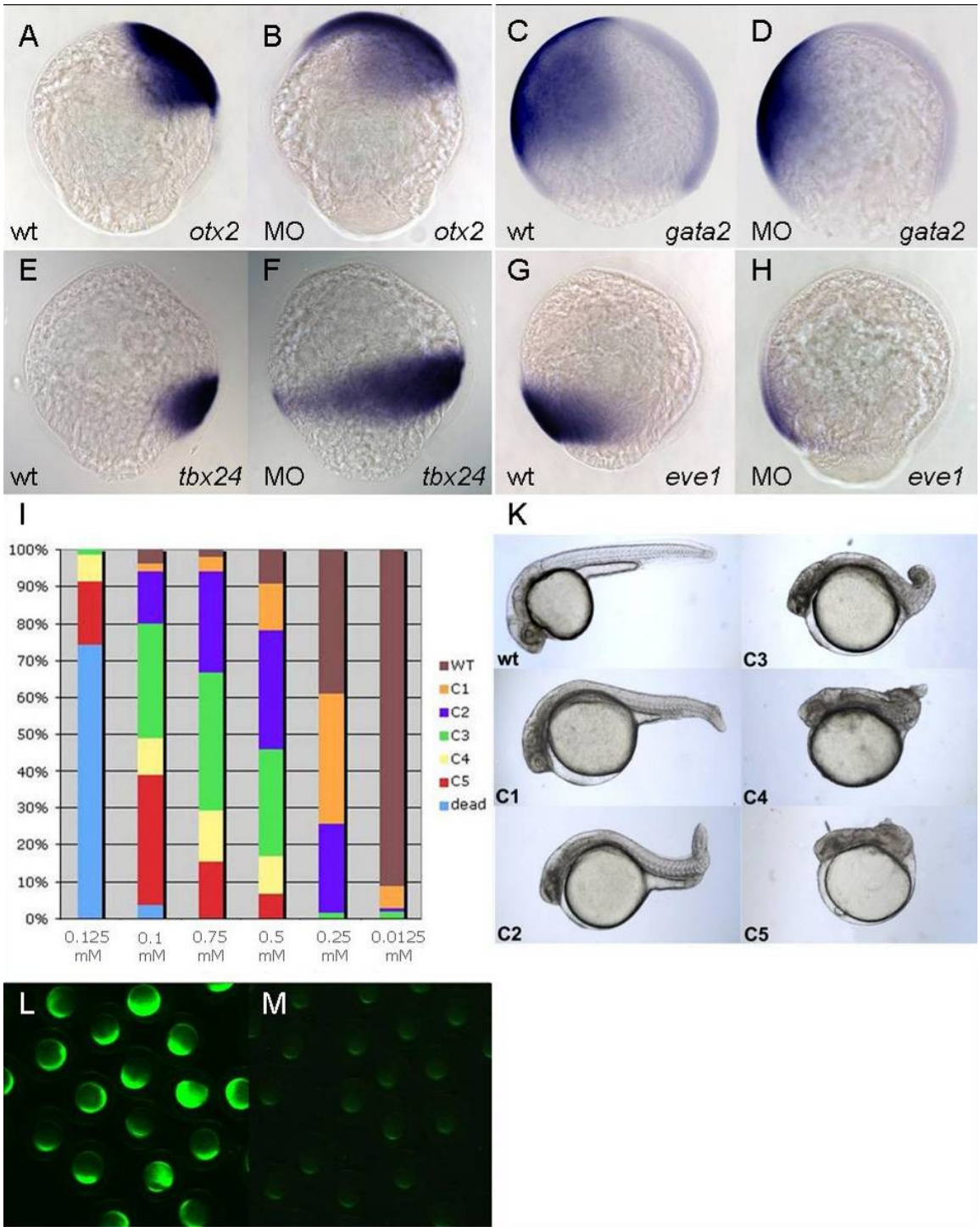


Figure 4-2: Knockdown of Acyl-CoA-Synthetase longchain family member 4a with morpholino SP3005a causes dorsalisation.
Whole-mount *in situ*-hybridisation with markers previously described in DV patterning, *otx2* (A, B) as marker for dorsal ectoderm, *gata2* (C, D) for ventral ectoderm, *tbx24* (E, F) for paraxial mesoderm and *eve1* (G, H) for ventral mesoderm; at 80% epiboly in uninjected embryos (A, C, E, G) and embryos injected with 0,1 mM MO SP3005a (B, D, F, H).
(I) Graphical illustration of obtained strengths of dorsalisation upon injection of SP3005a MO at concentrations ranging from 0.125 mM to 0.0125 mM. Dorsalisation strengths were classified at 24 hpf, using morphological criteria as described in Mullins et al. (1996) and as shown in panel (K).
(L, M) GFP expression after injection of *acsl4a-UTR-gfp* fusion mRNA (L); GFP expression is lost after injection of the mRNA together MO SP3005a (M).

4.2.2. ACSL4a seems to be required for the maintenance of BMP signaling

Several signaling molecules are involved in dorsal-ventral patterning; some of the most prominent are the BMPs (Bone Morphogenetic Proteins) (Hammerschmidt and Mullins, 2002). Because the knockdown of *acyl-CoA-Synthetase longchain family member 4a* with morpholino SP3005a cause dorsalisation, as observed after loss of BMP signaling (Bauer et al., 2001; Kishimoto et al., 1997), I investigated to which extent BMP signaling is affected in *acsl4a* morphants.

First, I studied the expression patterns and intensities of *bmp2b*, *bmp4* and *bmp7* by whole-mount *in situ*-hybridisation at the onset of gastrulation and at mid- to late-gastrula stages. At the onset of gastrlation (shield stage; 6 hpf), I could not see any differences in the expression of any of the three *bmp*s in the injected embryos compared to the uninjected control (Fig. 4-3 A -M). But the expression domains and levels were strongly reduced in the *acsl4a* morphants at the 80% epiboly stage (8 hpf; Fig. 4-3). To further confirm the loss of active BMP signaling in the morphants, I performed whole mount immunostainings against phosphorylated SMAD1/5/8 proteins. These are the receptor-SMADs expressed during early zebrafish development, which become phosphorylated through active BMP-signaling. Knockdown of *acsl4a* caused a dramatic reduction of SMAD protein phosphorylation at 80% epiboly (Fig. 4-3 N, O), indicating the loss of active signaling. Because the expression of *bmp*s is normal at shield stage, the initiation of the BMP gradient along the dorsal-ventral axis seems to be unaffected by Acsl4a knock-down. But later during gastrulation the knockdown of *acsl4a* seems to affect the maintenance of *bmp2/4/7* expression, possibly by promoting the aforementioned positive feedback of BMP signaling on *bmp* gene expression (see General Introduction).

To investigate whether *acsl4a* and *bmps* might indeed regulate dorsoventral patterning by acting together in one linear pathway, or independent of each in parallel pathways, I performed genetic interaction and epistasis experiments. For the test of genetic interaction, I carried out synergistic enhancement studies, co-injecting MO SP3005a and *bmp7* MO at suboptimal concentrations and classified the embryos at 24 hpf by their morphology. Upon coinjection of MO SP3005a at 0.025 mM and *bmp7* MO at 0.02 mM, I obtained 44% (n=47/106) dorsalised embryos, with phenotypes ranging from C1 to C5. In contrast, in the single injections, I observed only 3% (n=3/90) C1 for *bmp7* MO and 9% (n=9/99) phenotypes ranging from C1 to C3 upon *acsl4a* knock-down (Fig. 4-3 P). Thus, the dorsalisation obtained upon concomitant partial inactivation of *acsl4a* and *bmp7* is stronger than the sum of the two

single effects, pointing to a synergistic enhancement, rather than an additive effect, and a genetic interaction of the two genes during dorsoventral patterning.

As a first step to address the epistasis between *acsl4a* and *bmp* genes, I combined loss of *acsl4a* with gain of *bmp2b,* injecting *acsl4a* MO at a concentration of 0.1 mM into embryos from a cross between a wild-type fish and a transgenic fish carrying the transgene hsp70::*bmp2b*. This transgenic line had been generated in the Hammerschmidt laboratory by a former postdoctoral fellow, Fabian Rentzsch (Rentzsch et al., 2006). In the transgene, *bmp2b* is under the control of the temperature-sensitive *hsp70* promoter, leading to ubiquitous *bmp2b* expression in transgenic embryos within 30 minutes after the application of a heatshock (incubation of embryos at 39°C instead of the regular 28°C). Upon heatshock at shield stage the ectopic transcript was stable for several hours and caused strong ventralisation of the whole embryo. In the control clutches 50% (n=31/63) of the embryos showed V3 ventralsation at 24 hpf (Kishimoto et al., 1997), the other 50% (n=32/63) had wild-typic morphology. Without heatshock, all embryos were wild type. This indicates that, as expected, half of the embryos from the cross carried the transgene, and that the transgene was functional. Injection of *acsl4a* MO without subsequent heatshock led to the expected dorsalisation (wildtype 8/37, C1 6/37, C2 9/37, C3 11/37, C4 2/37, C5 1/37). When the embryos injected with *acsl4a* MO were heatshocked at shield stage, 50% (n=26/52) of the embryos showed V3 phenotypes as in the control clutches without MO injection. The rest of the clutch showed dorsalised phenotypes in a similar ratio as the injected controls without heatshock (wildtype 3/26, C1 4/26, C2 8/26, C3 10/26, C4 1/26). Thus, the forced expression of *bmp2b* during gastrula stages overcomes the dorsalising effects of *acsl4a* knock-down. This indicates that BMPs do not require and do not act via Ascl4a to fulfil their essential ventralising role during dorsoventral patterning of the zebrafish embryo. In reverse, this mean that Ascl4a might either act upstream of *bmps* in a linear pathway, or independent of BMPs in a parallel pathway. To distinguish between these two possibilities, it would be necessary to combine gain of Acsl4a function with loss of BMP function (but see below).

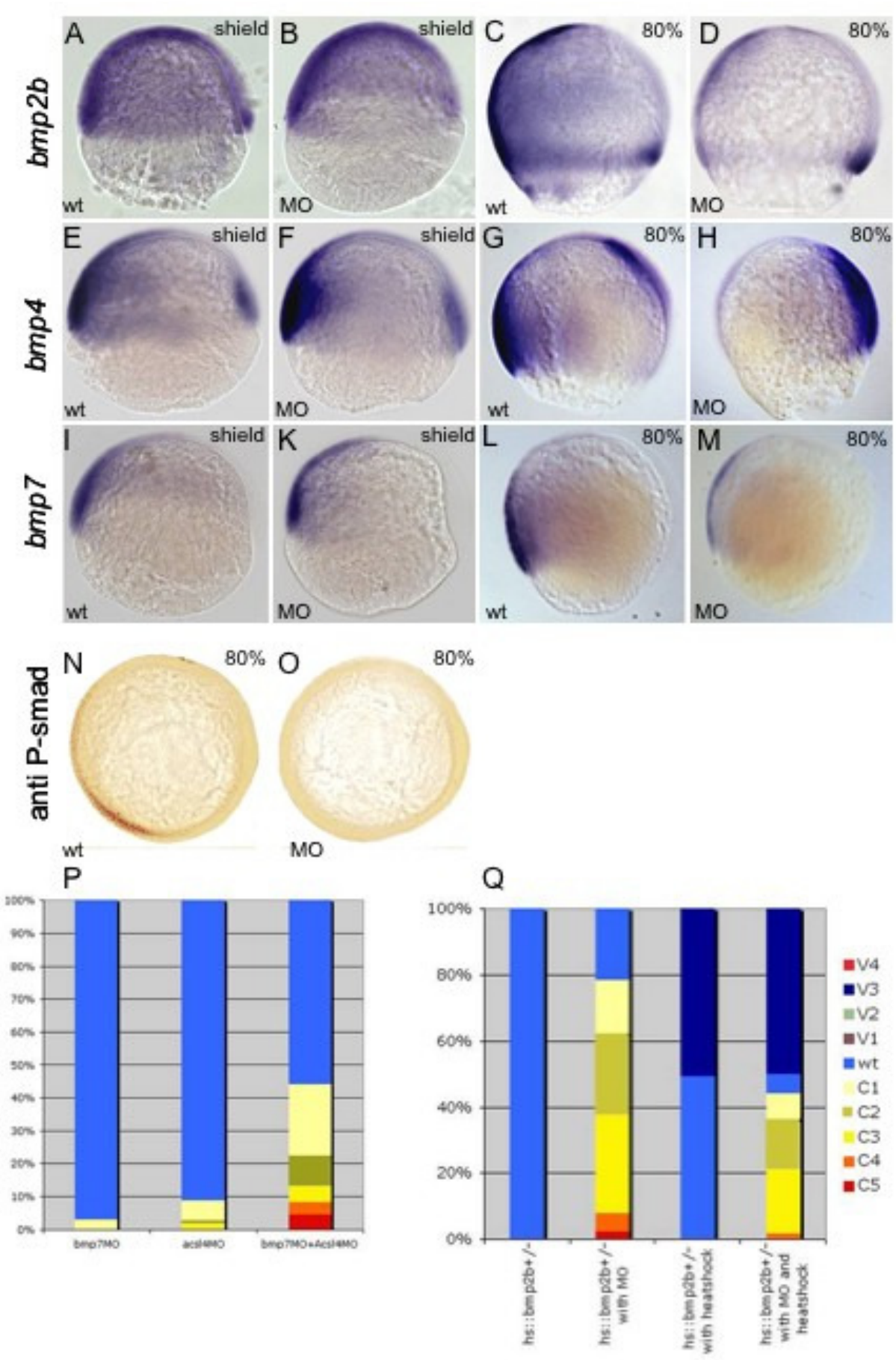

bmp2b
bmp4
bmp7
anti P-smad
A
shield
wt
B
shield
MO
C
80%
wt
D
80%
MO
E
shield
wt
F
shield
MO
G
80%
wt
H
80%
MO
I
shield
wt
K
shield
MO
L
80%
wt
M
80%
MO
N
80%
wt
O
80%
MO
P
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
bmp7MO
acsl4MO
bmp7MO+Acsl4MO
Q
100%
80%
60%
40%
20%
0%
hs::bmp2b+/-
hs::bmp2b+/- with MO
hs::bmp2b+/- with heatshock
hs::bmp2b+/- with MO and heatshock
V4
V3
V2
V1
wt
C1
C2
C3
C4
C5

Figure 4-3 (above): *acsl4a* may act upstream or in parallel to BMP-signaling during mid-gastrulation.
(A –M) Expression pattern of *bmp*2b /4 /7 by whole-mount *in situ*-hybridisation. No difference in expression domains and intensities were detected at shield stage between uninjected (A, E, I) and with 0.1mM MO SP3005a injected embryos (B, F, K), but a strong reduction in the morphants at 80% epiboly (D, H, M) compared to the wild-type situation (C, G, L). Active BMP-signaling, indicated by anti-Phospho-Smad stainings at 80% epiboly is strongly reduced in SP3005a morphants (O), (P) uninjected control.
(P) Test for synergistic enhancement between *bmp7* and *acsl4a*; phenotypic classification was done after 24 hpf. Partial *bmp7* and *acsl4a* inactivation by suboptimal MO concentrations result only in a small amount of dorsalised embryos in the single MO injections (lane 1 and 2), but in stronger dorsalisation upon co-injection (lane 3) of the two MOs.
(Q) Epistatic analysis, combining MO-mediated loss of Ascl4a function with gain of Bmp2b function in *hsp70::bmp2b* transgenic embryos; phenotypic characterisation was done at 24hpf; (lane 1) *hsp70::bmp2b* embryos without heatshock shows no phenotype, (lane 2) MO SP3005a injected in *hsp70::bmp2b* embryos without heatshock resulted in dorsalisation, (lane 3) *hsp70::bmp2b* embryos with heatshock at shield stage resulted in strong ventralisation in 50% of the embryos, (lane 4) MO SP3005a injected in *hsp70::bmp2b* embryos with heatshock at shield stage resulted in strong ventralisation in 50% of the embryos and dorsalisation in the other 50%.

4.2.3. During gastrulation, *acsl4a* is mainly expressed and required in the Yolk Syncytial Layer

I performed whole mount *in situ*-hybridisations and reverse transcriptase-PCR (RT-PCR) to unveil the temporal and spatial expression pattern of *acsl4a*, obtaining the same results with two independent antisense riboprobes and PCR primer pairs, respectively.

Whole-mount *in situ*-hybridisations revealed prominent *acsl4a* expression in the Yolk Syncytial Layer (YSL) of gastrulating embryos (80% epiboly; 8 hpf), whereas expression in blastoderm was much weaker (Fig. 4-4 A, B). The YSL is an extraembryonic structure that is required for proper mesoderm and endoderm formation (Chen and Kimelman, 2000). Expression of *acsl4a* in the YSL also persisted during somitogenesis. At these later stages, additional *acsl4a* expression could be detected in the brain and the eyes (Fig. 4-4 C-E).

I also performed RT-PCR analyses for different developmental stages during the first 24 hpf. By RT-PCR I could obtain a strong signal in fertilized eggs, which declined during blastula and early gastrula stages and increased again from mid gastrula stages onwards (Fig. 4-4 H). This points to the presence of maternally provided *acsl4a* transcripts during early stages of zebrafish development, whereas zygotic expression is first initiated after the onset of gastrulation. Consistently, I could not find significant expression of *acsl4a* via whole mount *in situ*-hybridization at 50% epiboly and shield stage (5 – 6 hpf), indicating that at late blastula and early gastrula stages, when BMP signaling is actively patterning the dorsoventral axis (Pyati et al., 2005), *acsl4a* mRNA levels are minor.

Because the most prominent expression of *acsl4a* during gastrulation is in the YSL, I wondered whether the phenotype of morpholino SP3005a is due to knockdown of ACSL4a in the YSL. To address this question I injected 0.8 mM of *acsl4a* MO into the yolk cell of high stage embryos (3-3.5 hpf). Since MOs cannot cross cell membranes, MOs delivered this way will only target mRNAs present in the YSL. To indirectly visualise the confinement to the yolk cell and the success of the injection, the MO was co-injected with rhodamin-dextran (Fig. 4-4 F, G). Injection of SP3005a into the yolk cell successfully blocked the translation of co-injected Acsl4a-GFP (not shown). However, only a comparably low number of successfully injected embryos successfully injected with SP3005a MO and rhodamin-dextran (n=5/37, data from three different experiments) showed mild to moderate dorsalisation phenotypes, while all embryos injected with rhodamin-dextran alone had wild-type morphology (Fig. 4-4 F, G). This indicates that Acsl4a is indeed required in the YSL, where it displays its strongest expression. I can only speculate about the reasons for the rather low penetrance and strength of dorsalisation obtained by mid blastula yolk injections compared to injections at the 1-cell stage. They could point to an additional requirement of low level Acsl4a in the YSL during earlier (blastula) stages, and/or to an additional requirement of low level Acsl4a in the blastoderm.

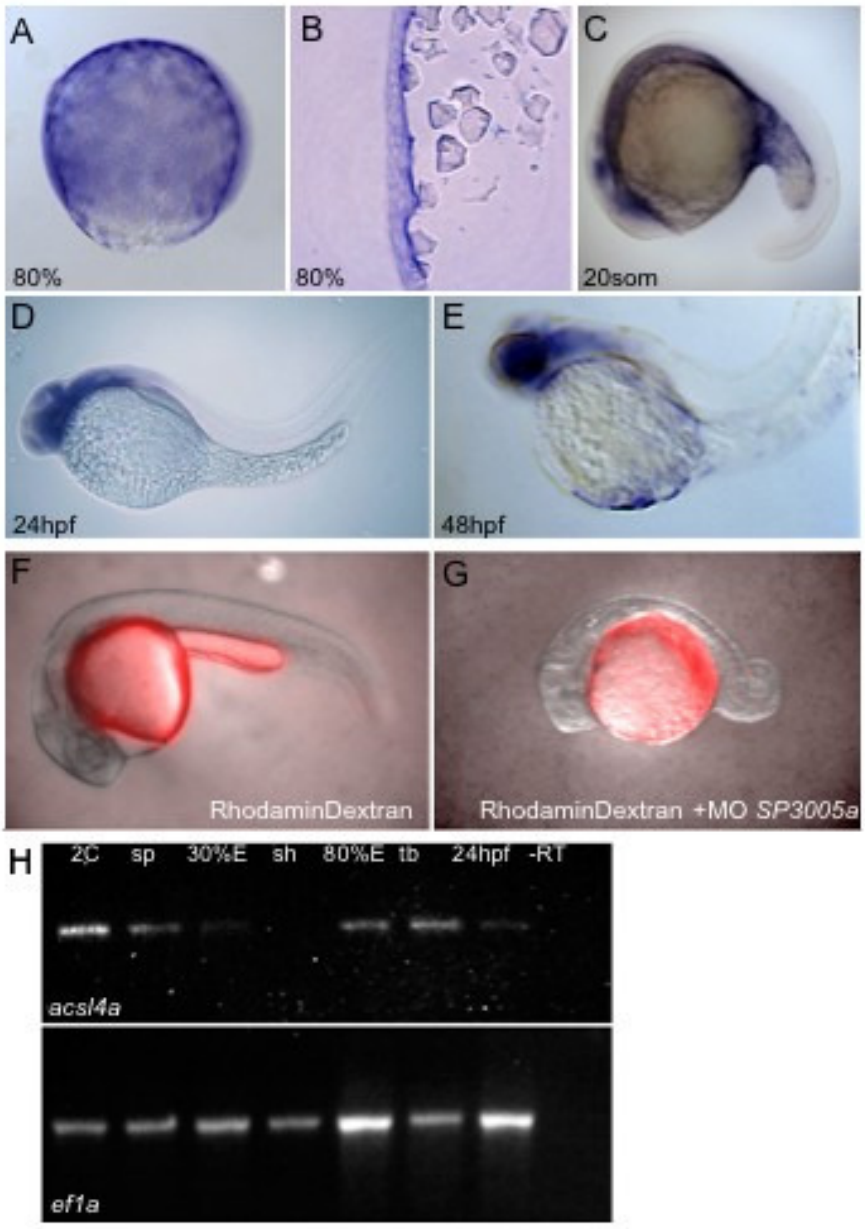


Figure 4-4: *acsl4a* is expressed and required in the yolk synticial layer during gastrulation stages
(A –E) Whole-mount *in situ*-hybridisations of wildtype embryos, with a probe against *acsl4a* (stages are indicated in the lower left corner); (A) during gastrula acsl4a is expressed in the YSL, (B) shows a transversal section of (A), (C-E) from somitogenesis onwards expression is detected in brain tissues, too, the YSL expression persists.
(G) Live image of a 24hpf embryo, injected into the yolk cell at high stage with 0.8 mM MO SP3005a /RhodamineDextran, shows dorsalisation phenotype, (F) control embryo injected with solely Rhodamine Dextran.
(H) RT-PCR for *acsl4a* at different developmental stages (2-cell stage, sphere, 30% epiboly, shield, 80% epiboly, tailbud, 24 hpf) detects a strong expression of *acsl4a* in fertilised eggs, a declining expression in blastula stages, no expression at shield stage and increased expression from mid-gastrula onwards. *ef1a* as control PCR.

