Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 331 (2005) 303–308
BBRC
www.elsevier.com/locate/ybbrc

Wortmannin induces zebrafish cardia bifida through a mechanism independent of phosphoinositide 3-kinase and myosin light chain kinase

Yuexiang Wang [a], Tao Zhong [a,b], Linxi Qian [a], Yongxin Dong [a], Qiu Jiang [a], Li Tan [a], Houyan Song [a,*]

[a] *Department of Molecular Genetics, Shanghai Medical School and Key Laboratory of Molecular Medicine, Ministry of Education, Fudan University, Shanghai 200032, PR China*
[b] *Department of Medicine and Cell Biology, School of Medicine, Vanderbilt University, Nashville, TN 37232, USA*

Received 21 March 2005
Available online 2 April 2005

Abstract

Cardia bifida is an anomaly of the embryonic heart in which the bilateral myocardial rudiments fail to travel to the midline, resulting in the formation of two separate hearts in lateral positions. In zebrafish, eight loci responsible for the cardia bifida phenotype were identified in the large-scale genetic screen. Wortmannin has been reported to be a highly selective inhibitor of phosphoinositide 3-kinase and myosin light chain kinase activity. We provide the first evidence that wortmannin treatment of zebrafish embryos can induce cardia bifida in a dose-dependent manner and that wortmannin alters cardiac development between 6 and 16 h post-fertilization. In addition, we demonstrate that wortmannin induces zebrafish cardia bifida through a mechanism independent of phosphoinositide 3-kinase and myosin light chain kinase. Our findings may provide new insights into the cardiomyocyte function and disfunction.

Keywords: Cardia bifida; Zebrafish; Wortmannin; Chemical genetics

Wortmannin was originally isolated from *Penicillium wortmannii* and has been reported to directly inactivate phosphoinositide 3-kinases (PI3 kinases) by covalent modification of the catalytic subunit with a low nanomolar IC_{50} [1–6]. Wortmannin can also interact directly with the catalytic domain of myosin light chain kinase (MLCK) at micromolar concentrations [36,37]. It has been widely used as a selective inhibitor of PI3 kinase or MLCK for the study of signal transduction pathways in different systems [2,3,7–10,35–37].

The zebrafish, *Danio rerio*, offers several distinct advantages as a genetic and embryological model system, including the external fertilization, rapid development, and optical clarity of its embryos [11,12]. In addition, owing to their small size, zebrafish embryos are not completely dependent on a functional cardiovascular system. Even in the total absence of blood circulation, they receive enough oxygen by passive diffusion to survive and continue to develop in a relatively normal fashion for several days, thereby allowing a detailed analysis of animals with severe cardiovascular defects [13–16]. By contrast, avian and mammalian embryos die rapidly in the absence of a functional cardiovascular system. In all vertebrates, the formation of the heart initiates soon after gastrulation, and the heart is the first organ to form and function during embryogenesis. The heart tube, which consists of a myocardial and an endocardial layer, is the result of the fusion of the bilateral heart primordial. During somitogenesis, the myocardial

* Corresponding author. Fax: +86 21 6403 3738.
E-mail address: hysong@shmu.edu.cn (H. Song).

0006-291X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2005.03.145

precursors appear to converge at the midline in concert with the anterior lateral plate mesoderm and fuse to form the heart tube [17,18]. Large-scale genetic screens in zebrafish have identified mutations in eight loci that have been disrupting this process so far, resulting in the formation of two separate hearts, a phenotype known as cardia bifida. The fusion of the primitive myocardial tubes results in the formation of the definitive heart tube. The molecular events underlying this process are not well understood [13,14,16–19].

Here, we show for the first time, to our knowledge, that wortmannin treatment of zebrafish embryos can induce cardia bifida in a dose-dependent manner. Wortmannin alters cardiac development between 6 and 16 hpf. In addition, we demonstrate that wortmannin induces zebrafish cardia bifida through a mechanism independent of PI3 kinase and MLCK.

Materials and methods

Zebrafish strains and maintenance. Wild-type (AB* strain) zebrafish stocks were obtained from International Zebrafish Research Center. Embryos were obtained from natural spawning of wild-type adults. Zebrafish were raised, maintained, and staged as previously described [20,21].