4.2.4. Further morpholinos targeting *acsl4a* do not lead to dorsalisation

To confirm the phenotype caused by morpholino SP3005a I designed further morpholinos targeting *acsl4a* (for sequences, see Materials and Methods). Injected embryos were analysed for dorsalisation at 24 hpf (Fig. 4-5). Each morpholino was injected at least three times with a concentration of 0.5 mM, and into two different wild-type strains, Tl/Ek and AB. One morpholino, named SP3005b, targets the region around the translational start site (AUG) site of the *acsl4a* mRNA. Upon coinjection with the same GFP-construct that I used for the efficacy check of SP3005a, SP3005b MO abolished GFP expression, indicating that it is functional (Fig. 4-5 G, H). However, in contrast SP3005a, SP3005b did not cause any dorsalisation of injected embryos (Fig. 4-5 C). Another morpholino named "splice site MO" targets the splice donor site of exon 4 of the *acsl4a* pre-mRNA. The functionality of this MO was tested by RT-PCR, revealing almost complete absence of the wild-type *acsl4a* transcript, whereas I could amplify a new transcript with an intron-specific primer (not shown). Like SP3005b, the splice site MO failed to yield any sign of dorsalisation. Instead, embryos displayed a rather subtle upward bending of the tail (Fig. 4-5 F). Also, a coinjection of SP3005b and the splice site morpholino did not cause dorsalisation. Finally, I tested two additional morpholinos, named acsl4C and acsl4D, which target the 5'UTR of the *acsl4* mRNA further upstream of SP3005a, at the positions -107 and -154, respectively of the transcript sequence. These two morpholinos did not cause any morphological defects, even when I coinjected them (Fig. 4-5 D, E). In sum, of a total of five sequence-independent MOs targeting *acsl4a*, only the orginal one, SP3005a, caused dorsalisation, although two of the remaining four were shown to be functional. This strongly suggests that the dorsalisation obtained by SP3005a may be an off-target effect.

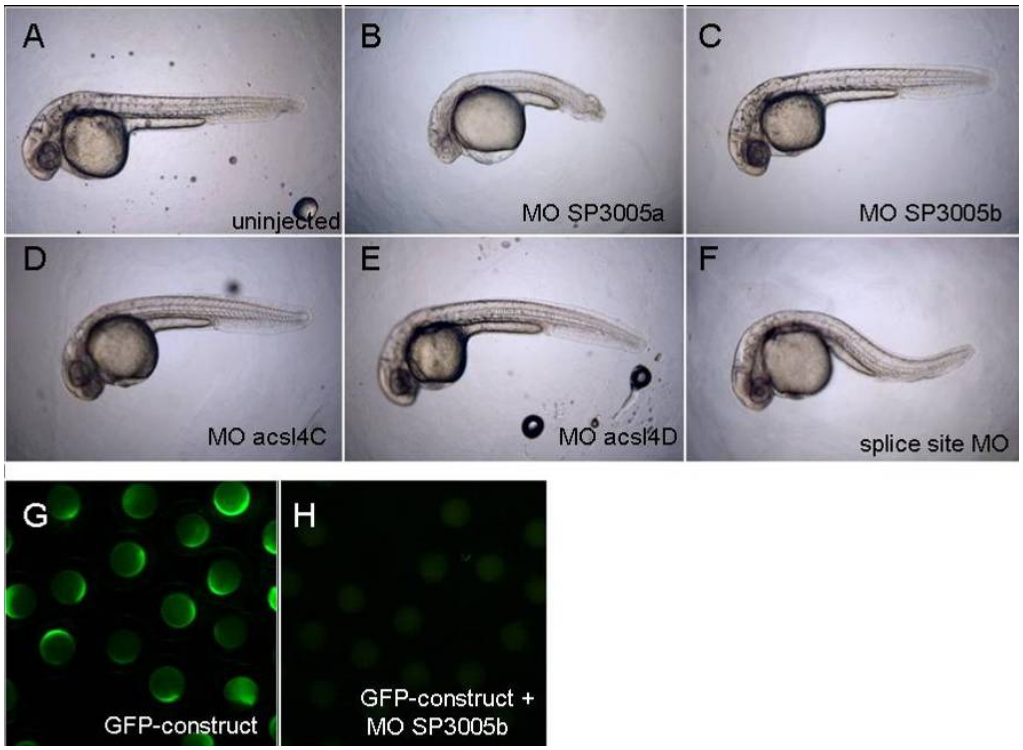


Figure 4-5: Knockdown of Acyl-CoA-Synthetase longchain family member 4a with several morpholinos did not phenocopy the phenotype of MO SP3005a.
Examination of injected embryos at 24 hpf for morphological changes, (A) uninjected control, (B) MO SP3005a show dorsalisation, (C) MO SP3005b, (D) MO acsl4C and (E) MO acsl4D do not show any morphological defects, (F) the splice site MO show no dorsalisation, but a slight bend up tail.
Coinjection of *acsl4a*-5'UTR-GFP construct with MO SP3005b diminished GFP expression (H) in comparison to the control without MO.

4.2.5. Injection of *acsl4a* RNA does not lead to a rescue of the dorsalisation caused by morpholino SP3005a

As a second approach to investigate the specificity of the phenotype caused by morpholino SP3005a, I tried to rescue the dorsalisation of morphants by re-applying *acsl4a* mRNA. For this purpose, I amplified the zebrafish *acsl4a* coding region via PCR from cDNA of 80% epiboly embryos (for primer sequences and cloning see Material and Methods), and cloned it into the pCS2+ expression vector (Rupp et al., 1994). Also I constructed a corresponding mouse *Acsl4* expression construct in pCS2+.

Upon injection of zebrafish *acsl4a* RNA into wild-type embryos various phenotypes could be observed at 24 hpf (Tab. 4-1). Most frequently, embryos displayed a small head and small eyes, always in combination with blood pooling in the ventral fin. In some cases, these defects were combined with the loss of an eye on one side of the embryo. Approximately 50% of the

embryos showed these phenotypes. Interestingly, single embryos showed a fusion of the eyes (Fig. 4-6 A-C), mostly combined with a shortened axis and malformations or absence of the notochord. Embryos injected with RNA in a concentration below 0.125µg/µl did not show any phenotype and grew up as normal wildtypes. The injection of the mouse *Acsl4* construct did not lead to any discernible phenotypes at any concentration.

In several zebrafish mutants, fusion of the eyes results from the absence of the prechordal plate, the anterior-most part of the dorsal mesoderm which is the source of signaling molecules inducing the separation of the eye field during early segmentation stages (Schier and Talbot, 2005). Therefore, I carried out *in situ*-hybridisations of injected embryos for the prechordal plate marker gene *goosecoid (gsc)* (Schulte-Merker et al., 1994a). During normal development, prechordal precursors ingress in marginal regions of the early gastrula embryos, and migrate towards the animal pole during further gastrulation. In contrast, in many of the *acsl4a* RNA-injected embryos, the *gsc*-expressing cells cells remained close to the margin (Fig. 4-6 D-L; n=15/ 23), indicating defects in prechordal plate formation or migration. This disturbed development of the prechordal plate visualised by *gsc* expression at mid gastrula stages was obtained more frequently than the corresponding fusion of the eyes, suggesting that defects could partially heal during further development. However, even for the *gsc in situ*-hybridisations, the strength and penetrance of prechordal plate defects varied from injection to injection, and results could not be consistently repeated.

injected concentration	wildtype	fused eye	fused eye with malformed notochord	small head and eyes, blood pooling in ventral fin	SHE, Blood pooloing and loss of one eye	necrotic or dead embryos at 24hpf
0.125µg/µl	65	3	7	77	29	15
0.2µg/µl	32	6	16	67	35	46

Tabelle 4-1: Morphological phenotypes observed after 24hpf upon overexpression of *acsl4a* (absolute numbers of obtained embryos)

For *SP3005a* MO specificity controls, I co-injected the morpholino with *acsl4a* RNA (which does not contain the endogenous 5'UTR and should therefore not be targeted by the MO). The coinjected embryos were compared with single injected embryos after 24hpf, applying morphological criteria to classify the strength of dorsalisation. In addition, I carried out *in situ*-hybridisations with dorsoventral marker genes at mid-gastrulation. I tested various combinations of concentrations of morpholino *SP3005a* and the zebrafish *acsl4a* RNA.

Morpholino concentrations ranged from 0.05mM to 0.2 mM for the morpholino, thus doses that cause only mild phenotypes up to doses that were nearly toxic. The RNA was injected from 0.02 µg/µl, a concentration that in single injected embryos never yielded any phenotype, to 0.2 µg/µl, the concentration resulting in the range of defects described above. However, in a total of 26 independent co-injection experiments, I never obtained a significant alleviation of dorsalisation strength compared to the controls injected with SP3005a MO alone. Similarly, no sign of rescue was obtained in three additional experiments, in which I co-injected *SP3005a* MO and mouse Acsl4 mRNA.

Consistent results were obtained via *in situ*-hybridisation at mid gastrulation, for instance with a *bmp4* probe, which marks both ventral cell types and prechordal plate precursors (Fig. 4-6 H). Embryos co-injected with MO and RNA displayed a marginal shift of the prechordal plate domain, indicative of the aforementioned effects of forced *acsl4a* expression on prechordal plate precursor cell migration (Fig. 4-6 L). In addition, they displayed a reduction of the ventral *bmp4* domain, indicative of dorsalisation, that was indistinguishable from the phenotype of control embryos injected with the MO alone (compare Fig. 4-6 L with Fig. 4-6 I). This indicates that the RNA failed to rescue the defects caused by the MO.

As further controls of these crucial experiments, I tested the stability and functionality of the injected mRNA. Thus, I carried out agarose gel electrophoresis of the injection solution after its recovery from the injection needle, and obtained normal sized RNA bands, ruling out RNA degradation. Furthermore, I cloned the zebrafish *acsl4a* in-frame into the N-terminus of the *gfp* coding region of the vector Xlt.GFPltCS2+, a derivative of pCS2+ containing *gfp* (Miller et al., 1999). Upon injection of *in vitro*-synthesized RNA of the *acsl4a-gfp* fusion contruct, I could not see GFP expression, which is worrisome. However, the sequences of the *acsl4a* nd *acsl4a-GFP* expression constructs did not reveal major mutations, except for a suboptimal kozak consensus sequence. Furthermore, I could detect transient expression of GFP in embryos injected with linearised *pCS2-acsl4a-GFP* plasmid-DNA, which should be transcribed *in vivo* driven by the CMV promoter of the plasmid (Fig. 4-6 M, N). I conclude that the construct itself is functional, but that the injected RNA might not be stable enough to express GFP. Further overexpression studies will be necessary to obtain more conclusive results. However, for the time being, I do not have any positive results on a rescue of the SP3005a morphant phenotype and thereby for the specificity of the MO effect. Together with the aforementioned failure of phenocopy with other MOs, it seems likely that the dorsalisation caused by the SP3005a MO is an unspecific off-target effect, rather than pointing to an essential ventralising role of *acsl4a* during dorsoventral patterning of the zebrafish embryo.

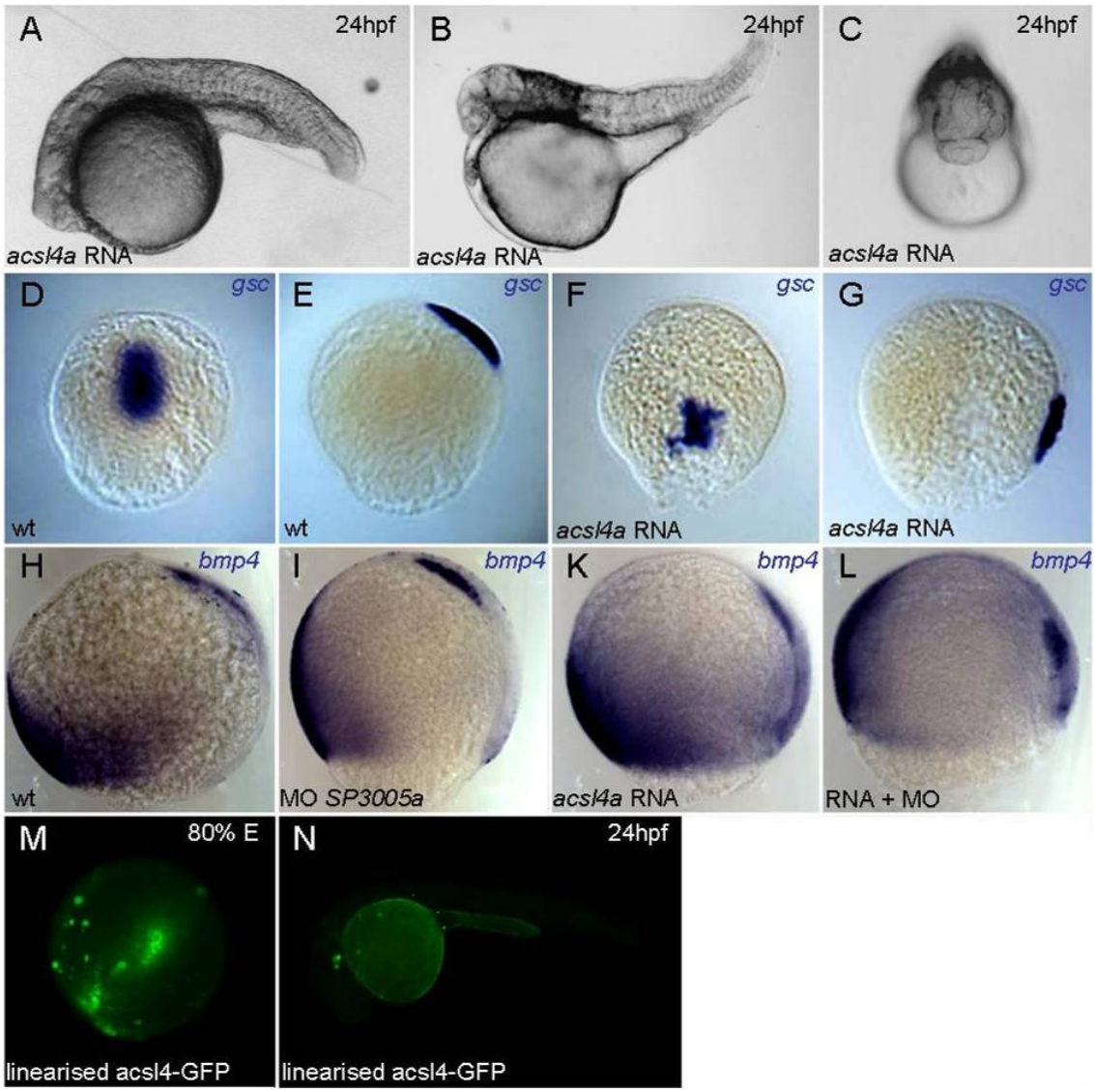


Figure 4-6: Overexpression of *acsl4a* cause defects in the prechordal plate formation, but cannot rescue the phenotype of MO SP3005s

(A-C) live pictures of 24hpf embryos injected with *acsl4a* RNA in a concentration of 0.2µg/ µl.

(D-E) whole-mount *in situ*-hybridisations embryos at 80% epiboly, with a *gsc* probe to mark prechordal plate cells. Prechordal plate cells stay close to the margin in embryos injected with *acsl4a* RNA (F dorsal view, G lateral view) n comparison to the uninjected control (D dorsal view, E lateral view).

(H-L) whole-mount *in situ*-hybridisations using *bmp4* at 80% epiboly in an approach to rescue the dorsalisation phenotype of MO SP3005a by a forced *acsl4a* expression. (H) Uninjected embryos, (I) embryos injected with MO SP3005a show loss of ventral *bmp4* expression, (K) embryos injected with *acsl4a* RNA show a marginal shift of prechordal plate cells, (L) embryos co-injected with of RNA and MO show no restoration of the ventral *bmp4* domain, but still the marginal shift of prechordal plate cells.

(M, N) embroys injected with linearised plasmid of full-length *acsl4a* GFP-fusion construct show transient GFP expression.

4.2.6. Knockdown of *acsl4a* shows no specific defects during gastrulation comparable to loss of PGE_2 signaling

It was shown by Cha et al. that prostaglandin E_2 (PGE_2) plays a role during gastrulation for proper convergence and extension (C&E) and epiboly movements (see General Introduction). Because ACSL4a is supposed to be specifically involved in arachidonic acid metabolism and arachidonic acid is a precursor molecule for prostaglandin synthesis, I compared *acsl4a* morphants to embryos with blocked PGE_2 synthesis by *ntl* (Schulte-Merker et al., 1994b) whole mount *in situ*-hybridisations at the end of gastrulation. *ntl* marks the notochord anlage, which during gastrulation becomes longer and narrower, driven by C&E movements. Thus, the shape of the *ntl* expression domain reflects possible defects during C&E.

As control for disturbed PGE_2 synthesis I treated embryos with 50µM Indomethacin, which is a nonselective inhibitor of cyclooxygenases 1 and 2 (Cha et al., 2005). In addition, I injected the morpholino targeting *prostaglandin E2 synthetase* (*ptges*) as described previously (Cha et al., 2006a). The embryos injected with SP3005b, *acsl4a* MO C or *acsl4a* MO D (each at a concentration of 0.5 mM) showed no difference in *ntl* expression compared to the wild-type control (Fig. 4-7 C-E). The SP3005a morphants displayed a moderately shorter and broader *ntl* expression domain, pointing to slightly compromised C&E movements (Fig. 4-7 B). But defects were much weaker than in Indomethacin-treated and *ptges* MO-injected embryos (Fig. 4-7 H, L). However, C&E defects of a comparable strength as in Indomethacin and *ptges* MO controls were obtained by injection of the *acsl4a splice site* MO (n=33/58 embryos) (Fig. 4-7 F).

To test whether the C&E defects of the *acsl4a splice site* morphants are due to insufficient prostaglandin E2 synthesis, I tried to rescue the gastrulation phenotype by incubating morphants with PGE_2. Treatment with 10µM PGE_2 (from Sigma-Aldrich) led to a reduction of the phenotypic strength of Indomethacin-treated and *ptges*-MO injected controls, indicated by a narrowing of the *ntl* expression (compare Figs. 4-7 I, L with Figs. 4-7 H,K). In contrast, parallel treatment of *acsl4a splice site* morphants with PGE_2 failed to alleviate the defects in the shape of the *ntl* expression domain (compare Fig. 4-7 G with Fig. 4-7 F). In sum, I failed to obtain conclusive results for an essential role of Acsl4a during prostaglandin synthesis in gastrulating zebrafish embryos.

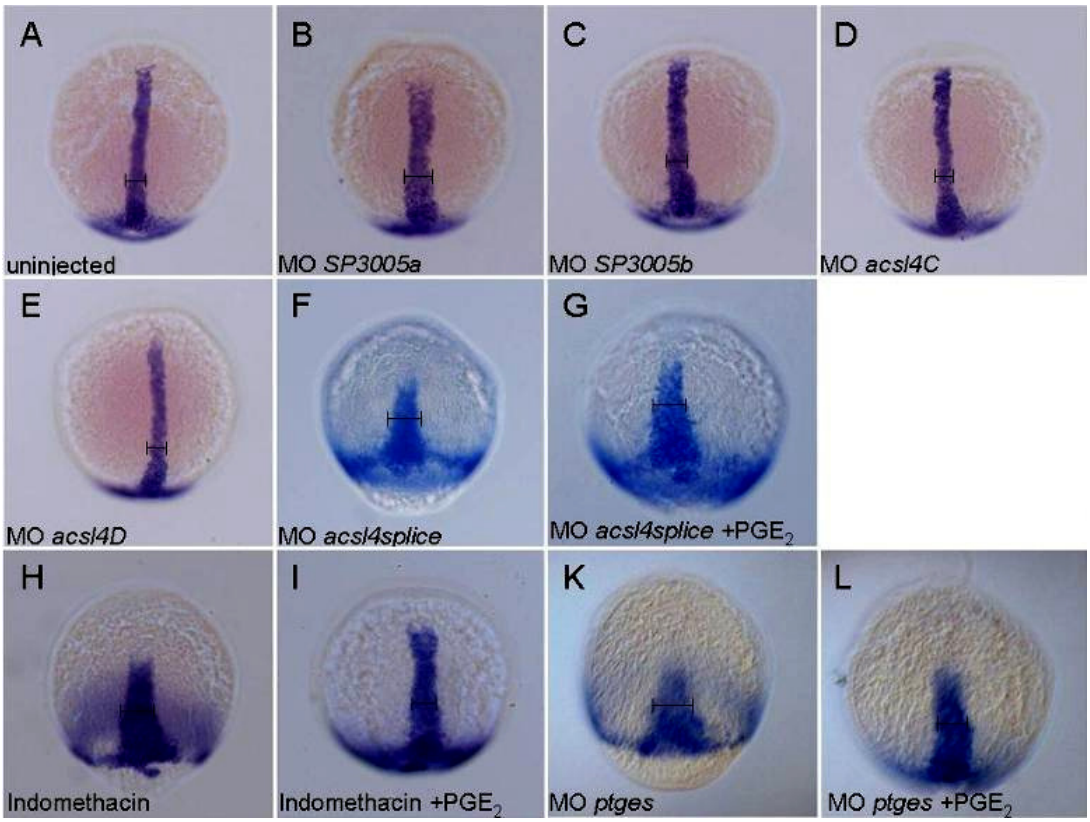


Figure 4-7: Test for epiboly defects of *acls4a* morphants in comparison with inhibition of PGE_2 synthesis.
Whole-mount *in situ*-staining using *ntl*, a marker for the notochord anlage to show compromised C&E movements, in late gastrulation stage embryos. (A) Uninjected control, embryos injected with (B) MO SP3005a, (C) MO SP3005b, (D) MO acsl4C, (E) MO acsl4D, (F) splice site MO, (G) splice site MO and treated with PGE_2. Embryos with affected C&E movements show a shorter but expanded (indicated by bars) expression of *ntl*.
As control embryos were treated with Indomethacin (H, I) or injected with *ptges* MO (K, L), and respective rescue experiment with PGE_2 (I, L) were done as previously described.

4.2.7. Morpholinos targeting genes downstream of *acsl4a* show no phenotype during early development

Arachidonic acid is the precursor molecule for eicosanoids, which are subdivided into four families, the prostaglandins, the prostacyclins, the thromboxanes and the leukotrienes.

Apart from the prostaglandin PGE_2, very limited data exist about the roles of the different eicosanoids during animal development, whereas their roles in cancer and inflammation are well investigated (Moreno, 2009; Patel et al., 2008; Wang and DuBois, 2008).

In collaboration with Steven A. Farber (Carnegie Institution, Baltimore) I searched the zebrafish genome for potential genes involved in the arachidonic acid pathway and eicosanoid production, to be included as potential targets in our large-scale morpholino screen.

The cytosolic Phospholipase A2 (zcPLA2) is an intracellular Phospholipase required for the release of arachidonic acid from phospholipid membranes, the initial step of eicosanoids production from stored arachidonic acid sources. I investigated the effect obtained by injection of *zcPLA2* MO via whole mount *in situ*-hybridisations with probes for different dorsoventral marker genes. No difference between control and morphant embryos could be seen during mid gastrula stages in the expression domains of *otx2*, a marker for dorsal ectoderm (see above) and *tbx6*, a marker for ventrolateral mesoderm (Hug et al., 1997). Furthermore, no difference in the expression of in *pax2.1* (Krauss et al., 1991) in the central nervous system, spinal chord or the pronephric duct, a derivative of the ventral mesoderm, was visible at mid segmentation stages (Fig. 4-8 A-D). Therefore zcPLA2 seems to have no role during early patterning.

My collaborator Steven A. Farber provided me with some evidence from mass-spectrometry analysis of *acsl4a* morphants, according to which levels of 5(S)-Hydroxyeicosatetraenoic Acid (HETE) are altered. Hydroxyeicosatetraenoic acids, which are made from arachidonic acid by lipoxygeneases (see above), were already described to regulate head formation and regeneration in *Hydra spec.* (Hassel et al., 1996; Muller et al., 1998). Therefore morpholinos against ESTs encoding lipoxygenases were included in the screen. Three different morpholinos targeting potential *5-lipoxygenases* (*lox5*), two designed by myself, and two morpholinos targeting *12-lipoxygenase* (*lox12*), one designed by myself, did not show any phenotype when injected in wild-type embryos. Furthermore, co-injection of one of these morpholinos with MO SP3005a did not change the strength of dorsalisation (not shown). In sum, by screening morpholinos, no further member of the arachidonic acid and eicosanoid pathway could be identified to play a role during early developmental stages of zebrafish.

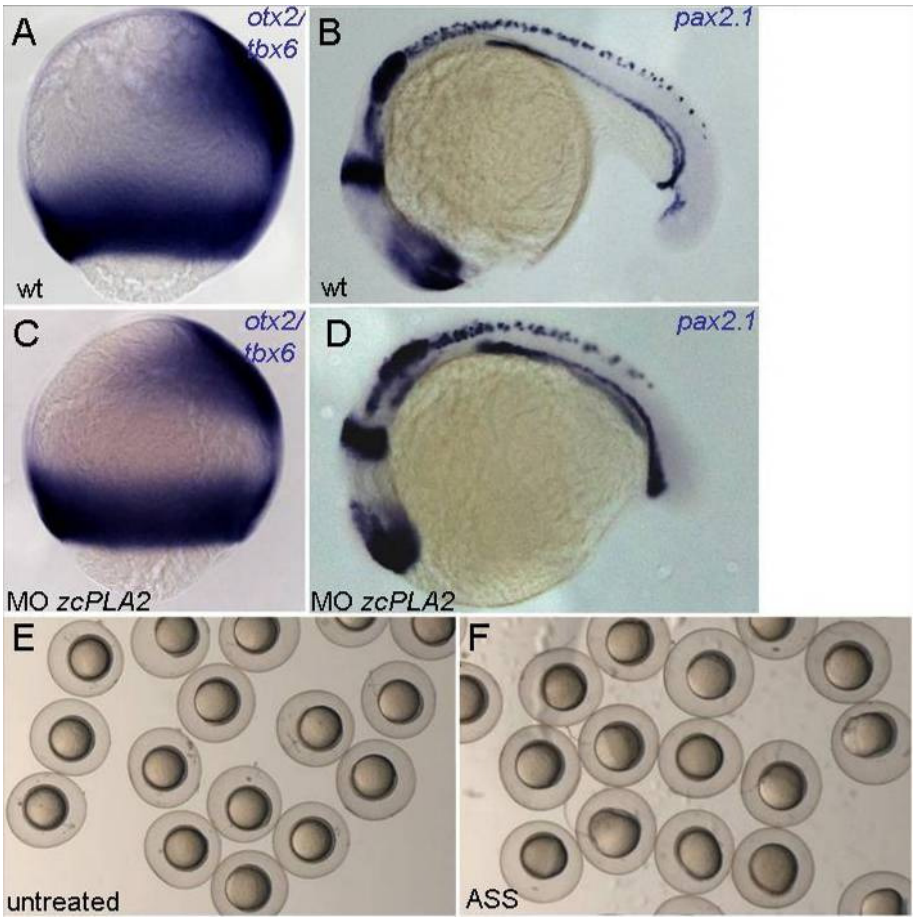


Figure 4-8: Only components that directly interfere with prostaglandin synthesis show defects in early embryonic development
whole-mount *in situ*-hybridisations of uninjected (A, B) and with *zcPLA2* MO injected (C, D) embryos. (A, C) Markers for DV patterning *otx2* for dorsal ectoderm and *tbx6* for ventrolateral mesoderm in a combined approach at 80% epiboly. (B, D) *pax2.1* as marker for the central nervous system, spinal chord or the pronephric duct during somitogenesis.
(E, F) live images of embroys treated with acetylsaliacylacid (ASS) show delayed or stopped gastrulation (F) compared to the untreated control (E).

4.2.8. Treatments with agonist and antagonist of the arachidonic acid pathway

In addition to the morpholino-based approach, I started an analysis of known agonist and antagonist of the arachidonic acid and eicosanoids pathways. One of the best-known antagonists of prostaglandin synthesis is Acetylsalicylsäure (ASS, also known as Aspirin). Embryos incubated during gastrulation stages with 1 mg/ml ASS solution showed delayed or stopped gastrulation (Fig. 4-8 F). The effectiveness differed, depending on the supplying company of ASS, the time point of administration and also the used wild-type strain. Thus, embryos from the AB line could tolerate double as much ASS as embryos of the Tl/Ek line. Furthermore, when ASS was administered after the shield stage (6 hpf; early gastrulation), the survival rate was much higher and phenotypes weaker than upon treatments starting before

the onset of gastrulation (data not shown). Since ASS inhibits the Cyclooxgenases COX-1 and COX-2, these results are in accordance to previous results obtained with *cox* morpholinos (Cha et al., 2006a; Cha et al., 2005). In the following, I used ASS as a positve control for experiments testing further compounds. All of these compounds were tested in at least two independent experiments with freshly prepared solutions. Incubation of embryos with arachidonic acid (Calbiochem), tested in dilution series from 2.5 µg/ml to 2.5 mg/ml, caused no specific developmental defects, but displayed general cellular toxicity leading to embryonic death when applied above 1 mg/ml concentrations. At these higher concentrations, the embryos became necrotic soon after the AA-solution was added, however, no specific developmental defects, e.g. during gastrulation, were observed. Similar results were obtained using arachidonic acid from Sigma-Aldrich, except that this solution was toxic from 25 µg/ml upwards. Finally, treating SP3005a morphants as well as zebrafish *acsl4a* RNA injected embryos with sub-lethal concentrations of AA did not lead to any shifts in the strength or penetrance of their defects.

Because of the mass-spectrometry data supplied by Steven A. Farber indicating higher r5(S)-HETE levels in SP3005a morphants, I also incubated wild type, SP3005a MO-injected and *acsl4a* mRNA-injected embryos in solutions of 5(S)-HETE, as well as the related 12(S)-HETE, ranging from 3 nM to 150 µM concentrations. These concentrations cover all thus far described biological active concentrations. But in six independent experiments, I never obtained an effect of one of the HETEs on early zebrafish development, including dorsoventral patterning.

Furthermore, I tested inhibitors of phospholipases. The sPLA2-IIA Inhibitor I (Church et al., 2001) has reported working solution from 100 nM to 10 µM. However, incubation of embryos in a solution concentrated up to 35µM did not harm the development in any way, indicating that the function of secreted phospholipase is dispensable during early development. Next I tested $AACOCF_3$ (Arachidonyltrifluoromethyl Ketone), an analogue of arachidonic acid that selectively binds and inhibits cytosolic Phospholipase A2 (Street et al., 1993). The treatment with 20 µM $AACOCF_3$ slowed down gastrulation, but the embryos recovered during further development, and did not unveil any morphological differences compared to the control at 24 hpf. Also it did not alter the strength of the SP3005a morphant phenotype.

Finally, I treated embryos with a bunch of antagonists of leukotriene and hydroxy-eicosa-tetraenoic acid production. The benzoquinone dervivate AA-861 is a competive inhibitor of 5-Lipoxygenase (Ashida et al., 1983; Wang et al., 2000). In a series with highly concentrated

compound (3 mM to 30 mM), I found that embryos incubated in a 6 mM solution showed slight developmental retardation at 24 hpf, but no morphological malformations. Also, AA-861 did not rescue SP3005a morphants, although one would expect this, if the dorsalisation of was due to the observed increase in 5-(S)-HETE levels in morphant embryos. Another inhibitor of 5-lipoxygenase is MK-886, which binds FLAP (5-lipoxygenase activating protein) (Ford-Hutchinson, 1991). MK-886 was tested up to concentrations of 5 µM, five times higher than the reported working solutions, but did not give a phenotype. Caffeic acid (3,4-Dihydroxycinnamic Acid) is a natural anti-oxidant in plants and has been reported to be a potent 5-lipoxygenase inhibitor (Koshihara et al., 1984). At a concentration of 5 mM, it caused slowed down gastrulation and caused developmental retardation at 24hpf. Lower concentrated solutions (2.5mM) led to the formation of smaller heads and smaller eyes. However, they did not lead to a reduction of dorsalisation in SP3005a morphants. Instead, embryonic lethality was increased, indicating an enhanced toxicity. A selective inhibitor of 12-lipoxygenase, named Baicalein (Sekiya and Okuda, 1982), was tested from 40 to 400µM without any effect.

In summary, none of the tested chemical compounds interfered with early patterning in a specific manner like MO SP3005a. Therefore, I was not able to further unveil the role of arachidonic acid pathways during axis specification of zebrafish.

4.3. Discussion

4.3.1. Knockdown of *acsl4a* create a new role for arachidonic acid metabolism during early embryonic development

While I was screening for morpholinos causing phenotypes during early development I found a morpholino that led to a loss of dorsal structures similar to phenotypes described in mutants with compromised or absent BMP signaling (Hammerschmidt and Mullins, 2002). This morpholino, named SP3005a, targets the 5'UTR of the mRNA of *acyl-CoA synthetase longchain family member 4a* (*acls4a*). Zebrafish Acsl4 genes and proteins had not been described so far, but some information was available from their mammalian orthologues.

I characterised the phenotype of SP3005a morphants as a general dorsalisation of the embryo, as evidenced by the shifts of the expression domains of dorsal and ventral ectodermal and mesodermal markers at mid-gastrula stages and by morphological criteria at 24 hours post fertilisation (Fig. 4-2). Consistent alterations were observed in the expression of the three bmp genes that are essential for dorsoventral patterning of the zebrafish embryo, *bmp2b*, *bmp4* and *bmp7* (Fig. 4-3). Because the BMP signaling gradient depends on its own positive feedback loop, these data did not allow to distinguish whether the SP3005a MO interfered with dorsoventral patterning upstream or downstream of BMP signaling. For this purpose I had to perform epistatic analyses, combining the two opposite effects obtained by gain of function of one gene and loss of function of the other. In one combination, with constitutive BMP signaling (ventralisation) combined with loss of Acsl4a activity (dorsalisation), I could show that *Acsl4a* does not act downstream of the BMP signaling pathway (Fig. 4-3). To distinguish whether it acts upstream of BMP signaling or in a parallel pathway, it would have been necessary to combine opposite effects obtained by gain of Acsl4a function and loss of BMP signaling. However, unfortunately, overexpression of *acsl4a* did not cause a ventralised phenotype. So, two scenarios are possible. In the first, Acsl4a acts upstream of and via BMP-signaling. Acsl4a may mediate the production of certain metabolites of the arachidonic acid cascade that either directly promote the transcription of *bmp* genes or components of the transduction pathway or that affect their gene products at the post-translational level. Recently, the canonical Wnt signaling and prostaglandin signaling pathways were linked, where prostaglandin can activate ß-catenin-dependent transcription via Axin (Castellone et al., 2005). A similar mode of action might be true for the BMP pathway, for example on the level of phosphorylation of SMADs. I showed that the initiation of *bmp* expression is unaffected and the expression is progressively lost during gastrulation. A possible scenario

would be that expression of *bmp*s is initiated in an Acsl4a-independent fashion, but an arachidonic acid-derived signaling pathway interferes with SMAD-dependent signal transduction and thereby the maintenance of *bmp* expression. Interestingly, a similar connection between ACSL4 and BMP might also exist in the fruit fly Drosophila, melanogaster. Thus, according to a recent report (Zhang et al., 2009), Drosophila mutants in the fly ACSL4 homologue display reduced production of the Dpp, the fly BMP2/4 homologue, and corresponding defects in the development of glial cells and neuronal wiring in the larval brain. However, the exact mechanism underlying this connection between Ascl4 and Dpp/BMP remained elusive. Eicosanoids are known to act mainly via G-Protein coupled receptors that opens wide field of downstream signaling mechanism (Dey et al., 2006). One of the possible mediators, MAPK, has been shown recently to inhibit SMAD signal transduction (Pera et al., 2003).

Cooperative functions of BMPs and eicosanoids are also known from other examples. Various Prostaglandins, the major eicosanoid subgroup, have been shown to cause an augementation of bone mass and induction of osteoblastic differentiation. *In vitro* studies describe an upregulation of BMP levels upon prostaglandin treatment (Arikawa et al., 2004; Damrongsri et al., 2006a; Damrongsri et al., 2006b; Li et al., 2007; Paralkar et al., 2003; Toyoda et al., 2005). It is likely that similar pathways are functional during early embryonic development. The exact mechanisms by which the eicosanoid and BMP pathways interact with each other are still unknown. Cha et al. (2005) have indirectly excluded BMPs as potential downstream targets of the Prostaglandin E2 pathway, showing that PGE_2 regulates cell migration processes, rather than dorsoventral patterning of the zebrafish gastrula.

In a second scenario, *acsl4a* and its downstream effectors could affect dorsoventral patterning independently of and in parallel to the BMP pathway. Because the expression of *bmps* depends on a feedbackloop, the down-regulation of *bmp* expression would in this case just be a secondary consequence of the dorsalisation. Eicosanoids can bind and activate Peroxisomal proliferator-activated receptors (PPARs) (Forman et al., 1995; Gupta and Dubois, 2002; Kliewer et al., 1995; Wahli et al., 1999). PPARs are nuclear receptor that function as transcription factor, usually working together with coactivators or corepresosors, upon binding to free fatty acid or eicosanoids (Berger and Moller, 2002; Feige et al., 2006; Schroeder et al., 2008). PPARs have diverse biological functions, e.g. they are known to be involved in adipocyte development (Moldes et al., 2003; Park et al., 2008). No investigations have been performed on the role of PPARs during early development, keeping this option speculative.

The knockdown of *acl4a* by morpholino SP3005a shows a possible new role for acyl-coA Synthetase longchain family member 4 and its derivates in patterning the dorsal-ventral axis during gastrulation. Dorsalised embryos, as for instance obtained by loss of BMP signaling, in addition to dorsoventral patterning also display defects during convergence, one of the morphogenetic movements of gastrulation (Myers et al., 2002; von der Hardt et al., 2007). Thus, the dorsalisation of SP3005a morphants includes some, but not all of the phenotypic traits caused by loss of PGE2 function (compromised convergence, extension and epiboly; Cha et al., 2005). In this light, it remains unclear why the loss of an arachidonic acid derivative can cause additional defects that are not obtained upon loss of its upstream substrate. Also, it remains unclear via which eicosanoid arachidonic acid affects dorsoventral patterning (in case the effect of the SP3005a MO is specific; see below).

4.3.2 *acsl4a* is expressed in the yolk syncytial layer of the gastrulating embryo, from where it might regulate dorsoventral patterning of the blastoderm

Zebrafish *acsl4a* is predominantly expressed in the yolk syncytial layer (YSL) during gastrulation stages. The YSL origantes from marginal blastomeres that do not form a membrane towards the yolk cell during cleavage stages, and release their nuclei into the yolk during blastula stges, so that a yolk syncytial layer consisting of multiple nuclei is formed beneath the deep cells. The YSL plays an important role in establishing the dorsoventral axis, since the accumulation of ß-catenin in some nuclei determines the future dorsal side (Nojima et al., 2004; Pelegri and Maischein, 1998). But obviously the initiation of the dorsal side in SP3005a morphants is not affected, because the gradient of BMP-expression is normal until the onset of gastrulation. Thus, acsl4a seems to be largely required for later steps of BMP gradient modification and maintenance during gastrulation, conistent with the time course of zygotic *acsl4a* expression, which is initiated at the shield stage and strongest throughout gastrulation (Fig. 4-4.). By RT-PCR I also found maternally supplied *acsl4a* transcript, however, there is no functional indication that *acsl4a* might play a role before the onset of gastrulation. During later development, *acsl4a* transcripts became apparent in the embryonic brain, which is along with of the reported expression of mammalian and Drosophila ACSLs (Soupene and Kuypers, 2008; Zhang et al., 2009).

It is not known, where and it which form arachidionic acid is stored in the zebrafish embryo. From the *acsl4a* expression pattern, it seems very likely that a major source and storage

compartment is the yolk. The yolk cell provides the embryo with all necessary nutritions and is its energy source until the larva starts eating at day five of development (Poupard et al., 2000). I tested if expression of *acsl4a* in the YSL is necessary for dorsoventral patterning of the blastoderm by injection of MO SP3005a solely into the yolk cell of blastula stage embryos. Using this approach of spatially restricted MO application, I obtained embryonic dorsalisation as upon MO application into the entire embryo, suggesting that Acsl4a protein is necessary in the YSL for dorsoventral patterning. However, the penetrance of the phenocopy was very low. This could have technical reasons. But it also cannot be ruled out that Ascl4a acts in both the YSL and the blastoderm.

How could Ascl4a function in the YSL affect *bmp* gene expression and dorsoventral patterning in the blastoderm? ACSL4 proteins do not seem to possess specific membrane tags or transmembrane domains; however, based on *in vitro* data, they appear to act in the cytoplasm close to nuclear membranes. Thus, Ascl4a present in the YSL of zebrafish embryos could regulate the availability of arachidonic acid (AA) by might promoting its storage as integral parts of phospholipids within the outer membrane of YSL nuclei, in close neighborhood of the blastoderm. Upon phospholipase-dependent release from the nuclear membranes, free AA or its derivatives could diffuse or be brought to the blastoderm to fulfil their signaling function. It is unclear which of AA's derivatives mediates its effect on dorsoventral patterning, and whether it is directly generated in the YSL or in the blastoderm. Cyclooxygenases, which are crucial for prostaglandin synthesis from AA, seem to be exclusively expressed in the blastoderm. At least no specific expression in the YSL is mentioned in the corresponding publication (Cha et al., 2005). So, either the blastoderm cells have an intracellular level of arachidonic acid that is sufficient for prostaglandin production or free arachidonic acid from the yolk cell is provided to the blastoderm by an unknown mechanism. However, things could be different for other AA derivatives, which might be generated in the YSL itself. Attractive candidates mediating AA and Ascl4 effects downstream of eicosanoids of the lipoxygenase pathway are the steroids. Thus, release of AA in a cPLA2-independent pathway is required for the expression of StAR protein (steroidogenic acute regulatory protein), a critical regulator of steroid biosynthesis (Cho et al., 2000;Wang et al., 2000; Maloberti et al., 2005; Stocco et al., 2001). Future studies have to reveal whether zebrafish *star* genes are expressed during gastrulation, and, if so, whether expression is confined to the YSL or the blastoderm.

4.3.3 The phenotype of SP3005a morphants might be caused by off-target effects

Dorsalisation is usually a very specific phenotype that has not been obtained as an artifact in any of the over 300 antisense MOs used in the Hammerschmidt laboratories. Nevertheless, there are substantial doubts about the specificity of the phenotype of *SP3005a* morphants, In attempts to confirm that the dorsalisation by the *SP3005a* MO is indeed due to the loss of Acsl4a function, I tested further *acsl4a* MOs and tried to rescue the phenotype of *SP3005a* morphants by co-injecting the MO with *in vitro* synthesized full-length *ascl4a* mRNA that cannot be targeted by the MO. However, none of four other *acsl4a* MOs generated dorsalised phenotypes, even when injected together to compensate for possible problems in the efficacy of Acsl4a knock-down. Two of these other MOs were even found to efficiently knock down *acsl4a* via scoring of GFP suppression or RT-PCR analysis, respectively. In sum, only one out of three MOs with *acsl4a* confirmed targeting *acsl4a* mRNA caused a dorsalisation phenotype, whereas the other two and two further, uncharacterized MOs did not..

For rescue and overexpression studies, I cloned the coding region of the *acsl4a* cDNA into an expression vector that can be used for *in vitro* mRNA synthesis. The SP3005a MO sequence is complementary to nucleotides -38 to -13 in the 5' UTR of the *acsl4a* mRNA. Since this sequence is absent from the *acsl4a* expression constructs, the injected *in vitro* synthesized mRNA will not be targeted by the MO. Injection of *acsl4a* mRNA into wild-type embryos caused in few cases defects in the prechordal plate migration, as revealed by *in situ*-hybridisation for the prechordal plate marker gene *goosecoid* during midgastrulation stages. Since the PGE_2 pathway interferes with PI3Kinase activity (Cha et al., 2006a) and PI3Kinase is involved in the transduction of chemotatic signals in migrating cells at the anterior edge of the prechordal plate (Montero et al., 2003), it is tempting to speculate that *acsl4a* overexpression might cause the prechordal plate defects by interfering with PI3Kinase activity. Thus, forced expression of *acls4a* could lead to ectopic generation of arachinoic acid derivatives and ectopic PI3Kinase activation in more posterior prechordal plate cells, thereby confusing chemotaxis and directed cell migration. However, this phenotype was obtained at rather low frequencies, and it is unclear whether it is specific (see also below).

When coinjected at variable concentrations together with the *SP3005a* MO, *acsl4a* mRNA never caused a significant alleviation of the dorsalisation, providing further support for the suspision that the dorsalisation might be caused by an off-target effect of the MO. On the other hand, I have to admit that the failed rescue experiment is not fully conclusive, because the used mRNA might not be fully functional. To test its functionality, I had modified the used *acsl4a* expression construct by fusing the GFP coding region in frame to the 3' end of

the *acsl4a* coding region. However, mRNA from this fusion construct failed to give GFP fluorescence in mid gastrula stage zebrafish embryos after injection at the 1-cell stage. In addition, sequencing revealed a missense mutation in the Kozak sequence directly adjacent to the *ascl4a* start codon. Therefore, it is possible that the injected *acsl4a* mRNA is not properly translated, which would explain the failed rescue of the morphant phenotype, and the low frequencies with which specific defects were obtained upon *acsl4a* overexpression in wild-type embryos. Similar experiments with *acsl4a* mRNA after repair of the Kozak site mutation have to be carried out (see also below; outlook and perspectives).

4.3.4 Further components of the arachidonic acid cascade can most likely be excluded as regulators of dorsoventral patterning

Beside their role in inflammation, where they were initially described, eicosanoids are known to be involved in many developmental processes. Two of the best studied developmental processes regulated by eicosanoids are ovulation and implantation of mammalian blastocysts (Dey et al., 2004; Wang and Dey, 2005). The specific role of prostaglandins during oocyte maturation and ovulation is evolutionary conserved and has been found in many organisms, including non-mammalian species, like *Xenopus* (Sena and Liu, 2008) and several teleosts including zebrafish (Lister and Van Der Kraak, 2008; Sorbera et al., 2001), and even invertebrates like the fruitfly *Drosophila melanogaster* (Tootle and Spradling, 2008). The major prostaglandins involved are PGE_2 and PGF_2, which act via their G-Protein coupled receptors (GPCR) on several signal transduction pathway members like PI3K-Akt, PKA, JNK-NFkappaB or MAP kinases (Takahashi et al., 2006). The regulation of steroidgenesis by eicosanoids is a very important mechanism. In this context, a direct role of ACSL4 has been described, which together with the mitochondrial acyl-CoA thioesterase 1 (MTE-1, also called AA-related thioesterase involved in steroidogenesis ARTISt) was shown to be crucially involved in the regulation of steroid production (Stocco et al., 2005). Consistently, *Acsl4* knock-out in mice causes reproductive defects in heterozygous females, while the phenotype of homozygous mutants has not been reported as yet. However, hemizyous Ascl4-/Y males, which also should be Ascl4 nulls, appear normal (Cho et al., 2001). Homozygocity for amorphic mutations in Drosophila causes lethality during larval stages, and mutants are characterized by glial and neuronal wiring defects in the brain (Zhang et al., 2009), in line with the non-syndromic X-linked mental retardation (MRX) associated with ACSL4

mutations in human.

The *Drosophila* work also points to a role of Ascl4 in the regulation of Dpp/BMP production in some, but not all Dpp expression sites (Zhang et al., 2009). Since mutants make it through embryogenesis, it is likely that early Dpp required for embryonic D-V patterning is normally expressed. In sum, there is no explicit linkage of ACSL proteins to axis and body plan formation described in any organism thus far.

In zebrafish, little is known about the arachidonic acid cascade and the eicosanoid signaling pathways in general. Only the cyclooxygenase and prostaglandin pathway has been investigated so far (Cha et al., 2006b; Grosser et al., 2002; Lister and Van Der Kraak, 2008; North et al., 2007; Pini et al., 2005; Teraoka et al., 2009; Yeh and Wang, 2006). The most prominent prostaglandin in the zebrafish embryo is PGE_2, whereas other prostaglandins are not or only weakly expressed (Cha et al., 2006a; Yeh and Wang, 2006).

Mass-spectrometry data from my collaborating laboratory of Steven A. Farber showed increased levels of 5-S- hydroxy-eicosa-tetraenoic acid (HETE), a product of the 5-lipoxygenase pathway, during somitogenesis (Steven A. Farber, personal communication). Studies in the past showed patterning effects of HETEs in *Hydra spec*. These studies, mainly done by Werner Müller in Heidelberg, revealed a role of the arachidonic acid cascade in pattern formation along the body axis of hydrozoans. Arachidonic acid and Diacylglycerol were shown to induce ectopic head formation with supernumerious tentacles, via Proteinkinase C as key regulator. In *Hydra*, arachidonic acid seems to work as morphogen, which appears to act at least partly via its main hydrozoan metabolites, the hydroxy-eicosa-tetraenoic acid (HETE) lipoxygenase products, predominately 12-S-HETE (Hassel et al., 1996; Muller et al., 1998). More recent studies also revealed the presence of an active BMP gradient along the head to foot axis of *Hydra*, with the head constituting an organizer-like structure that expresses a chordin ortholgue (Rentzsch et al., 2007). A link or cooperative role of the arachidonic acid cascade and BMP signaling even in such an evolutionary ancient phyla, would be perspicuous, but has not been shown.

Based on these findings, HETEs and further lipoxygenase products were excellent candidates as the effective mediators of arachidonic acid and Acsl4a during zebrafish D-V patterning. Therefore, I performed an exhaustive test series, trying to phenocopy or rescue the dorsalisation of SP3005 morphants with 5(S)-HETE or a range of different lipoxygenase antagonists. As positive controls, I used the previously described cyclooxygenase drugs Indomethacin and Acetylsaliacyl acid. However, none of the lipoxygenease products or inhibitors yielded positive results. I also identified putative zebrafish *5-lipoxygenase* and *12-*

lipoxygenase genes, and designed MOs against them. But neither injections into wild-type nor into *SP3005a* morphant embryos had any effect. A further detailed examination of lipoxygenases in zebrafish is necessary, since it is possible that there are further lipoxygenases that could be redundant to the two I have identified and knocked down.

However, the results obtained with both the chemical drugs and the MOs suggest that hydroxy-eicosa-tetraenoic acids are dispensable for early zebrafish patterning. Because the targeted lipoxygenases are also required for the synthesis of leukotrienes, leukotrienes can be ruled out as potential effectors as well.

Finally, to get more functional data for the requirement of arachidonic acid itself, I targeted phospholipases with chemical antagonists and MOs. The phospholipases, mainly the Cytosolic Phospholipase A2 (cPLA2) are the key enzymes in releasing arachidonic acid from phospholipids and making it available for eicosanoid synthesis. The laboratory of Steve Farber identified the Zebrafish homologue of zcPLA2 and designed a specific MO. However, in none of the approaches, I obtained dorsoventral or gastrulation defects, or an alleviation of the dorsalization of SP3005a morphants, as would be expected if zcPLA2 or related phospholipases played an essential role during early zebrafish development.

As in the case of the lipoxygenases described above, one reason for the lack of phenotype of the morphant could be the existence of paralogues with redundant functions. To circumvent this problem, I also used antagonists that should target all enzymes of one class. However, they were ineffective as well. This approach might have suffered from technical problems, since the embryos had to be treated for at least five hours at 28°C in aqueous solution, which might have affected the stability of the compounds, decreasing the effectivity and maybe producing toxic by-products. The same might be true for the treatment with the metabolites like the HETEs themselves. I tried to prevent such problems by using titration series and by regular exchange of the solutions. Also, the treatments with the cyclooxgenase inhibitors interfering with prostaglandin signaling did work, leading to the same defects as in *cox* morphants, suggesting that technical problems might only play a minor role. Nevertheless, they cannot be completely ruled out, which means that my negative results are not fully conclusive.

In summary, I could not obtain any positive results pointing to a role of arachidonic acid metabolits during early patterning of the zebrafish embryo, which would be consistent with the dorsalised phenotype of *SP3005a* morphants.

4.3.5 Outlook and perspectives

There is a bunch of data suggesting that the dorsalisation caused by the SP3005a MO might be an off-target effect. On the other hand, dorsalisation is a very clean phenotype, which usually is not obtained by unspecific effects. Thus, and in light of the reported roles of BMP-eicosanoid cooperations in multiple other instances, it remains possible that Acsl4 enzymes do play an essential role during dorsoventral patterning of the zebrafish embryo. One explanation for the failed rescue of SP3005a morphants with *acsl4a* mRNA could be problems with the translation rates of the exogeneous mRNA (see above). An explanation of the failed phenocopy by four other *ascl4a* MOs could be functional redundany between *ascl4a* and its paralogue. Indeed, a second *ascl4* gene has been identified in zebrafish and is annotated in the Zebrafish Model Organism Database Zfin as *acsl4l*. Like *acsl4a*, *acsl4l* is strongly expressed in the YSL of gastrulating zebrafish embryos (http://zfin.org). Unfortunately, the 5' region of the *acsl4l* cDNA has not been identified as yet, making it impossible to design *acsl4l*-specific ATG or 5' UTR MOs. Also, I could not look up whether the SP3005a MO, but not SP3005b and the other *ascl4a* MOs, might target both *acsl4* paralogues. My initial experiments with an MO targeting an *acsl4l* splice site (generous gift of Steven A. Farber) indicate that *ascl4l* is dispensable for early zebrafish development. However, considering a redundant function of the two Acyl-CoA synthetase longchain family member 4 enzymes, it will be important to knock down both concomitantly in co-injection experiments.

To identify the relevant arachidonic acid metabolites, more systematic approaches might be more fruitful. First, a more thorough investigation of the lipids present in the early embryo during blastula and gastrula stages should be carried out. The obtained profile should give hints about the pathways of lipid signaling active in the early embryo. This profiling has to be done by mass-spectrometrie. Second, a more detailed analysis of enzymes and receptors present in the early embryo has to be performed. Via the expression patterns one could nail down the site of signal production and the sites of action. Also the identification of possible paralogues will be helpful for later knock-down experiments. Finally, large-scale chemical compound screens similar to the one that led to the identification of PGE_2 as regulator of hematopoetic stem cell homeostasis (North et al., 2007), could be carried out as an unbiased approach to identify lipid regulators of early zebrafish development. Results obtained in zebrafish will most likely also be relevant for other species. Clearly, the patterning and regeneration phenotypes observed in *Hydra spec.* strongly suggest that lipid signaling might be an evolutionary conserved patterning mechanism.

5. Studies for cell commitment during early gastrulation stages

5.1. Introduction

5.1.1. During gastrulation cells are specified towards their final fate in a region- and time-dependent manner

During development, a multi-cellular organism consisting of a whole range of different cell types is formed from a single cell, the fertilized oocyte, also called zygote. Thus, the zygote and its direct progenitors, the germ cells, are omnipotent, and can give rise to any cell of the later body. The same is usually true for most cells of the early embryo, which for instance revealed by the plasticity of "embryonic stem cells" derived from early stage mouse embryos. However, during the course of early development, most embryonic cells become progressively specified and less plastic, until they eventually undergo terminal differentiation, with their characteristic function, shape and gene expression. Important specification steps take place shortly before and during gastrulation, such as induction of mesodermal and endodermal cell fates, and determination of differential dorsal-ventral fates. Different approaches have been undertaken to determine the future fate and specification status of the cells of pre-gastrula embryos. Results on future cell fates are summarised in so-called "fate maps". Such fate maps are generated by cell lineage analyses, as done for example in the nematode *Caenorhabditis elegans* by directly following the development of cells *in vivo* via time-lapse microscopy (Sulston et al., 1983), or by comparing subsequent developmental stages, as for example done in the fruitfly *Drosophila* (Hartenstein and Campos-Ortega, 1985). A second method is to ablate cells and to analyse the structural defiencies caused by this, as already done in the late 1970s in *Drosophila* (Lohs-Schardin et al., 1979). A third method is the lineage tracing with dye-labelled cells, a method that was intially used in the clawed frog *Xenopus laevis* (Heasman et al., 1984), but later also in zebrafish (Kimmel and Law, 1985a; Kimmel and Law, 1985b; Kimmel and Law, 1985c).

Full zebrafish fate maps have been generated for late blastula stages, revealing crucial spatial differences both along the animal-vegetal and the dorsal-ventral axis (see below). However, similar experimental approaches have also revealed particular differences during earlier stages of development, such as the establishment of dorsal-ventral polarity, which is already obvious during early cleavage stages (Abdelilah et al., 1994; Helde et al., 1994), and the segregation of the Yolk Syncitial Layer (YSL) and Enveloping Layer (EVL), which occurs during early blastula stages (Kimmel and Law, 1985b,c). Compared to Xenopus, specification steps in

zebrafish occur rather later. Thus, the D-V axis of Xenopus embryos is determined by cortical rotation of the fertilised egg, before the first cell cleavage (Moon and Kimelman, 1998). Also, the different cell fate domains of the zebrafish late blastula embryo are broader and more overlapping, with comparably many cells that can still give rise to more than one cell type (Fig. 5-1). The cells located closer to the animal pole will give rise to the ectoderm. Cells on the ventral side of the ectoderm will contribute to the epidermis, whereas cells on the dorsal side form the neuroectoderm. The anterior-posterior axis in the neuroectoderm is established according to the animal-vegetal axis. Cells are directed towards forebrain fates when they are closer to the animal pole, and cells further marginal will become mid- and hindbrain. Precursors of the spinal cord and neural crest can be also found on the ventral half, but in more marginal zones than the epidermal precursors. The region close to the margin gives rise to the mesodermal derivates. Here, dorsal-most cells will form prechordal plate and notochord, which are later positioned in the midline of the body axis. Muscles of the head derives from dorsal-lateral positions, while somite-derived muscles of trunk and tail stems from more lateral and ventral positions, respectively. The heart comes from lateral zones, while blood and pronephros derive from the ventral mesoderm. The presumptive endoderm is intermingled with mesodermal progenitors in most marginal positions. The pharynx is the most dorsal endodermal derivate, stomach, intestine and liver are derived from more lateral and ventral zones (Kimmel and Law, 1985a; Kimmel and Law, 1985b; Kimmel and Law, 1985c; Kimmel and Warga, 1986; Kimmel et al., 1990; Shih and Fraser, 1995; Warga and Nusslein-Volhard, 1999; Woo and Fraser, 1995; Woo et al., 1995).

However, fate maps do not necessarily say much about the actual specification status of investigated cells. They just describe to which cell types they, or better to say their progeny, will develop when left at their normal position and in their normal environment. However, crucial cell fate-determining processes, driven by both intrinsic and extrinsic factors, might occur after the time point of cell labelling. To address the specification status at a particular developmental stage, other approaches have to be used. For Xenopus, specification maps have been generated by cutting out small tissue pieces and culturing them in minimal medium. Compared to the blastula fate map, the blastula specification map shows some crucial differences. For example, dorsal ectoderm, which according to the fate map will give rise to neural tissue, forms epidermis according to the specification map.

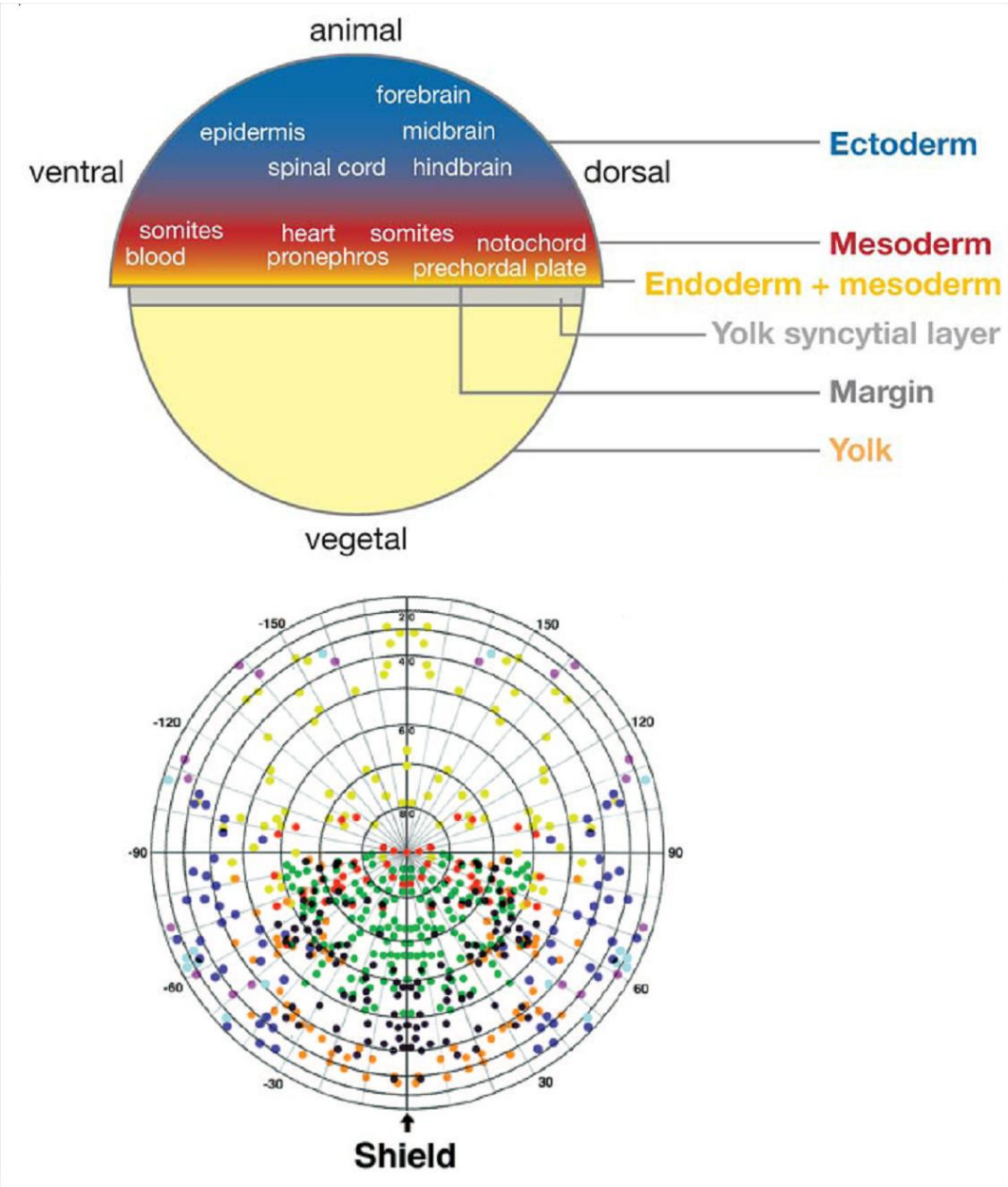


Figure 5-1: Fate maps of zebrafish
(Upper panel) Fate map at 50% epiboly. At the animal pole the ectoderm is forming, whereas at the margin mesodermal and endodermal precursors are found. Dorsal ectoderm gives rise to neural tissue, thereby the forebrain develops close to the animal pole and the hindbrain closer to the margin, resembling the A/P axis. Ventral ectoderm forms the epidermis. The dorsal mesoderm will form notochord and prechordal plate, more lateral and ventral mesoderm gives rise to trunk and tail somites and blood derivates. (adapted from Schier and Talbot, 2005)
(Lower panel) schematic view at a shield stage embryo from animal. Indicated are the different fates of ectodermal cells; (red) telencephalon (including the olfactory placode), (black) diencephalon (excluding the retina), (green) retina; (orange) midbrain, (dark blue) hindbrain, (light blue) spinal cord, (purple) somite, (yellow) neural crest, ear placodes and epidermis. (adapted from Woo and Fraser 1995)

This indicates that at blastula stages, neural fates have NOT been determined in the dorsal ectoderm as yet, and that neural development requires some neural-inducing extrinsic factors that normally reach the dorsal ectoderm after the investigated blastula stage. If these signals

are absent, as in the tissue explants, cells specify according to their intrinsic program. On the other hand, for the same reasons, the specification revealed by such transplantation experiments does not necessarily correspond to the actual final fate, and does therefore not give any information as to whether the final fate of a cell has already been determined or not. To address this question, so-called cell commitment studies have to be carried out. In case of ectoderm explants, for instance, treatment with factors like the BMP inhibitor Noggin and/or FGFs would make them acquire neural, rather than epidermal fates, indicating that at the time point of explantation, ectodermal cells had not been committed to their intrinsically programmed, epidermal fate as yet. In zebrafish, similar explantation experiments have suggested that at the onset of gastrulation, forebrain fates in the anterior dorsal ectoderm have already been specified, because the tissue will express forebrain-specific markers like *opl* even after explantation and cultivation in minimal medium *in vitro* (Grinblat et al., 1998). However, again, this does not necessarily mean that at the onset of gastrulation, cells are already irreversibly committed to their forebrain fate. Indeed, beads of BMPs, which can block neural induction, prevented the maintenance of forebrain marker expression even when implanted into the anterior neuroectoderm after the onset of gastrulation (Grinblat et al., 1998).

Another way to test for cell commitment is via heterotopic transplantation of cells into an ectopic embryonic environment. Woo and Fraser addressed the question of hindbrain specification by whole tissue grafting experiments (Woo and Fraser, 1998). Prospective hindbrain tissue was microsurgically dissected and transplanted from dorsal into ventral regions. Transplanted presumptive hindbrain tissue from shield stage embryos (early gastrula) was still able to differentiate into epidermal cells of the tail and fins as well as somitic tail muscle, indicating at this developmental stage, hindbrain progenitors were not committed to their fate. In contrast, transplanted hindbrain tissue from mid gastrula embryos maintained its fate, leading to the formation of ectopic hindbrain structures and demonstrating that at this stage, the tissue was committed (Woo and Fraser, 1998). However, also in this case, conclusions about the irreversible determination of final cell fates have to be taken with caution, as cells were transplated as entire and intact tissues. This could lead to some kind of community effect, via which cells protect each other from inductive, cell fate-modifying signals coming from the ectopic environment (for a review on community effects, see Gurdon et al., 1993). To circumvent this problem, individual cells have to be transplanted with a polished glass capillary. Using this technique, Ho and Kimmel (1993) investigated the timing of mesoderm induction, showing that at 5 hpf (onset of gastrulation), marginal cells

transplanted into ectopic animal positions could still adopt ectodermal fates according to their new environment and were not committed to their mesodermal fate as yet, whereas they did become committed during the further course of gastrulation. Furthermore, transplanted presumptive mesodermal and endodermal cells directly ingressed from their ectopic superficial positions into deeper cell layers, where the endogenous mesoderm and endoderm are located, revealing an intrinsic "homing" effect (David and Rosa, 2001; Aoki et al., 2002).
The differential timing of fore- versus hindbrain specification, although revealed via different and not fully conclusive techniques (Grinblat et al., 1998; Woo and Fraser, 1998; see above) is consistent with recently obtained data with heat-shock inducable, temporally controlled overexpression of the BMP inhibitor Chordin, demonstating that dorsoventral patterning of the animal ectoderm (from which the forebrain derives) occurs earlier than dorsoventral patterning of the more marginal ectoderm (from which the hindbrain is formed) (Tucker et al., 2008). This points to differential timing of cell fate specification along the animal-vegetal (anterior-posterior axis), with vegetal/posterior cells becoming specified later, possibly due to a specification-delaying effect of FGFs from the marginal mesoderm. However, the timing of cell specification and commitment along the dorsoventral (D-V) axis of zebrafish embryos had not been investigated as yet.

5.1.2. Aim of the project

One of the main interests of the Hammerschmidt Lab is to unveil the different functions of BMPs during development. A D-V BMP signaling gradient is established during early embryonic development. This gradient is mainly responsible for D-V patterning, which means the establishment of differential cell fates along the D-V axis of the gastrulating embryo. Taking into account that BMPs also work as stem cell factors (see General Introduction), I hypothesized that similar to the proposed effect of FGFs along the animal-vegetal axis (see above), BMPs might regulate the timing of cell specification along the D-V axis, so that ventral cells that are exposed to high levels of BMPs are kept in a pluripotent and uncommitted state longer than cells in dorsal positions with low or absent BMP activity. Such a scenario would be consistent with results obtained for Drosophila embryos, in which ventral cells (low Dpp/BMP) transplanted into the dorsal side (high Dpp/BMP) maintained their ventral fate, whereas ventrally transplanted dorsal cells did not (Technau, 1987).
To test whether such differential and possibly BMP-dependent timing of cell commitment also occurs in zebrafish embryos, I carried out heterotopic and – for technical reasons -

heterochronic cell transplantations of ventral versus dorsal ectodermal cells from early to mid gastrula embryos, followed by the analysis of cell fates several hours after the tranplantation, using whole mount *in situ* -hybridisations with cell type-specific probes.

5.2. Results

5.2.1. Dorsal and ventral cells do not show significant commitment differences after heterotopic transplantations at shield stage

To address the question of cell commitment along the D-V axis, I started with heterotopic cell transplantations at the shield stage (early gastrula). Thereby I compared the behaviour of dorsal ectodermal cells (presumptive neuroectoderm) after transplantation into the ventral ectoderm (will give rise to epidermis) (Fig. 5-2 A) with the behavior of transplanted ventral ectodermal cells on the ectopic dorsal side (Fig. 5-2 F).

For transplantations, donor embryos were injected at one- to two-cell stages with a fluorescent cell marker (fluorescein or rhodamin dextran (1mg/ml)) to be able to trace the transplanted cells or their descendants. Cell transpantations were done as previously described (see Materials and Methods), and mosaic embryos were fixed at the 20-25 somite stage, a time point when the neural and epidermal tissue are specified and clearly distinguishable by specific marker gene expression.

At shield stage, when the transplantations were performed, the dorsal side can be readily recognized by a local thickening, the shield, where the future dorsal mesoderm is localized (the General Introduction). Thus, pending on proper orientation of the embryos, one can carry out the transplantations in a spatially controlled manner. However, since according to the fate map (see Fig. 5-1), incorrect embryonic orientation by just a few degrees can mean that cells are transplanted from or into a region with a different fate, I carried out some of my transplantations with transgenic donors and hosts expressing GFP under the control of the *goosecoid* (*gsc*) promoter. *gsc* is expressed in the presumptive prechordal plate cells of the dorsal mesoderm, thereby marking the shield and the dorsal midline (Schulte-Merker et al., 1994a). In my dorsal-to-ventral transplantations, I tried to hit animal positions on the opposite side, approximately 180° from the *gsc* expression domain (Fig. 5-2 B). As expected from the fate map (Fig. 5-1), these cells later usually ended up in the epidermis covering the the yolk sac, the ventral trunk, or the tail (Fig. 5-2 C; and data not shown). In the reverse, ventral-to-dorsal transplantations, I transplanted cells into positions slightly animal and lateral (up to 30°) of the *gsc* domain (Fig. 5-2 G), with cells later ending up in the telencephalon, diencephalon, the retinas of the eyes or, in some cases, in the midbrain (Fig. 5-2 H; and data not shown), consistent with the fate map (Woo and Fraser, 1995) (see also Fig. 5-1). I conclude that I was transplanting cells into the correct positions.

For the identification of neural cell fates in mosaic embryos at the 20-25 somite stage, I carried out fluorescent whole mount *in situ*-hybridisations for *sox19a* or *otx2*. The panneural marker *sox19a* is broadly expressed in the neuroectoderm from early gastrula stages onwards and the expression persists in neural tissues during further embryogenesis (Okuda et al., 2006; Vriz et al., 1996). *otx2* is a more specific marker for fore- and midbain cells, which also starts to be expressed at early gastrula stages. For the identification of epidermal cells, I used an antibody against DeltaNp63, which stains the nuclei of basal keratinocytes, the most abundant cell type in the larval fish epidermis (Lee and Kimelman, 2002). Furthermore, transplanted cells were visualised via anti-Fluorescein immunostaining. Stainings were analysed by confocal microscopy with a Zeiss LSM 510/ 710, searched for transplanted cell, and scanning them in single optic planes for colocalisation with the selected fate markers.

Of the cells transplanted form the dorsal ectoderm (*sox19a*-positive) into the ventral ectoderm (DeltaNp63-positive), 72% (n=119/ 166) displayed expression of DeltaNp63, the epidermal marker of their new environment (Fig. 5-2 D and Tab. 5-2). The remaining, DeltaNp63-negative cells (n=47/ 166 cells) were not integrated into the epidermis, and displayed either weak *sox19a* expression (n=34/ 166 cells) (Fig. 5-2 E), or were s*ox19a*-negative, thus lacking both ventral and dorsal marker gene expression (n=13/ 166 cells) (Fig. 5-2 D, arrow indicates a p63 negative cells).

After the reverse transplantation of ventral cells into the dorsal ectoderm, 79% of transplanted cells (n=126/ 160 cells) displayed expression of *sox19a* or *otx2*, the neuroectodermal markers of their new environment (Fig. 5-2I). In the *otx2* stained embryos some cells were observed that did not express *otx2* (n=34/ 160 cells). These cells were located in or close to the brain, but outside the endogenous *otx2* domain (Fig. 5-2 K). Thus, they might represent more posterior cell fates, which would have been positive in *sox19a in situ*-hybridisations. Clearly, none of these *otx2*-negative cells showed expression of DeltaN63 (Tab. 5-2).

In sum, my results indicate that at the onset of gastrulation, ventral ectodermal cells are not committed to their epidermal fate as yet, but can adopt neural fates when placed into the neuroectoderm. This was according to my working hypothesis and the concept of BMP signaling blocking cell specification / commitment. However, in contrast to my working hypothesis, I obtained similar results for the reverse transplantations, indicating that the majority of dorsal ectodermal cells are also not committed to their neural fate as yet, but able to respond to BMP signaling and to switch on DeltaNp63 expression. On the other hand, at least some of the dorsal cells remained positive for neural markers in ectopic ventral positions, whereas no cell at all maintained epidermal marker gene expression in ectopic

dorsal positions, suggesting at least some subtle differences in the commitment status between dorsal and ventral cells.

Also, I noticed that in my dorsal-to-ventral transplants, the absolute number of embryos carrying transplanted cells at the 20-25 somite stage was rather low., This raised the possibility that statistics might have been distorted by the death of possibly neurally specified cells in ectopic epidermal environments. To look closer into this possibility, I quantified the survival rate of the transplantated cells by comparing the number of successful transplanted embryos right after the transplantation (6 hpf) with the number of embryos with transplanted cells at 24 hpf (Tab. 5-1). For the heterotopic transplantations from dorsal ectoderm to ventral ectoderm, the survival rate was 43% (23 out of 54 embryos with labelled cells at 6 hpf still contained labelled cells at 24 hpf). As a control to show that this is not due to a general insuitablility of dorsal cells for transplantations, I performed homotopic transplantations from dorsal to dorsal, yielding a survival rate of 76% (22/29). Interestingly, in the reverse heterotopic transplantation of ventral ectodermal cells into the dorsal ectoderm, the survial rate was 95% (36/38) (Fig. 5-2 G, H), thus, significantly higher than for the dorsal-to-ventral transplants, while in the homotopic ventral-to-ventral controls, it was 90% (n=27/ 30 embryos).

It has been described that in cell culture systems, committed neural cells undergo apotosis when challenged with BMP (Gambaro et al., 2006). To test whether this is also true for the majority of my ventrally transplanted dorsal cells, I selected embryos that had contained labelled cells at the shield, but lacked labelled cells at the 20-25 somite stage, and performed TUNEL stainings for apoptotic cells. However, I could not detect striking differences in the pattern of TUNEL-positive cells between experimental and control embryos, suggesting that apoptosis of transplanted cells, if at all, must have occurred during earlier stages after the transplantation. To determine this time point, I decided to track some of the heterotopic transplanted cells via time-lapse recording, mounting mosaic embryos directly after the transplantation and monitoring labelled cells with a Zeiss Axioimmager microscope for 18 hours. However, in 4/4 examined embryos, I could track transplanted cells and their movements until the end of the recording time (24 hpf) without noticing the loss of any of the cells. Since not all of the cells were nicely separated from each other, I cannot rule out that some of them might have died during the recording. But if so, I have missed it, and could not determine the proper time point for further apoptosis. Therefore, it remains unclear whether or whether not dorsal cells undergo apoptosis in an ecoptic ventral environment (but also see below).

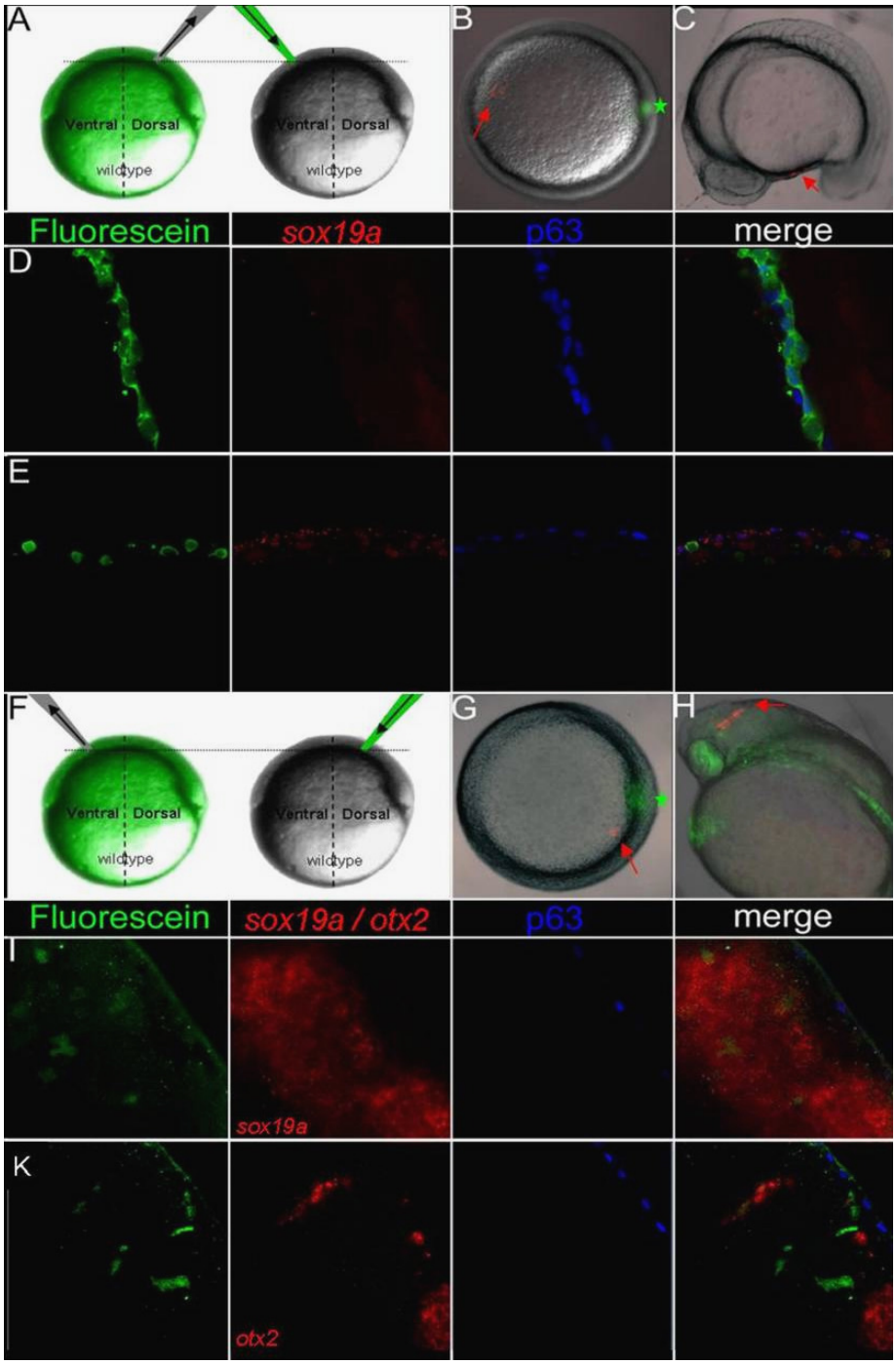


Figure 5-2: Heterotopic transplantation with embryos at shield stage do not unveil a difference between ventral and dorsal cells

Cells were transplanted from donor embryos injected with fluorescein or rhodamine dextran from the dorsal ectoderm to the ventral ectoderm of shield stage embryos as shown in the scheme (A) and the revesre,experiment (F); modified from Kimelman et al., 1990.

(B, C) show a successfully transplanted host embryo, transplanted cells are marked with rhodamine dextran (red arrow), (B) shows the dorsal cells on the ventral side of the embryo one hour after transplantation, the expression of GFP in gsc::GFP transgenic embryos (asterisk) marks the dorsal side; (C) shows the same embryos at the 25-somite stage, with transplanted red cells now in the epidermis covering the yolk sac. (G, H) show the reverse experiment, wth vetral cells on the dorsal side (G) hat eded up n the brain (H).

(D, E and I, K) show whole-mount fluorescent *in situ*-hydrisation against *sox19a* (red), followed by immunhistochemistry using antibodies against fluorescein dextran (green) and DeltaNp63 (blue); embryos were analysed by confocal microscopy. (D) shows transplanted cells expressing DeltaNp63; (E) shows dorsal cells weakly stained for *sox19a*. (I) show ventral cells integrated into the *sox19a* domain; (K) shows ventral cells in an *otx2* staining with only partial coexpression (arrow).

In summary, at the onset of gastrulation (shield stage), dorsal as well as ventral ectodermal cells seem to be able to transfate upon transplantation into an ectopic environment, and are thus not committed to their normal fates, although for the dorsal ectodermal cells, at least a small fraction (approximately 20%) might have been committed to their neural fate, indicated by persistent *sox19a* expression. Also, I cannot rule out that the percentage of neurally committed dorsal cells was even higher, but that most of them had undergone apoptosis at an unknown time point between the transplantation and the investigated late somitogenesis stage.

Transplantation	embryos containing labelled cells shortly after transplantation	embryos containing labelled cells at 24 hpf
Heterotopic		
Dorsal to Ventral		
at shield stage	54	23
at 60%epiboly	31	22
Ventral to Dorsal		
at shield stage	38	36
at 60% epiboly	25	14
Homotopic		
Dorsal to Dorsal	30	27
Ventral to Ventral	29	22
Heterochronic		
Dorsal to *alk8CA*		
at 60% epiboly	34	34
at 80% epiboly	10	10
Ventral to *tBRIa*		
at 60% epiboly	28	18
at 80%epiboly	9	6

Table 5-1: Survival rate of transplanted cells; embryos with successfully transplanted cells were raised until next morning and then examined for existence of labelled (transplanted) cells; absolute numbers of counted embryos.

5.2.2. Dorsal and ventral cells do not show significant commitment differences after heterotopic transplantations at the 60-70% epiboly stage

To investigate whether the subtle differences in the commitment of dorsal versus ventral cells described above become more obvious during the further course of gastrulation, I carried out similar heterotopic dorsal-to-ventral and ventral-to-dorsal transplantation experiments at a slightly later developmental stage (60-70% epiboly, 30-90 minutes after the shield stage analysed above).

Transplantations at these later stages are technically more difficult, since the mesendoderm becomes internalized at the margin of the embryo and starts to progressively move underneath the ectoderm. Thus, to avoid mixed transplants of ectodermal and mesodermal cells, one has to stay in animal regions and/or outer cell layers of the embryo. Furthermore, lateral cells move towards the dorsal midline (convergence and extension), leading to a progressive thinnening of the ventral side (Solnica-Krezel, 2006), which got accordingly more difficult to penetrate with the transplantation capillary. Therefore, the absolute number of investigated mosaic embryos after transplantation at the 60-70% epiboly stage was lower than the number at shield stage. However, I also carried out control transplantations with *gsc::GFP* transgenic donors and hosts, as described above, revealing that cells with proper fate were transplanted into, and ended up in, proper positions (Fig. 5-3 B,C, G, H). In addition, I determined survival rates of transplanted cells. For dorsal-to-ventral transplantations, it was 71% (22/31), higher than the rate obtained at shield stage (43%), and for ventral-to-dorsal transplantations, it was 56% (14/25), lower than at shield stage (95%). Finally, I selected a few of the mosaic embryos after dorsal-to-ventral transplantations for time-lapse analysis, as described above. Again, in 3/3 recordings, I could follow the transplanted cells over night without observing any loss of cells.

Analysis of marker gene expression at the 20-25 somite stage revealed that 62% of cells (n= 81/130) transplanted from the dorsal into the ventral ectoderm expressed DeltaNp63, indicating that these cells had obtained the epidermal fate according to their new environment. The other part of transplanted cells (n= 49/ 130 cells) either diplayed weak expression of the neural marker *sox19a* or lacked both DeltaNp63 and *sox19a* expression (Fig. 5-3 D, E), very similar to the behavior of cells upon transplantation at the shield stage.

After transplantation of ventral ectodermal cells into the dorsal ectoderm, only 36% (n= 22/ 60) of the cells expressed the neural marker, whereas 53% (n= 32/ 60 cells) lacked *sox19a* or *otx2* expression, although they were located close to neural tissues (Fig. 5-3 I). Strikingly, a

minority of cells (n= 6/ 60 cells) were found in the epidermis of the head region expressing DeltaNp63 (Fig. 5-3 K). This could be interpreted as a sign of commitment of cells to an epidermal fate. However, as pointed out above, transplantations had to be carried out in rather animal positions to avoid mixing with mesoendodermal fates. In regions close to the animal pole, forebrain cells are located in close proximity to placodal and head epidermal cells (see Fig. 5-1). Thus, the obtained DeltaNp63-positive cells after ventral-to-dorsal transplantations could result from slightly mispositioned animal transplants.

In conclusion, the heterotopic transplantation experiments at 60-70% epiboly stage (early to mid gastrulation) did not reveal any clearer differences in the commitment of dorsal versus ventral cells than the studies carried out at the onset of gastrulation. If at all, results were more inconsistent, which could be due to the aforementioned larger difficulties of the applied transplatation techniques at these later stages. Therefore, in the following experiments, I applied a different technique, transplanting cells from 60-80% epiboly stage host embryos heterochronically into younger hosts that were either completely ventralised or dorsalised.

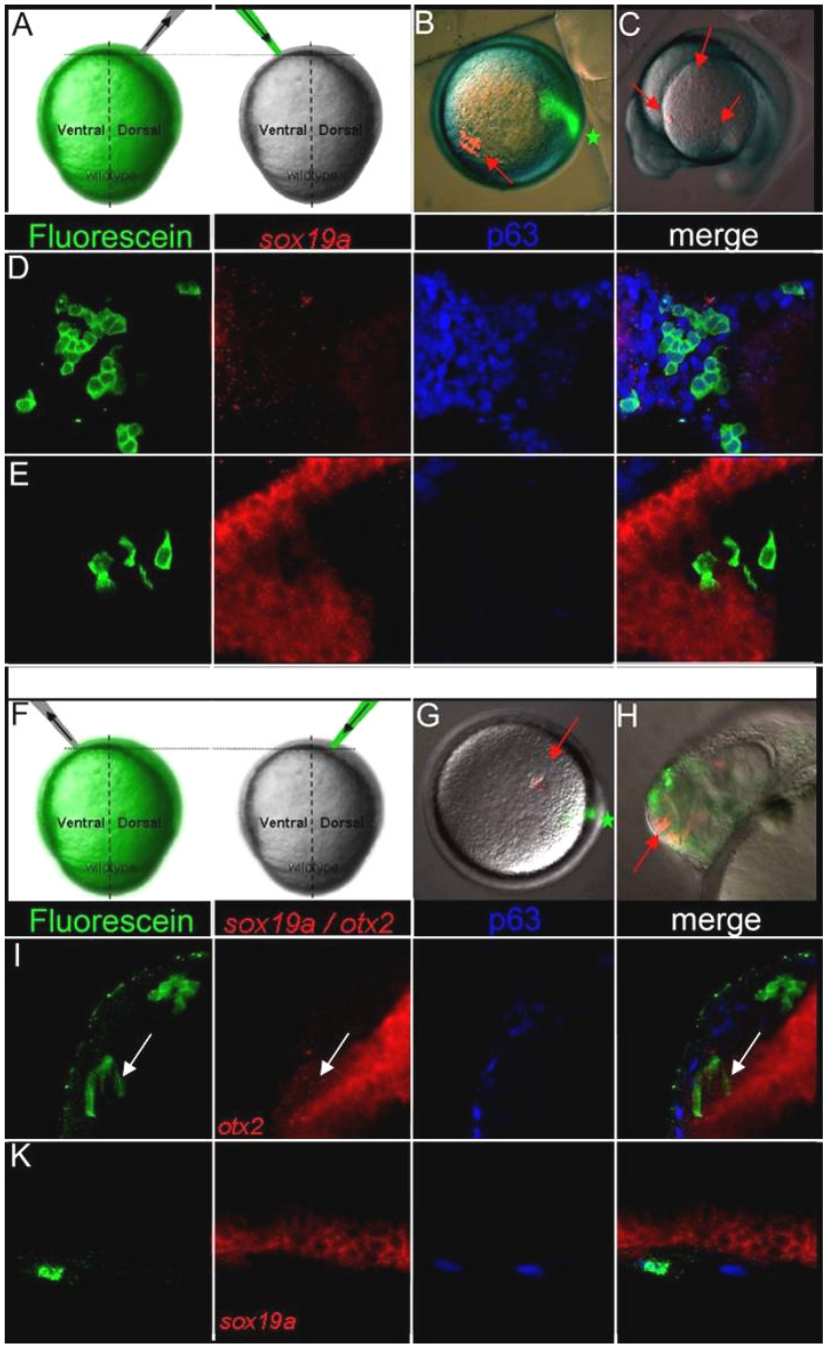


Figure 5-3: Heterotopic transplantation with embryos of the 60% to 70% epiboly do not unveil striking differences between ventral and dorsal cells

Panels (B to E) show mosaic embryos after transplantation of dorsal cells from 60-70% epiboly stage embryos donors into the ventral ectoderm of hosts, as illustrated in (A); panels (F to K) show the corresponding reverse ventral-to-dorsal transplantations.

(B,C) and (H,I) show two successfully transplanted host embryos directly after transplantation (B,H) and at the 20-25 somite stage (C,I); transplanted cells are marked with rhodamine dextran (red arrow), the dorsal side in B and H is marked by the expression of GFP in *gsc::GFP* transgenic host embryos (asteriks). (B) shows transplanted cells on the ventral side of the embryo, which later are located in the skin covering the yolk cell (C). (G) shows transplanted cells on the dorsal side, which ended up within brain tissues (H; green shows again gcs::GFP transgene expression.

(D, E, and K, L) show whole-mount fluorescent *in situ*-hydrisation against *sox19a* (red), followed by immunhistochemistry using antibodies against fluorescein dextran (green) and DeltaNp63 (blue), as noted of the dorsal to ventral (D-F) and ventral to dorsal (K, L) transplantation; (D) shows dorsal cells expressing p63, (E) shows dorsal cells close to the *sox19a* expression domain. (I) shows ventral cells close to and in the *otx2* domain (arrow), and (K) shows a single ventral cell positive for DeltaNp63.

5.2.3. Heterochronic transplantations of dorsal ectodermal cells from 60% epiboly stage donors into ventralised blastula stage embryos

Because of the low efficiency of the late heterotopic transplantations, I started heterochronic transplantations that are technically easier to handle. I transplanted cells of donor embryos at 60% epiboly (7 hpf) into the animal pole of sphere stage (4 hpf) embryos. Epiboly has not started yet in embyos at sphere stage, and the deep cells form a compact, but rather loose tissue; for this reason it is easy to integrate ectopic cells. Indeed, survival rates of cells transplanted this way was 100% (n=34/ 34 embryos) (Tab. 5-1), and cells ended up properly integrated into host tissue. However, at this pregastrula stage, the dorsoventral axis is not morphologically visible as yet, which makes it impossible to transplant cells in a spatially controlled fashion into dorsal or ventral regions of the embryo. To circumvent this problem, host embryos were injected at the one to two-cell stage with mRNA encoding either constitutively active or dominant negative BMP receptors (Bauer et al., 2001; Graff et al., 1994). This leads to a broad hyper-ventralisation or hyper-dorsalisation of the entire embryo, respectively.

When examined at 24 hpf by morphological criteria, embryos injected with *caAlk8* mRNA (Bauer et al., 2001) showed ventralisation of the V3 classification. This means that from late blastula stages onwards most, although not all, of the embryonic cells are ventrally specified. Transplanted dorsal cells will therefore be surrounded by ventral cell types and challenged by BMP signals emanating from them. .

After heterochronic transplantation of dorsal ectodermal cells from 60% epiboly donors into ventralised sphere stage hosts, mosaic embryos were raised until 24 hpf, when uninjected control siblings had reached the 20-25 somite stage, and were analysed via confocal microscopy after whole mount *in situ*-hybridisations and immunostainings, as described above for the heterotopic transplantations.

56% (161/287) of the transplanted dorsal cells were negative for both the neural marker *sox19a* and the epidermal marker DeltaNp63 (Fig. 5-4 C, D), while 41% (119/287) expressed the neural marker *sox19a*. In the ventralised host embryo the endogenous expression domain of *sox19a* was strongly reduced due to the reduction of the neuroectoderm. However, a small *sox19a* domain remained. Nearly all transplanted cells that expressed *sox19a* were found within this domain, and might therefore not have been properly challenged by environmental BMP signals (Fig. 5-4 E, F). Only in two embryos I found clusters of transplanted cells that were completely surrounded by host epidermal cells. These clusters contained *sox19a*-positive cells in the middle of the clusters, whereas cells at the periphery lacked both *sox19a*

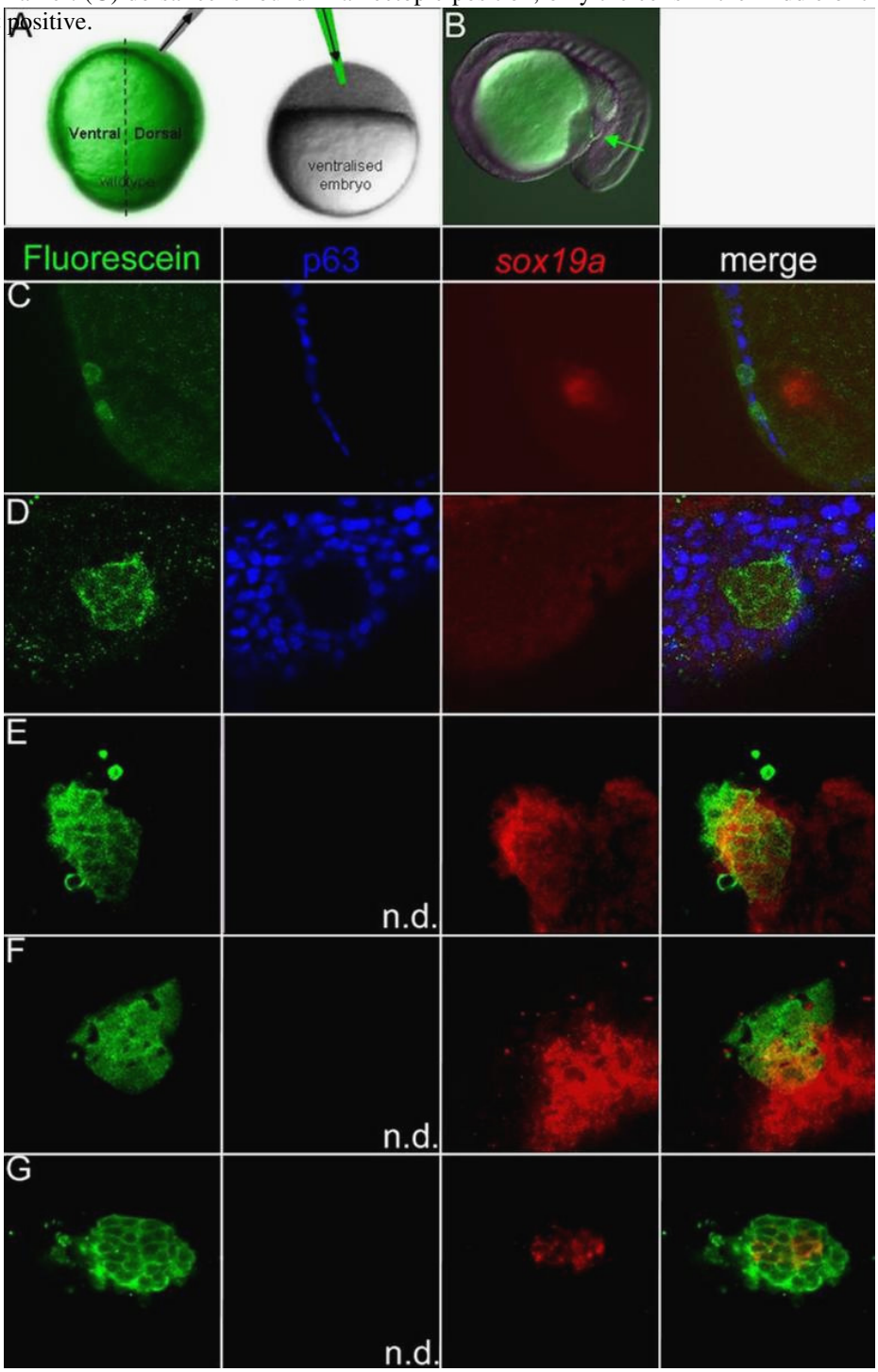
and DeltaNp63 expression (Fig. 5-4 G). In very few cases (7/ 287 cells), I also found DeltaNp63-positive transplanted cells (Tab. 5-2).

In conclusion, these results indicate that dorsal ectodermal cells directly exposed to BMPs from host cells could not maintain their neural fate, whereas cells in a distance of two- to three cell diameters could. Strikingly, however, almost all transplanted cells failed to acquire an epidermal fate, indicating that they had lost their trans-fating capacity.

Next, I wanted to find out at which time point the dorsal cells loose their initial fate. To address this, I fixed and analysed embryos at different time points after the transplantation. One hour after transplantation, when the host embryos had reached 50% epiboly, only 25% (18/71) of transplanted cells were *sox19a* positive. Six hours after transplantation, when the host embryos were around tailbud stage, 44% (80/180) of the transplanted cells expressed *sox19a*. They were usually organised in clusters surrounded by or directly attached to the remaining endogenous *sox19a* expression domain of the host, whereas again most of the transplanted cells (56%) lacked *sox19a* transcripts (Fig. 5-4 E). This is a similar percentage as obtained at 20-25 somites or later (44% *sox19a*-positive at tailbud stage, 41% at 20-25 somite stage or later). It remains unclear why the fraction of *sox19a* positive cells was lower directly after the transplantation (25%). Higher proliferation rates of neural versus non-neural ectodermal cells could be one explantation.

I conclude that most dorsal ectoderm cells of 60% epiboly stage embryos, when challenged in a ventral, BMP-expressing environment, loose their initial neural marker expression within one hour after transplantation. Unlike in the heterotopic transplantations, however, they do not become epidermis, but remain in the embryo in an unspecified or thus far unresolved state. My results also show that maintenance of neural fate requires some kind of community effect (see Introduction; 5.1.1), and shielding/insulating of transplated cells from direct contact with the BMP-expressing environment in large clones (Fig. 5-4 G).

stage (C,D,F,G), after whole mount fluorescent *in situ* hybridisation against *sox19a* (red), and immunostainings of fluorescein dextran, the used lineage tracer (green), and DeltaNp63 (blue). For (E, F, G) embryos were not stained for DeltaNp63.
(C, D) dorsal cells were surrounded by DeltaNp63 positive cells, but itself they were negative for DeltaNp63 as well as *sox19a*. (E, F) dorsal cells found in the *sox19a* domain partially coexpressed the neural marker. (G) dorsal cells found in an ectopic position, only the cells in the middle of the cluster are *sox19a* positive.



5.2.4. Heterochronic transplantations of ventral ectodermal cells from 60% epiboly stage donors into dorsalised blastula stage embryos

Next, I carried out the reverse experiment to test the behaviour of ventral cells in a dorsal environment, transplanting ventral ectodermal cells from of 60% epiboly stage embryos into the animal pole of dorsalised sphere stage embryos that had been injected at the 1 to 2-cell stage with mRNA encoding dominant negative, truncated version of the Xenopus BMP receptor 1a (Graff et al., 1994). At 18 hpf, injected host embryos displayed strong dorsalisation of C4/C5 strength, with many of them lysing during mid somitogenesis.

Therefore, mosaic embryos were raised at low temperature and fixed when controls had reached the 15-somite stage.

Whole mount *in situ*-hybridisation and immunostaining revealed that within the host tissue, the neural markers *sox19a* or *otx2* were strongly upregulated, displaying a radialised expression domain throughout the entire embryos, whereas DeltaNp63 was absent. This indicates that endogenous epidermal tissue was completely lost.

Similarly, all of the heterochronically transplanted ventral cells (228/228) lacked DeltaNp63, indicating that they were unable to maintain their initial fate. Of those, 61% (138/228) showed expression of one of the neural markers tested, indicating that they had adopted fates according to their new environment (*sox19a*: 57%, n=86/151; *otx2*: 68%, n=52/77) (Fig. 5-5 C -E). However, the remaining 39% (90/228) of transplanted cells of ventral ectodermal origin did not express any of the tested epidermal and neural markers (*sox19a* 43%, n=65/ 151 cells; for *otx2* 32%, n=25/ 77 cells), leaving their fate unsolved (Fig. 5-5 F).

The survival rate of heterochronically transplanted ventral cells was around 64% (n=18/ 28 embryos). I assume this might be due to technical difficulties, because the ventral ectodermal side becomes progressively thinner during gastrulation, which means that it becomes more difficult to get intact cells out of the embryo.

In sum, my results indicate that ventral cells are not committed to epidermal fate, since I never detected ectopic expression of p63 with the counterstain. Also, the majority of cells can adopt the neural fate of the new environment. Thus, they are still multipotent. This is in striking difference to the results obtained for dorsal cells described above (5.2.2). Nevertheless, also a significant number of transplanted ventral cells lacked expression of both epidermal and neural markers. Future experiments have to show whether they are unspecified, or whether they might have acquired a different fate (see Discussion).

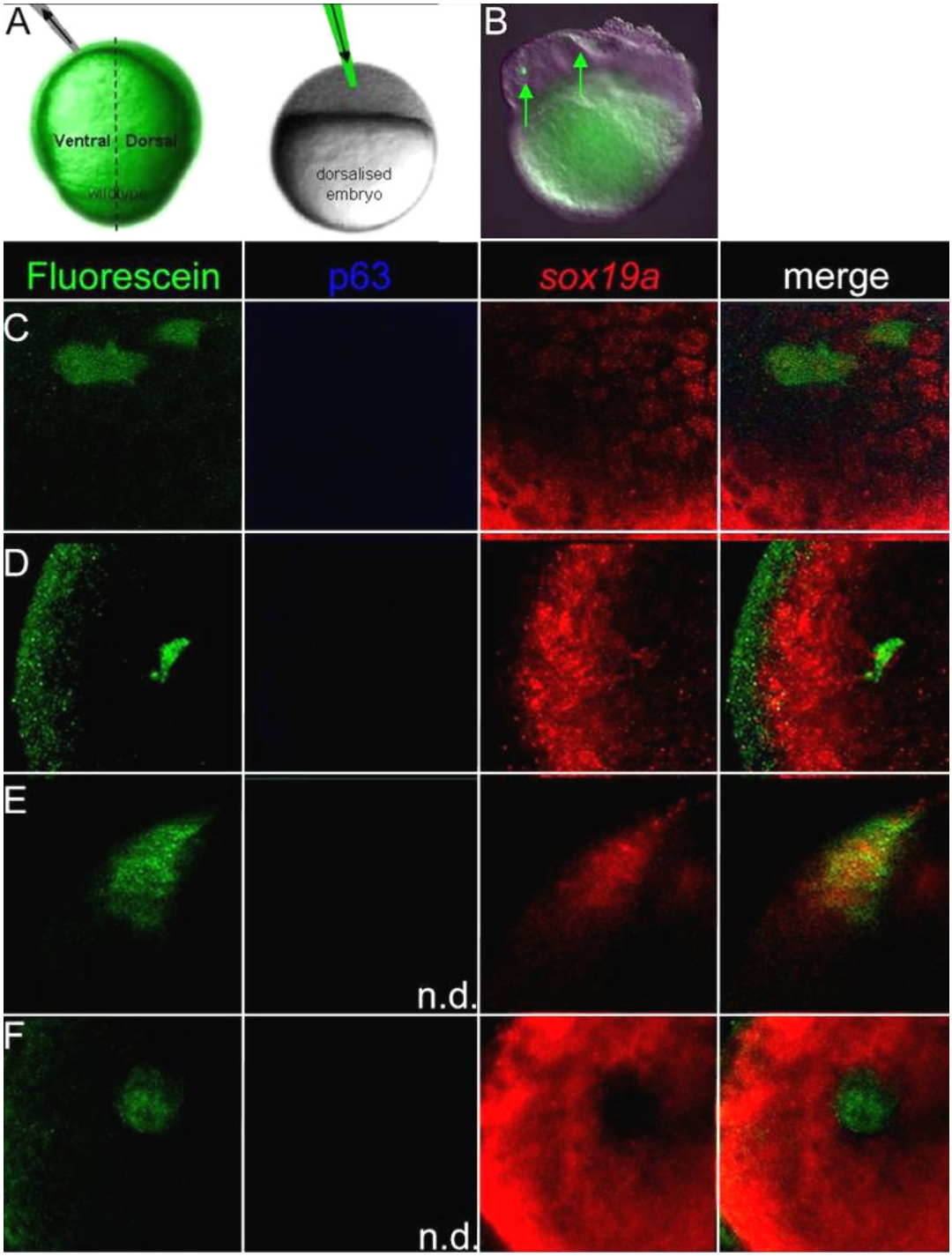


Figure 5-5: Heterochronic transplantation of ventral cells from 60% epiboly stage embryos into dorsalised hosts

(A) illustrates the transplantation procedure: ventral ectodermal cells from labeled donor of the 60% to 70% epiboly stage are transplanted into late blastula (sphere) stage embryo that has been dorsalised by injection of constitutive dominant negative, truncated BMP receptor RNA. (B) shows a successfully transplanted dorsalised host embryo at the 15-somite stage; transplanted cells are marked with fluorescein dextran and located in brain (green arrows).

(C to F) show confocal microscopy images of mosaic embryos at the 15 somite stage, after whole mount fluorescent *in situ*-hybridisation against *sox19a* (red), and immunostainings of fluorescein dextran, the used lineage tracer (green), and DeltaNp63 (blue).

(C, D, E) show ventral cells integrated into the *sox19a* domain and they did adopt the neural fate. (F) shows ventral cells integraed n the *sox19a* domain, but did not stain for the marker.

5.2.4 Heterochronic transplantations of dorsal and ventral ectodermal cells of 80% epiboly stage embryos

The results described above show that at the 60% epiboly stage, dorsal ectodermal cells are less plastic than ventral cells. However, both cell types are not committed to their intrinsic fate as yet, indicated by the loss of initial marker gene expression when integrated into ectopic tissues. This suggests that cell fate commitment must occur later. Therefore, I next carried out heterochronic transplantations as described above with dorsal or ventral ectodermal cells from embryos of the 80% epiboly stage (mid gastrulation; 8.5 hpf).

However, also at this later stage, both dorsal and ventral cells appeared uncommitted. 93% (37/40) of the dorsal cells transplanted into ventralised hosts neither expressed *sox19a* nor DeltaNp63 (Fig. 5-6 A, B), while only 7% (3/40) of the transplanted cells maintained the expression of *sox19a*. This indicates that as at early gastrula stages, neuroectodermal cells of mid gastrula embryos are still not committed to their neural fate, while they have lost their capacity to become epidermal. Of the ventral ectodermal cells transplanted into dorsalised embryos, 86% (31/36) were negative for both the epidermal and neural markers (Fig. 5-6 C, D), while 6% (2/36) showed expression of *sox19a*, the marker of the new environment. This indicated that in contrast to dorsal cells, at least some of the ventral cells could transfate and specify according to their new environment. However, compared to the 60% epiboly stage, the fraction of such multipotent cells has dropped from 61% to 6%, pointing to some advances in cell fate specification. Along the same lines, it remains puzzling, however, why the fraction of uncommitted neuroectodermal cells at the 80% epiboly stage is higher (93%) than at the than at 60% epiboly stage (56%). Differences in the sizes of transplanted cell clusters can be most likely ruled out as a possible explanation. As shown above (Fig. 5-4 G), such clusters have to reach a certain size to maintenance of *sox19a* expression in the centre of the clone. However, clusters of this size were also obtained upon transplanation of dorsal cells at the 80% epiboly stage, while cells lost *sox19a* expression even in the middle of the cluster (Fig. 5-6 A). Also, survival rates of transplanted cells were reasonable (10/10 embryos for transplantation of dorsal cells into ventralised hosts; 6/9 embryos for transplantation of ventral cells into dorsalised hosts; Tab 5-1). Nevertheless, I have to admit that the transplantation of cells out of 80% epiboly embryos was a technical challenge, due the advanced gastrulation, so that only a few transplantions with comparably low numbers of transplanted cells were successful. Thus, obtained numbers are difficult to compare with those from the previous transplantations.

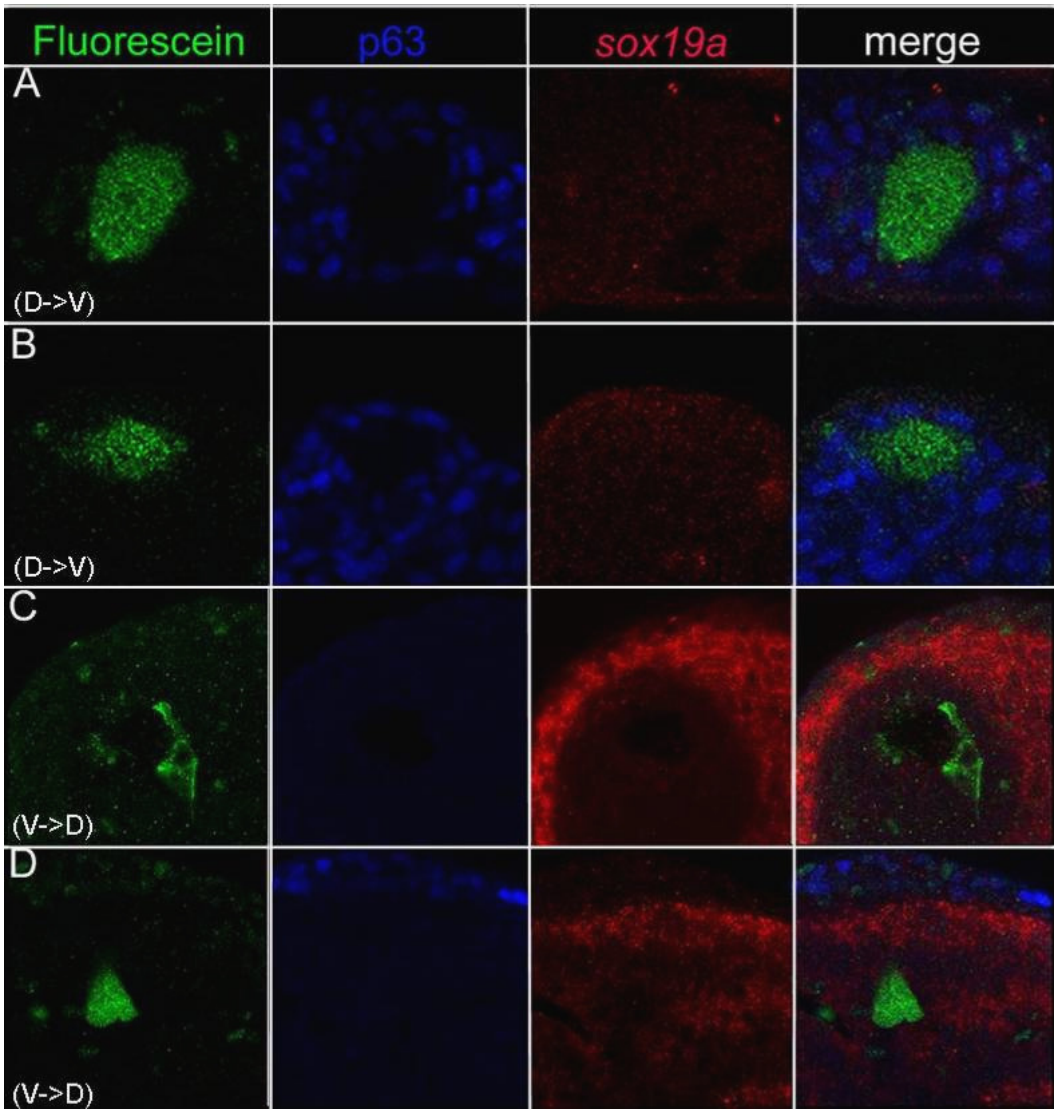


Figure 5-6: Heterochronic transplantations of dorsal (A,B) or ventral (C,D) ectodermal cells from 80% epiboly stage embryos into ventralised (A,B) or dorsalised (C,D) sphere stage hosts, respectively

All panels show confocal microscopy images of mosaic embryos at the 15-25 somite stage, after whole-mount fluorescent *in situ*-hydrisation against *sox19a* (red), followed by immunhistochemistry using antibodies against fluorescein dextran (green) and DeltaNp63 (blue) as noted.

(A, B) dorsal cells were surrounded by DeltaNp63 positive cells, but itself they were negative for DeltaNp63 as well as *sox19a*. (C, D) show ventral cells close to the *sox19a* domain, but did not stain either for the neural marker nor the epidermal marker.

Transplantation assay	number of cells counted	*sox19a/ otx2* positive cells	DeltaNp63 positive cells	Cells negative for both markers
Heterotopic				
Dorsal to Ventral				
at shield stage	166	34	119	13
at 60%epiboly	130	19	81	30
Ventral to Dorsal				
at shield stage	160	126	0	34
at 60% epiboly	60	22	6	32
Heterochronic				
Dorsal to *alk8CA*				
at 60% epiboly	287	119	7	128
at 80% epiboly	40	3	0	37
Ventral to *tBRIa*				
at 60% epiboly	228	138	0	90
at 80%epiboly	36	2	3	31

Table 5-2: Overview of all transplantations analysed by whole mount fluorescent stainings with *sox19a/ otx2* and DeltaNp63 and further analysed by confocal microscopy. Shown are the absolute numbers of counted cells.

5.3. Discussion

5.3.1. After heterotopic transplantations, both dorsal and ventral cells do not maintain their initial fate and can integrate into the new tissues

The BMP gradient with its from ventral to dorsal progressively dropping BMP concentration most likely fulfils multiple functions during early embryonic patterning and morphogenesis. First, BMPs are supposed to act as a morphogen, inducing differential cell fates along the dorsoventral axis in a dose-dependent fashion. According to this concept, high BMP levels in the ventral ectoderm induce epidermal fates, intermediate levels in the lateral ectoderm levels cause neural crest and preplacodal specification, while lowest levels in the dorsal ectoderm lead to neural development (see for review; Mullins and Hammerschmidt, 2002). A second function is concerned with guiding the migration of cells from ventrolateral into dorsal regions (von der Hardt et al., 2007). Here, I addressed a possible third function, which is related to the known role of BMPs in stem cell biology, keeping cells in an undifferentiated and pluripotent status under certain conditions (Hogan, 1996; Varga and Wrana, 2005) (see general introduction). My working hypothesis was that the high levels of BMPs on the ventral side keep cells in a multipotent and undifferentiated status, whereas on the dorsal side, where BMP signaling is weak or absent, cells start to differentiate and become committed to their final fate. Interestingly, the dorsoventral BMP gradient is highly dynamic over time, and the BMP expression domain becomes more and more restricted to ventral-most regions of the gastrulating embryo, suggesting that cells might only be released from the BMP-mediated specification block once BMP levels have dropped below a certain threshold. To study differential timing of cell specification along the dorsoventral axis of zebrafish embryos, I carried out cell commitment studies, using heterotopic transplantations of ventral ectodermal cells (normally given rise to epidermis) into the dorsal ectoderm (normally giving rise to neural tissue), and *vice versa*. Similar heterotopic transplanations have been previously performed for the *Drosophila* embryo, which like vertebrate embryos is patterned along its dorsoventral axis under the control of the BMP-like signal Decapentaplegic (Dpp) and its Chordin-like inhibitor Short gastrulation (Sog). However, in line with the concept of axis inversion during bilaterian evolution (Arendt and Nübler-Jung, 1994; De Robertis and Sasai, 1996), Dpp is most strongly expressed on the dorsal side of *Drosophila* embryos, whereas Sog is strongest in ventrolateral regions, where the neuroectoderm is formed. Correspondingly, and consistent with my working hypothesis, ventrolateral cells of the *Drosophila* embryo displayed a higher degree of commitment than cells in more dorsal

positions (Technau, 1987; Udolph et al., 1995). However, my own results obtained for zebrafish embryos were less clear. According to my working hypothesis, I would have expected that after transplantation into the ventral ectoderm, dorsal ectodermal cells specify according to their original positions, forming ectopic neuroectodermal domains within the epidermis. In contrast, ventral cells transplanted into the dorsal domain should be uncommitted and more plastic, and should acquire neural fates according to the instructions of their new environment, rather than becoming epidermal, the fate according to their original position.

I carried out heterotopic transplantations at two subsequent developmental stages, at shield stage, an early gastrula stage, when the dorsoventral axis becomes morphologically visible, and approximately 1 hour later, at the 60% epiboly stage. For both time points, I obtained rather similar results: the majority of dorsal ectodermal cells lost expression of the neural markers *sox19a* and *otx2* (both of which are already expressed in the neuroectoderm at shield stage), integrated into the epidermis and started to express DeltaNp63 (72% after transplantation at shield stage; 62% after transplantation at 60-70% epiboly stage). DeltaNp63 is a direct transcriptional target gene of BMP signaling and one of the key factors towards epidermal commitment (Bakkers et al., 2002; Medawar et al., 2008). This indicates that at early gastrula stages, dorsal ectodermal cells are not committed to their neural fate as yet, but are still able to receive and respond to BMP signals and to acquire epidermal fates (but see below). In reverse, the same is true for ventral cells, which after transplantation into the dorsal ectoderm started to express neural marker genes (79% after transplantation at shield stage; 39% after transplantation at 60-70% epiboly stage). There was one subtle difference in the behavior of dorsal and ventral cells: while at least some of the transplanted dorsal cells maintained neural marker gene expression in the ectopic ventral environment (20% after transplantation at shield stage; 15% after transplantation at 60-70% epiboly stage), not a single ventral cell displayed persistent DeltaNp63 expression after transplantation into the dorsal ectoderm. This suggests that dorsal cells might have reached a more advanced, although not fully committed status than ventral cells.

Nevertheless, the differences between dorsal and ventral cells are less striking that I would have expected according to my working hypothesis. How do my findings correlate to previous data obtained for the specification status within the zebrafish neuroectoderm? Presumptive forebrain tissue had been shown to display persistent expression of the anterior neural marker genes *opl* and *otx2* even when explanted at the shield stage (Grinblat et al., 1998; Sagerstrom et al., 1996). This, however, does not mean that cells were committed to the neural fate, since

the explants were cultured in saline, meaning that cells were not really challenged by extrinsic factors that could induce trans-fating. And indeed, when the same explants were treated with BMPs, neural marker gene expression was lost (Sagerstrom et al., 2005). Consistently, *in vivo* expression of *opl* within its endogeneous presumptive forebrain domain was lost when BMP-loaded beads were implanted into the dorsal side of embryos even after the shield stage (Grinblat et al., 1998). This is in line with the data obtained in my transplantation experiments, indicating that at early gastrula stages, dorsal ectodermal cells can still respond to BMPs and give up neural specificity. In addition to the exposure to neural-blocking BMPs, a second reason for the loss of neural identity could be the loss of neural-inducing or –maintaining factors in the new environment. Thus, in contrast to data obtained for animal caps from Xenopus embryos, it has been shown that the expression of neural genes in neuroectodermal zebrafish explants is lost when the tissue is dissociated into a single cells suspension (Sagerstrom et al., 2005). This could be due to diluting out autocrine pro-neural factors when dissociating the tissue. Likewise, dorsal ectodermal cells become at least transiently dissociated when transferred in a narrow capillary into the new tissue.

In addition to losing the neural fate, transplanted dorsal cells also seem to acquire epidermal fates. This is at least suggested by the integration of transplanted cells into the epidermis, as observed in multiple mosaic embryos, and by the initiation of DeltaNp63 expression. DeltaNp63 is known to be required for basal keratinocyte proliferation in zebrafish embryos (Lee and Kimelman, 2002), and for BMP-induced commitment of embryonic stem cells to an epidermal fate (Medawar et al., 2008; Aberdam et al., 2007). However, although co-expressed with *keratin* genes in differentiated keratinocytes, its expression is initiated much before the onset of *keratin* expression, in zebrafish during earliest gastrula stages (Bakkers et al., 2002), when ventral ectodermal cells are not committed to their epidermal fate as yet. Therefore, DeltaNp63 cannot necessarily be regarded as a bona fide marker of differentiated keratinocytes. For this purpose, it would have been necessary to carry out *in situ*-hybridizations for *keratin* genes. Nevertheless, my data show that most likely induced by BMP signaling (Bakkers et al., 2002), dorsal cells not only lose neural marker gene expression, but also initiate the expression of genes that are exclusively expressed in epidermal cells and their precursors.

Interestingly, such trans-fating from neural to epidermal was not observed when dorsal cells were heterochronically transplanted into hyper-ventralised embryos, although also in this case, cells lost neural marker gene expression (see also below). Furthermore, according to a previous report, stable neural specification can be obtained when presumptive hindbrain tissue

is microsurgically transplanted into ventral regions of 80% epiboly stage embryos (Woo and Fraser, 1998). Sizes of transplanted cell clusters and community effects might account for these differences, as will be discussed in more detail below.

But what is the exact status of transplanted cells which at mid segmentation stages lacked both neural and epidermal marker gene expression. They were obtained, although with slightly different frequencies, upon transplantation of dorsal cells into the ventral ectoderm, and transplantation of ventral cells into the dorsal ectoderm, and upon heterotopic and heterochronic transplantations. Since cells were transplanted into the animal / ectodermal half of hosts, it is very unlikely they that might have acquired mesodermal fates. They could have returned to naive, completely unspecified embryonic cells, comparable to Oct4-positive embryonic stem cells of the mouse, which can be obtained by BMP treatment of ES cells that would otherwise form neurons (Tropepe et al., 2001). Alternatively, they might have acquired an intermediate ectodermal fate. Ventral-most cell types are known to require sustained BMP signaling beyond early gastrula stages (Pyati et al., 2005). Thus, a ventral cells that is transplanted dorsally during early gastrula stages might have already received too much BMP signaling to acquire a neural fate (since BMPs are switched off in this domain before the onset of gastrulation), but not enough to acquire an epidermal cell (since it misses BMP signaling after being moved out of the tissue and onto the dorsal side). Accordingly, the would have received intermediate levels of BMP signaling, which according to the fate map should make them become preplacodal or neural crest derivatives (Barembaum and Bronner-Fraser, 2005; Huang and Saint-Jeannet, 2004; Nguyen et al., 1998). Likewise, dorsal cells transplanted to the ventral side would be exposed to additional BMP signaling after their transplantation to the ventral side, preventing them from becoming or remaining neural. On the other hand, they would not acquire enough BMP signaling for epidermal specification, since they missed BMP signaling before their transplantation, when they were still on the BMP-negative dorsal side, again resulting in intermediate fates. Future experiments with placodal and neural crest markers have to test this notion. If correct, the data would be in line with a morphogenetic role of BMP signaling that is integrated over time.

5.3.2. After heterochronic transplantations, both dorsal and ventral cells show an increased tendency to lack the neural as well as the epidermal marker

Because of technical reasons I decided to carry out heterochronic transplantations at 60% epiboly and, around two hours later, at 80% epiboly into late blastula (sphere) stage embryos. At late blastula stage the D-V axis is morphological not visible, yet. Therefore, I transplanted the dorsal cells into a hyper-ventralised and the ventral cells into a hyper-dorsalised host embryo to challenge them.

I obtained higher ratios of *sox19a* positive cells in the heterochronic transplantation of 60% epiboly donors than in the corresponding heterotopic approach (41% after heterochronic transplantation from dorsal to ventral compared to 15% in the heterotopic approach; 61% after hetrochronic transplantation from ventral to dorsal compared to 37% in the heterotopic transplantation). These results are in line with my working hypothesis that dorsal cells are committed at an earlier timepoint and keep their initial marker expression (neural), whereas the ventral cells are more plastic and can adopt the new fate (neural). But these results are still not as striking as I expected, because many cells stayed in an unspecified state, as I already mentioned.

Nearly all dorsal cells, that were positive for the neuroectodermal marker, were found in or close to the remaining natural *sox19a* domain of the ventralised host embryo. Therefore one cannot conclude that these cells maintained the neural fate on their own. However, this remaining domain is rather small in a hyper-ventralised embryo. But 41% of the cells ended up in this neuroectodermal region and this is a relative high ratio. Therefore I cannot rule out a directed migration of the heterochronically transplanted cells. Such an intrinsic “homing” effect has been shown for mesodermal and endodermal precursors. Transplanted marginal cells directly migrate from their ectopic positions towards their endogenous domain (David and Rosa, 2001; Aoki et al., 2002). One would have to perform timelapse analysis of the heterochronically transplanted cells to see if the cells directly migrate towards the neuroectodermal domain.

Another result of the heterochronically transplanated dorsal cells is even more puzzling. The majority of dorsal cells in ectopic positions were neither positive for *sox19a* nor for DeltaNp63, even when they were surrounded by DeltaNp63 postive cells. The lack of the epidermal maker is contradicting my own results that I obtained after the heterotopic transplantation (2% DeltaNp63 positive cells and 56% marker-negative cells after the heterochronic transplantation; in comparison 62% of DeltaNp63 positive cells, 23% marker-negative cells after the heterotopic transplantation). Why do the dorsal cells after the

heterotopic transplantation respond to BMP signaling and express DeltaNp63; and after heterochronic transplantation not? In both cases, they are challenged with high levels of BMP signaling, the cells receive even a higher level of BMP signaling after the heterochronic transplantation into a hyper-ventralised embryo. So, if the level BMP signaling would be the sole trigger for the cell fate decision, I would have expected that the ratio of DeltaNp63 positive cells after the heterochronic transplantation is similar or even higher than the ratio after the heterotopic transplantation. Other effects beside a morphogenetic role of BMP seem to be important for cell fate specification.

Community effects might account for the differences between the two approaches. After the heterotopic transplantation the cells are directly intermingled with an epithelium of cells, while in the heterochronic approach the cells are inserted between loosely associated cells. Could it be, that the close contact of heterotopically transplanted cells to epidermal precursors promotes the epidermal specification? It has been shown in explant studies of *Xenopus*, that the specification of the epidermis requires cell contact or close cell proximity (Jones and Woodland, 1986). It is speculative whether direct cell-cell contacts are required for epidemal specification or this is due to a community effect (Gurdon et al., 1993). To test the effect of cell-cell contact one would need to repeat the experiment with cells lacking the ability to form cell contacts, but it will be difficult to prove this idea in *in vivo* experiments like cell transplantations.

However, the results of explantations differ between Xenopus and zebrafish. In zebrafish, undissociated explants initiate and maintain the expression of neural markers, but it is lost after dissociation of the cells or upon challenge with BMPs (Grinblat et al., 1998; Sagerstrom et al., 1996, 2005). So far, no report describes the exact fate of the dissociated cells in zebrafish. So, it is speculative whether these cells stay undifferentiated or become epidermis. But on the other side, and in contrast to the data from Xenopus, neural precursors seem to require close proximity to each other to maintain neural commitment in zebrafish (Sagerstrom et al., 2005). A community effect of neuroectodermal cells will be a possible explanation of the behaviour of heterochronically transplanted dorsal cells, as I will discuss in more detail below.

I found some populations of dorsal cells with *sox19a* positive cells in the center of the cluster, but the cells at the margin of the population were unspecified. The formation of cellular clusters becomes evident by the analysis of the stainings, but I did not clarify whether it is an active migration of transplanted cells towards their siblings or this formation is due to the transient dissociation of the cells when they were transferred in a narrow capillary into the

new tissue. However, a group of dorsal cells can form its own community (population/ cellular cluster) in the ventralised blastula stage embryo. The transplanted cells are in direct contact to each other and protect themselves from signals of the ectopic environment (BMPs) via anatgonistic factors sectered by themselves, e.g. Fibroblast Growth Factors (FGFs). FGFs are antagonising BMP-signaling during early gastrulation and are involved in patterning of the neuroecoderm along the A-P axis (Furthauer et al., 1997; Pera et al., 2003; Rentzsch et al., 2004). All cells within such a population will emit this anti-BMP/ pro-neural signal, but the effective concentration of this factor can be reached only if the population of cells achieves a certain size. In the center of the cluster the concentration of the pro-neural factor will become high enough to drive neural differentation. Also the concentration of BMPs, emitted from the surrounding host tissue, will be at the lowest in the center of the population (Freeman and Gurdon, 2002). The cells at the margin of the population, so far described as unspecified cells, would receive intermediate levels of BMP. Cells receiving intermediate levels of BMP signaling should become neural crest or preplacodal ectoderm according to the fate map and as I disccussed above.

So far, I mainly focussed on the behaviour of the heterochronically transplanted dorsal cells. The ventral cells do not maintain their initial fate, either they do adopt the neural fate or they stay unspecified. The tendency to exclude the neural marker, too, and to become unspecified is more evident after heterochronic transplantation of ventral cells at the 80% epiboly stage. So, ventral cells seem to be more plastic, and they adopt new fates depending on the received level of BMP signaling. As I already mentioned, it is crucial to see whether the unspecified cells become neural crest and preplacodal ectoderm or not. If so, these results would be in line with my working hypothesis and with a morphogenetic role of BMP signaling integrated over time.

There are differences between the heterochronic and the heterotopic transplantation of ventral ectodermal cells at the 60% epiboly stage (after heterotopic transplantation 53% of the cells are unspecified, 37% adopt the neural fate; in comparison 39% of the cells are unspecified and 61% adopt the neural fate after the heterochronic transplantation). Why are heterochronically transplanted cells more plastic than cells after heterotopic transplantation? A community effect, as described above, should shield the heterochronically transplanted cells from neural inducing factors and promote the unspecified or intermediate fates. But another explanation might be simplier than community effects. The heterochronically transplanted cells (into a hyper-dorsalised embryo) are exposed to high levels of neural-inducing factors, and achieve the neural fates in high ratios. In the situation after the

heterotopic transplantation, the cells received slightly lower levels of neural inducing signaling, and therefore the cells either stay undifferentiated or become an intermediate fate in a higher frequency. If correct, ventral ectodermal cells sense even very small changes in pro- and anti-BMP signaling at 60% epiboly. Therefore one has to propose a morphogenetic role of BMP signaling as well as of anti-BMP / pro-neural factor signaling integrated over time.

5.3.3. Outlook and further perspective

It is not clear when ectodermal cells become committed to their specific fate during the course of gastrulation. There is some data pointing into the direction that the dorsal ectodermal cells are specified and committed at an earlier point of time than the ventral cells. But the results of my transplantation experiments show differences between each appoach and are inconclusive; and a lot of open questions remain.

The cell fate specification of ectodermal cells, both dorsal and ventral, seem to depend on the level and the time of BMP signaling, proposing a morphogenetic role of BMP signaling. Thereby the fate of a cell depend on the achieved level of BMP signaling over time, low levels will promote neural fates, intermediate levels will give rise to neural crest and preplacodal ectoderm and high and sustained levels of BMP signaling are required for the ventral most fates (see also above). But the different results between heterotopic and heterochronic show that community effects and probably even more factors than sole BMPs are involved in cell fate specification along the D-V axis. Further experiments have to adress the role of cell contacts and community effects as well as the possible morphogenetic role of BMP signaling.

The most striking question is, whether the unspecified cells achieved a fate of neural crest and preplacodal ectoderm, as I have speculated, or if they have acquired a completely unspecified fate. The new analysis of the transplanted cells with neural crest and preplacodal markers is absolute crucial, to test the hypothesis of a morphogene concept.

During gastrulation the mesendoderm becomes internalised and moves anteriorly. Still, it is possible that the unspecified cells are of mesodermal origin, although I carried out the transplantations close to the animal pole and very carefully. However, the injection of *lefty* RNA in donor and host embryos would reduce the risk of mesodermal contaminations. Overexpression of Lefty completely abolishes mesoderm induction at blastula stage, via antagonising Squint signaling, without affecting the D-V patterning of the ectoderm (Thisse and Thisse, 1999; Chen and Schier, 2002).

One has to perform transplantations during the whole course of gastrulation, to nail down the exact timing of cellular commitment. The transplantation of dorsal cells until the end of gastrulation (tailbud stage, 10 hpf) will be necessary. This will technical be very challenging because the dorsal tissue becomes more compact and the ventral side becomes very thin. The injection of *has2* MO, to impair the gastrulation movements without affecting D-V patterning, would avoid these problems (Bakkers et al., 2004). This could be done in combination with the *lefty* RNA injection, to reduce the technical source of errors to a minimum.

As I speculated above, ventral cells sense even very small changes in pro- and anti-BMP signaling at 60% epiboly. If the aforementioned morphogenetic role of BMP signaling is correct, a fine-mapping of the plasticity of ventral cells from shield stage to the 70% epiboly stage might be worth to perform.

Further one could combine loss and gain of function approaches in the transplantation experiments to adress the question, which factors are required for cellular commitment. So, one could test dorsal cells with a gain of BMP signaling in a dorsal and ventral environment, as well as ventral cells with a loss of BMP signaling. One could also test additional factors that might be involved. For example, whether gain of FGF signaling in the donor cells can promote the neural fate of ectopically transplanted cells or not, this will show the relevance of these factors.

6. Large-scale screen using morpholino antisense nucleotides to identify new genes involved in early development, pituitary or skin development

6.1. Introduction

Genetic studies of loss-of-function mutations gain insights to the function of a gene. The Nüsslein-Volhard and Driever labs started in the 1990s large-scale screens using ENU-mutagenised zebrafish. The zebrafish is an ideal vertebrate model organism for large-scale screening. These and following forward genetic screens gave the first large collection of mutants for vertebrate development. The generation of mutagenised fish, the screening for phenotypes and positional cloning of the mutated gene is a time-expensive approach. In contrast the design of morpholinos and injections to obtain morphant embryos can be done in a little while. A further advantage of reverse genetics is that the targeted gene is already known. Stephen C. Ekker (University of Minnesota, Minneapolis) started an international consortium of zebrafish labs, funded by the NIH (USA) for screening a collection of morpholinos. The morpholino collection targeted either annotated full length genes or Expressed Sequence Tags (EST) of so far unknown function. The screen was organized and coordinated by the lab of Stephen C. Ekker (Pickart et al., 2006).

As a starting point of my PhD thesis work I joined this consortium and performed a large-scale screen using the collection of morpholino antisense oligonucleotides.

6.2. Results

Morpholinos were injected in different concentrations (0.01mM to 1.5mM); injected embryos were screened for the first three days of development by general morphology. To circumvent unspecific effects of the MOs, like small heads and eyes (SHE), brain necrosis, edema in the heart and the brain and retarded development, I reinjected some MOs together with p53 MO (Robu et al., 2007). Further the injected embryos were checked for proper pituitary development, by fixation at three days post fertilisation and analysis by whole mount *in situ*-hybridisations with the previously described pituitary gland markers *pomc* and *prl* (Herzog et al., 2004). A second batch of injected embryos was stained for DeltaNp63 to check keratinocyte and skin development (Lee and Kimelman, 2002).

I picked genes and EST with known or annotated expression pattern (ZFIN expression pattern Database). Thereby I concentrated on genes expressed in the developing pituitary gland or epidermis. A second criteria for selection was homology to genes that are annotated in OMIM (Online Mendelian Inheritance in Man), a database for human disorders, and could also cause developmental defects in zebrafish. The morpholinos were designed by members of the consortium themselves. Morpholinos named with BR…. were designed by myself as contribution to the screened MO collection.
In total I screened morpholinos targeting 60 different genes or ESTs. 36 out of these 60 morpholinos caused effects in general. In the following I describe the morpholino targets and observed effects of injected embryos.

BR0001 zgc:110343; an EST expressed in hypothalamus and epidermis. Its sequence is similar to *natural killer cell enhancement factor* (*nkef*), a factor of the antioxidant protein family that enhances natural killer cell activity (Zhang et al., 2001).
Embryos injected with this morpholino showed pleiotropic defects such as slight toxicity during the first 24 hours (17/88; 7/70), edemas along the body, small eyes, curved tail or reduced tail fins. A concentration of 0.1mM caused a significant developmental delay in an additional part of the clutch (11/70). No differences in skin or pituitary tests were observed compared to the uninjected control.

BR0002 *secretograninV* (*scg5*) is expressed in the zebrafish pituitary gland. Secretogranins belong to the chromogranin-secretogranin family and are realesed from neuroendocrine cells as granins, which are acidic proteins co-localized with peptides in secretory granules (Taupenot et al., 2003)
Injected embryos did not show any phenotype except for small edema, no phenotype during the development of the pituitary gland was observed.

BR0003 *secretograninIII* (*scg3*) is another member of the chromogranin-secretogranin family, which is also expressed in the zebrafish pituitary gland.
This morpholino was toxic at a concentration of 0.5mM; otherwise it did not cause any phenotype.

BR0004 and BR0019 *n-myc downstream related family member 3b* and *3a* respectively, are expressed in the zebrafish pituitary gland, a gene function is not reported so far.

The morpholino targeting *ndrg3b* (BR0004) caused SHE phenotype, heart edema, shortened axis or curved down tail with a penetrance of 50% in a concentration of 0.01 mM. The morpholino against *ndrg3a* showed similar phenotypes with a lower penetrance. Coinjection of both morpholinos increased the penetrance with no additional phenotypes. By *in situ*-hybridisation with pituitary gland markers the intensity of the staining was increased, but no additional cells were observed and the increased intensity occured solely in embryos with small heads. Injection of single or both morpholinos into transgenic pomc::GFP embryos did not show an increase in pomc expression, indicating that changes in intensity were staining artefacts.

BR0005 *inositol heyaphosphate kinase 2* (*ihpk2*) is expressed in the zebrafish pituitary gland and encodes an enzyme required in the production of inositol pyrophosphate and inositol trisphosphate (Saiardi et al., 1999).

The morpholino injected in a high concentration of 1mM caused edema, apart from that the embryos and analysis of pituitary gland markers revealed no phenotypes.

BR0006 *claudin h*; the gene target was chosen because of its expression domain in the zebrafish pituitary gland. Claudin h is a tight junction molecule that belongs to large family of claudins (Angelow et al., 2008).

A few of the injected embryos generated showed strong edema during the first five days at a morpholino concentration of 0.1mM (5/105). The tests with pituitary markers did not show any change in marker expression.

BR0010 *claudin 7,* another tight junction molecule, is expressed in the zebrafish epidermis, but also in several other tissues like pronephric duct, neuromasts, hatching gland and otic placode (Angelow et al., 2008).

This morpholino caused high toxicity and necrosis at a concentration of 0.05 mM, 4 out of 80 suffered from a gastrulation arrest and less affected embryos had uncoordinated swimming movements. Lower concentrations just caused brain necrosis. No obvious changes in DeltaNp63 stainings were observed.

BR0007 *spalt-like1*, is expressed in the zebrafish brain including the pituitary gland and the homologue to the homeotic gene *spalt* (*sal*) in *Drosophila.*

Injected embryos showed edema at the heart and in the brain; also a small head/ small eyes phenotype was observed; *pit1*, *prl* and *pomc* markers for the pituitary gland was misexpressed or the intensity of expression was altered, but only in embryos with SHE. Coinjection of p53 MO did prevent any phenotype.

BR0008 zgc:174890, an EST encoding a predicted *Zinc-finger protein*, that is expressed in the EVL and epidermis.

The morpholino caused high toxicity and pleiotropic phenotypes in a concentration of 0.05 mM; whereas 0.01 mM caused blisters and edema at day three of development.

BR0009 im:7142141, an EST of a predicted gene, encoding a *GA-binding-protein*, a putative transcription factor that is expressed in the epidermis.

In a high concentration of 1 mM the embryos became necrotic, developed edemas and smaller heads, the embryos did not survive until day five. Single cells were delaminating from the skin, but staining with DeltaNp63 did not confirm a skin phenotype.

BR0011 *cysteine-rich, angiogenic inducer, 61* (*cyr61*) encodes an extracellular protein, that promotes the adhesion of endothelial cells (Babic et al., 1998). The morpholino was ordered for tests of skin defects.

Injected embryos had an overall smaller body size and loss of tail fins together with kinked tail, they survived only for two days. The morpholino was toxic above 0.01mM concentration; coinjection with p53 morpholino in the same concentration reduced the strength of the phenotypes as well as toxicity. Staining against DeltaNp63 did not show a specific loss of keratinocytes.

BR0012 zgc:64101 is an unknown EST that was ordered by the lab of Stephen Ekker.

This morpholino caused edema along the body axis and loss of the tail tip, but had a low penetrance (15/76 embroys for 0.05mM; 5/60 for 0.1mM).

BR0013 *coagulation factor II (thrombin) receptor-like1* encodes a G-Protein-coupled receptor, also called *proteinase-activated receptor 2* (*par2*), was ordered in collaboration with Tom Carney to test for putative skin development.
Embryos injected with this morpholino showed no specific phenotype, but strong necrosis from 0.1 mM upwards.

BR0014 *ron protein tyrosine kinase*, also called *macrophage stimulating 1 receptor* (*c-met-related tyrosine kinase*) (*mst1r*) was ordered in collaboration with Tom Carney because of its presumptive role in skin development (Carney et al., 2007)

BR0015 *nidogen2*, a basement membrane protein described in mouse and human (Kohfeldt et al., 1998; Schymeinsky et al., 2002), It was annotated to be expressed in the zebrafish hypophysis. In the current annotation the expression pattern is not available anymore and in the new annotated sequence this morpholino is not targeting the 5'UTR.
This morpholino did not cause any effects and was obviously misdesigned.

BR0016 *transforming growth factor beta 1*, was picked because of its expression in the epidermis at day one of development. It was cloned and described in oocyte maturation (Kohli et al., 2003), meanwhile it was also used as marker in wound healing of adult zebrafish (Andreasen et al., 2007).
The morpholino caused pleiotropic defects with a low penetrance (6/45 and 14/51); the embryos without pleiotropic phenotypes were screened for keratinocytes, but did not show a phenotype. Around day 3 all embryos died.

BR0017 cellular retionoic acid binding protein 1a, is expressed in the neurohypophysis (Liu et al., 2005) and was picked for a collaboration with Hans-Martin Pogoda.

BR0018 sb:cb649, an EST expressed in the EVL during gastrulation and later in the pituitary gland. It is predicted to encode a SH3 domain binding protein.
The morpholino caused pleiotropic defects (SHE, edemas, brain necrosis) and showed up to 50% (41/82, 14/81) toxicity during the first 48 hours. At a concentration of 0.1mM the embryos did not move upon touching, too. No specific phenotype was found in the skin or pituitary test.

BR0020 *dyskerin*, found to be the gene responsible for dyskeratosis congenitautaneous pigmentation, dystrophy of the nails, leukoplakia of the oral mucosa, continuous lacrimation due to atresia of the lacrimal ducts, often thrombocytopenia, anemia, and in most cases testicular atrophy (Knight el al., 1999; see also for an over view OMIM). It was choosen for test of skin defects.

Concentrations of 0.5mM caused toxicity and very strong necrosis during the first day of development. At lower concentrations of 0.2mM embryos were smaller in body size and had SHE phenotype, the blood circulation was absent and heart edema did form, the embryos showed uncoordinated swimming movements and survived only until day four. But no specific phenotype in the keratinocytes was observed.

BR0021 *zinc metallopeptidase STE24*, This MO was designed because of its annotation in OMIM. Malfunctions of it can cause Mandibuloacral dysplasia, patients with this disorder suffer from skeletal abnormalities, cutaneous atrophy and lipodystrophy.

This morpholino caused no morphology or skin defects during the first five days of development.

BR0022 *zinc finger protein 750*, was found in OMIM to cause the human disease seborrhea-like dermatitis with psoriasiform elements (Birnbaum et al., 2006) and tested for skin phenotypes.

This morpholino did not cause a skin phenotype, even in a concentration of 1.5mM.

BR0023 zgc:85705, an EST encoding T-Box gene 2a, picked for the screen because of its predicted expression in the pituitary gland, but recently shown to be involved in heart development (Ribeiro et al., 2007)

This morpholino did not show phenotypes in pituitary development or general morphology. The morpholino was not tested for heart defects as control for proper morpholino functionality.

BR0024 *t-box gene2b*, is according to the ZFIN expression pattern database expressed in the epihysis, and was ordered to be checked for pituitary/neurohypophysis development (Fong et al., 2005; Gross and Dowling, 2005).

Also this morpholino did not show any phenotype by morphology or pituitary markers, even when coinjected with the *tbx2a* MO (BR0023).

BR0025 *ventral anterior homeobox gene* (*vax*), is expressed in the anterior forebrain and pituitary gland, before shown to be involved in the development of the optic stalk (Take-uchi et al., 2003).
This Morpholino did not cause any phenotype.

BR0026 *frizzled homolg8b*, is expressed in the zebrafish neurohypophysis. It is a predicted receptor of Wnt signaling with no published function so far.
0.05mM concentrated morpholino did cause some SHE phenotype (6/50), but no change in *pomc* expression.

BR0027 *sciellin*, in humans the protein is found in keratinizing tissues and functions as barrier protein (Champliaud et al., 2000; Champliaud et al., 1998) and was investigated for a potential role in zebrafish skin development.
Injection of high concentrations above 1mM caused edema, but with a low penetrance (5/49). No further skin phenotypes were observed.

SP3001 *plexin D1*; a gene that is described in development of the vasculature and nervous system (Gitler et al., 2004).
Embryos injected with raising concentrations of morpholino (from 0.05 to 0.1mM) did not show any phenotype in morphology, pituitary or skin development.

SP3002 TC285526, an EST for a hypothetical protein of unknown function.
Injection of this morpholino did not show any phenotypes in any test.

SP3003 TC269498, an EST of a novel Cysteine Proteinase, most similar to CathepsinK.
This morpholino showed SHE phenotype, edema, and a reduced hypothalamic *pomc* expression as well as a general lethality of 50%.

SP3004 *prepronociceptin*, encoding the precursor of the neuropeptide nociceptin, a natural agonist of opioid receptor in humans (Mollereau et al., 1996)

Concentrations of 0,02mM led to embryos with curled up tail, reduced blood flow and movement defects, higher concentration caused lethality during the first 24 hpf.

SP3006 TC280069, similar to *BBP-like protein2*, beta-amyloid peptide-binding protein (denoted BBP) containing a G protein-coupling module. This protein binds beta-amyloid peptide and induces apotosis via caspase activity, as seen in the degeneration of neurons in Alzheimer disease (Kajkowski et al., 2001)
Injection of 0.05mM morpholino caused SHE in 19% (n=14/71) of injected embryos, also it led to a loss of hypothalamic *pomc* expression, which might be due to the small head phenotype. A concentration of 0.1mM led to general necrosis.

SP3007 TC283881, an EST with similarities to *Cysteine knot secreted protein DAN*, a putative BMP-like protein.
Upon injection diverse phenotypes were observed like delayed gastrulation, loss of the tail fin or edema at the heart, but for every phenotype with a very low penetrance (below 5% per clutch).

SP3008 *renin*, human Renin catalyzes the first step in the activation pathway of angiotensinogen, which is further required for aldosterone release, vasoconstriction and the increase in blood pressure.
Embryos injected by this morpholino developed edema from day one onwards.

SP3009 TC284843, this EST is weakly similar to *nerve growth factor receptor* (*ngfr*) (Bothwell, 1996).
A concentration of 0.02mM led to high lethality during the first day after injection, the surviving embryos had a reduced blood flow and a ventral curvature of the body axis.

SP3010 TC271225, an EST of an unknwon protein with calcium binding properties.
This morpholino did not cause any phenotypes.

SP3011 *stanniocalcin2*, Stanniocalcins are glycoprotein hormones working as paracrine factor during follicular development (Luo et al., 2005)

10% of injected embryos showed retardation during development, edema or shorten tails.

SP3012 *microfibril-associated glycoprotein-1* (*magp1*), this morpholino and the role of Magp1 in vascular development is described by the lab of Stephen Ekker (Chen et al., 2006).

SP3013 TC299698; an EST with a weak similarity to *WNT1 inducible signaling pathway protein 2* (*WISP2*). WISPs belong to the subfamily of connective tissue growth factors. They are acting downstream of wnt signaling and might play a role in tumor biology (Pennica et al., 1998)
This morpholino show pleiotropic defects with strong necrosis and a high lethality upon injection of 0.05mM. In lower concentration I observed a reduction in DeltaNp63 staining, but could not confirm this phenotype in further injections.

SP3014 TC268808, is an EST of a hypothetical protein with no known similarities.
The injected morpholino did not cause any phenotype.

SP3015 *torsinA*, which is an ATPase of the AAA family and has got functional properties of a chaperone (Konakova et al., 2001)
Zebrafish injected with 0.1mM morpholino responded after touching with uncoordinated/ spasm movements of their body.

SP3017 TC281391 is an EST weakly similar to a member of the family of *Epidermal growth factor like proteins*.
10% of embryos injected with 0.05 mM concentrated MO showed delayed development at 24 hpf, but recovered after 48 hpf.

SP3019 *ephrinB2* (*epnb2*), EphrinBs are transmembrane ligands for Ephrin receptor tyrosine kinases, EphrinB2 is described to play a role in hematopoiesis (Sakano et al., 1996)
Injection of this MO caused no defects.

SP3020 TC282663, an EST for a novel protein with a weak similarity to Cystatins, a family of mammalian lysosomal cysteine proteinases.
Embryos injected with 0.1mM MO had movements defects and a shorten tail.

SP3021 TC269176, EST similar to *ER-Golgi intermediate compartment 53 kDa protein* (*ergic53*), in humans also called Lectin. The protein functions as chaperone and is important for proper coagulation (Nichols et al., 1998b)
Injection of 0.05mM led to pleiotropic defects like SHE and heart edema and 50% of the injected embryos showed movement defects, higher concentration led to a shorten axis, too.

SP3022 is targeting the gene *transforming growth factor-ß typeII receptor* (*tgfbr2*), which was before described in oocyte maturation (Kohli et al., 2003)
Injection of 0.1mM or higher concentrations led to shorten axis with blocky somits, always in combination with brain necrosis and SHE phenotype, even upon coinjection of the same amount of *p53* morpholino

SP3023 TC288467; an EST that is weakly similar to Cytochrome P450 2K1 (Cyp2K1), a novel member of the large family of cytochrome P450 proteins.
Injection of this morpholino showed no phentotype.

SP3024 TC294353, an EST similar to Esophageal cancer related gene 4 protein (ECRG4-A) (Yue et al., 2003)
This morpholino caused pleiotropic defects and high lethality from concentrations of 0.01mM upwards.

SP3026B *ApolipoproteinE*; which has multiple roles in lipid homeostasis, like interactions with lipoproteins. In zebrafish it is expressed in the YSL (Poupard et al., 2000).
Embryos showed no phenotypes compared to the control.

SP3054b CB578, an EST similar to *Neuroplastin*, a member of the Ig superfamily expressed in human brain (Bernstein et al., 2007).
The morpholino was toxic in concentrations of 0.1mM and caused strong necrosis in lower concentrations.

BB0006 *lipocalin-interacting membrane receptor*, the protein is working as endocytic receptor in human cell lines (Wojnar et al., 2003)
This morpholino caused pleiotropic defects (SHE phenotype, shorten tail or curved up, edema). In a concentration of 0.1mM also loss of blood circulation, edema in vasculature and blood pooling.

boc *brother of cdon*, BOC is decribed as cell surface receptors of the immunoglobulin (Ig)/fibronectin type III repeat family. It is involved in myogenic differentiation (Kang et al., 2002).
Embryos injected with 0.1mM morpholino were retarded after 24 hpf and showed pleiotropic phenotypes. Coinjection with p53 morpholino did increase the phenotypic strength and lethality.

cndp2 *carnosine dipeptidase2*, human Carnosine Dipeptidase2 is a nonspecific dipeptidase (Teufel et al., 2003).
Upon injection of this morpholino no phenotype was found.

elmo1 *engulfement and cell motility gene 1*, the *C.elegans* homologue *ced12* is required for phagocytosis and cell migration functioning via Dock180 and CrkII (Gumienny et al., 2001)
Upon injection of this morpholino no phenotype was found.

mcts1 Malignant T-Cell Amplified Sequence1, a putative oncogene that increases cell proliferation by decreasing G1-Phase (Prosniak et al., 1998)
This morpholino caused no phenotype.

npc2 *niemann-pieck C Disease 2*, encoding a cholesterol-binding protein involved in the Niemann-Pieck disease, a disorder of lipid storage affecting visceral and central nervous system (Naureckiene et al., 2000)
No phenotype was found in morphology and touch response.

nxph1 *neurexophilin 1*, a neuropeptide-like glycoprotein bindimg to alpha neurexins (Missler and Sudhof, 1998)

Upon injection of this morpholino no phenotype was found.

ppt1 *palmitoyl-protein thioesterase 1*, this enzyme is involved in catabolism of lipid-modified proteins (Camp et al., 1994)
This morpholino caused no phenotype.

rfng *radical fringe* is a mammalian homologue of *Drosophila mel. fringe*, the protein is modulating the activity of Notch receptor (Johnston et al., 1997).
No phenotype was found.

6.3. Discussion

The outcome of the screen was less efficient than of an ENU-mutangenesis in regard to the discovery of new gene functions. The selection of genes was just based on published expression data (ZFIN expression pattern database). But neither me nor the collaborating labs have done a confirmation of the expression pattern prior to morpholino design. Hence some morpholinos might target genes with wrong annotated expression pattern and could not cause any phenotype. Or they caused unexpected phenotypes that were overlooked. Also a further validation of the annotated sequences, like a 5'RACE (rapid ampification of cDNA ends), was not done. So morpholinos could be designed against wrong target sequences, especially in the case of ESTs. A more stringent validation of the targeted genes might decrease the number of false positives or morphants without any phenotype.
Morpholinos are chemical substances and can cause toxic side effects. Many of the morpholinos I screened, showed known unspecific phenotypes, like small heads and eyes (SHE), brain necrosis, edema in the heart and the brain and retarded development. Although simultaneous knock-down of p53 function can deplete toxicity (Robu et al., 2007), real phenotypes might be masked by either the unspecific defects or even by the knock-down of p53 function. Further the efficiency of morpholinos depends on their chemical composition and purification. Every gene/ EST was only targeted by one morpholino. So it might be that an inefficient MO let me miss a phenotype. During the screen several batches showed reduced efficiency of newly synthesised morpholinos, even in controls with known morpholinos. Because of this variability in morpholino synthesis I assume that some morpholinos giving no phenotype were just ineffective.

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8. Appendix

I also contributed substantial work during the revisions of following manuscript in addition to my thesis work.

Martina Jänicke, Björn Renisch and Matthias Hammerschmidt (2009). „Zebrafish grainyhead-like1 is a common marker of different non-keratinocyte epidermal cell lineages, which segregate from each other in a Foxi3-dependent manner“ **Int.J.Dev.Biol.,** *in press*

My contributions are to the final manuscript are in particular: Figure1, panels (P-T); Figure 2, panels (C-E); Figure 4 panel (F), Supplementary Figure S2 and Supplementary Figure S6.

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Abbreviations

AA	arachidonic acid
Alk	Activin receptor-like kinase
A-P	anterior-posterior
ASS	acetylsaliacylacid
bp	base pairs
BSA	bovine serum albumin
Bmp	bone morphogenetic protein
CA	constitutive active
C-terminal	carboxy-terminal
C&E	convergence and extension
cDNA	DNA complementairy to RNA
D-V	dorsoventral
ddH_2O	double distilled water
DMSO	dimetylsulphoxide
DNA	deoxyribonucleic acid
dNTP	Desoxyribonucleotide
dpf	day(s) post fertilization
DTT	Dithiothreitol
e.g.	example given
ENU	ethylnitrosourea
EST	expressed sequence tag
EVL	enveloping layer
Fgf	fibroblast growth factor
g, mg, µg, ng	gram, milligrams, micrograms, nanograms
GFP	green fluorescent protein
HETE	hydroxy eicosatetraenoic acid
hpf	hours post fertilization
kb	kilobase pairs
L, mL, µL	litres, millilitres, microlitres
M, mM, µM	moles, millimoles, micromoles per litre
MO	morpholino oligonucleotide
mRNA	messenger RNA
n	number
NSAID	non steroidal anti inflammatory drugs
PBS	phosphate buffered saline
PBST	phosphate buffered saline with 0.1% Tween
PCR	polymerase chain reaction
PFA	para-formaldehyde
PG	prostaglandin
PLA2	Phospholipase A2
PPAR	peroxisome proliferator-activated receptor
p-Smad	phosphorylated Smad1/5/8
RNA	ribonucleic acid
RT	reverse transcription
Smad	Sma and Mad family member
tBR	truncated BMP receptor
Tris	Tris-(Hydroxymethyl)-amino methane
Tween-20	polyoxyethylensorbitanmonolaurat
rpm	rotations per minute
w/v	weight per volume
v/v	volume per volume
YSL	yolk synticial layer

9. Acknowledgements

First of all I would like to thank Prof. Dr. Matthias Hammerschmidt for giving me the opportunity to work in his lab and for providing these interesting projects. I thank him for all the suggestions, proof-reading and comments that helped to improve my scientific skills and his never ending willingness to encourage me not to give up on a scientific problem.

I would also like to thank the second referee of this thesis, Prof. Dr. Siegried Roth .

I thank all present and past members of the Hammerschmidt Lab for the interesting scientific discussions, the good working atmosphere, for technical advise and also for cheering me up. In particular I would thank Kathrin and Hans-Martin for being great bench mates. A very special thank goes to Martina, you brought a lot of fun into my PhD.

I am very grateful to my collaborators; I thank Steven A. Farber and his coworkers for the interesting discussions about lipid signaling and for providing me with materials and methods. For many interesting and helpful telephone calls about morpholinos and for organising the screen, I thank Stephen C. Ekker and his coworkers.

Further I have to thank all members of the Max-Planck-Institute in Freiburg for providing an excellent scientic environment and for a lot of funny events. A special thank to Christine for listening.
For a nice welcome in Cologne and an interesting last year of my PhD, I would thank all members of the Institute of Developmental Biology.

At last, I thank my family and all my friends for sympathy and a lot of support during the last four years.

Erklärung

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