Drug treatments. Kinase inhibitors, wortmannin, LY294002, ML-7, and ML-9 (Sigma, USA), were dissolved in dimethyl sulphoxide (DMSO) at stock concentrations of 10 mM, and then diluted to final concentrations in embryo media [20] at the stages indicated. Control embryos were treated with the equivalent amount of DMSO solution. All embryos were incubated at 28.5 °C [20].

Whole-mount RNA in situ hybridization. Plasmids encoding zebrafish *ventricular myosin heavy chain* (*vmhc*) and *atrial myosin heavy chain* (*amhc*) were kindly provided by Tao Zhong (Vanderbilt University, Tennessee, USA). Whole-mount RNA in situ hybridization using digoxigenin (DIG)-labelled antisense RNA probes was performed using standard methods as previously described [20,22,23]. Briefly, DIG-labelled antisense RNA probes were produced using DIG-RNA labeling kit (Roche, IN) following the manufacturer's instructions. Hybridization and detection with an anti-DIG antibody coupled to alkaline phosphatase was performed with fixed zebrafish embryos.

Whole-mount immunofluorescence. Whole-mount immunofluorescence experiments were performed as previously described [23], using the monoclonal antibodies MF20 and CH1. MF20 and CH1 were obtained from the Developmental Studies Hybridoma Bank, University of Iowa.

Photography. Stained embryos were examined with Olympus BX61 and SZX12 microscopes, and photographed with a DP70 digital camera. Images were processed using Adobe Photoshop software.

Online supplemental material. Supplemental video material is available online. The videos show the heart beat of a wortmannin-treated embryo (Movie 1) and a control embryo (Movie 2) at 48 hpf.

Results

Cardia bifida in wortmannin-treated zebrafish embryos

To investigate the possible influence of wortmannin on zebrafish development, we observed zebrafish embryos' morphology under different concentrations of the drug from nanomolar to micromolar. Wortmannin causes two swollen pericardial sacs to form, one on each side of the body as occurs in zebrafish mutants with the condition cardia bifida, instead of a single heart forming in the middle. At 48 hpf, there is no blood flow in the wortmannin-treated embryos. Two hearts also beat independently and are located at either side of the body (see Movie 1). In control embryo, only one heart forms, and the ventricle and atrium exhibit vigorous, rhythmic contractions (see Movie 2).

To test whether the two separate hearts consist of distinct atrial and ventricular tissues, we carried out whole-mount RNA in situ hybridization to examine the expression pattern of zebrafish atrial and ventricular markers. Two hearts can be clearly visualized by in situ hybridization of either ventricular marker *vmhc* or atrial marker *amhc*. Wortmannin-treated embryos have two main populations of ventricular myocardial (Fig. 1, compare Figs. 1E and I with Fig. 1A) at variable distance from the midline (Fig. 1, compare Fig. 1E with Fig. 1I). Wortmannin can also induce two main populations of atrial myocardial (Fig. 1, compare Fig. 1F with Fig. 1B). We performed whole-mount immunofluorescence to examine the expression of genes known to be involved in cardiac contractility. Embryos were stained with the monoclonal antibody MF20 (TRITC), which recognizes a sarcomeric myosin heavy chain epitope found in both the ventricle and atrium. The anti-tropomyosin antibody CH1 (FITC) stains both chambers. It is clear that wortmannin-treated embryos display cardia bifida, with the two separate hearts (Fig. 1, compare Figs. 1G and H with Figs. 1C and D).

Wortmannin induces zebrafish embryos cardia bifida in a dose-dependent manner

We next examined whether wortmannin induces zebrafish embryos cardia bifida in a dose-dependent manner. Embryos were exposed to different concentrations of wortmannin at 6 hpf and then transferred to embryo media without wortmannin at 24 hpf. The presence of cardiac bifida was scored at 48 hpf. Fig. 2 shows that concentrations of wortmannin lower than 2 μM cannot induce cardia bifida. At a concentration of 8 μM, a partial biological activity was observed with a 20–30% induction of cardia bifida. In several experiments, 16 μM of wortmannin is able to induce a significant percentage of cardia bifida (50–60%). At concentrations above 32 μM, wortmannin treatment caused nonspecific defects.

Wortmannin alters cardiac development between 6 and 16 hpf

We took advantage of the temporal control afforded by small molecules to define the developmental stage at

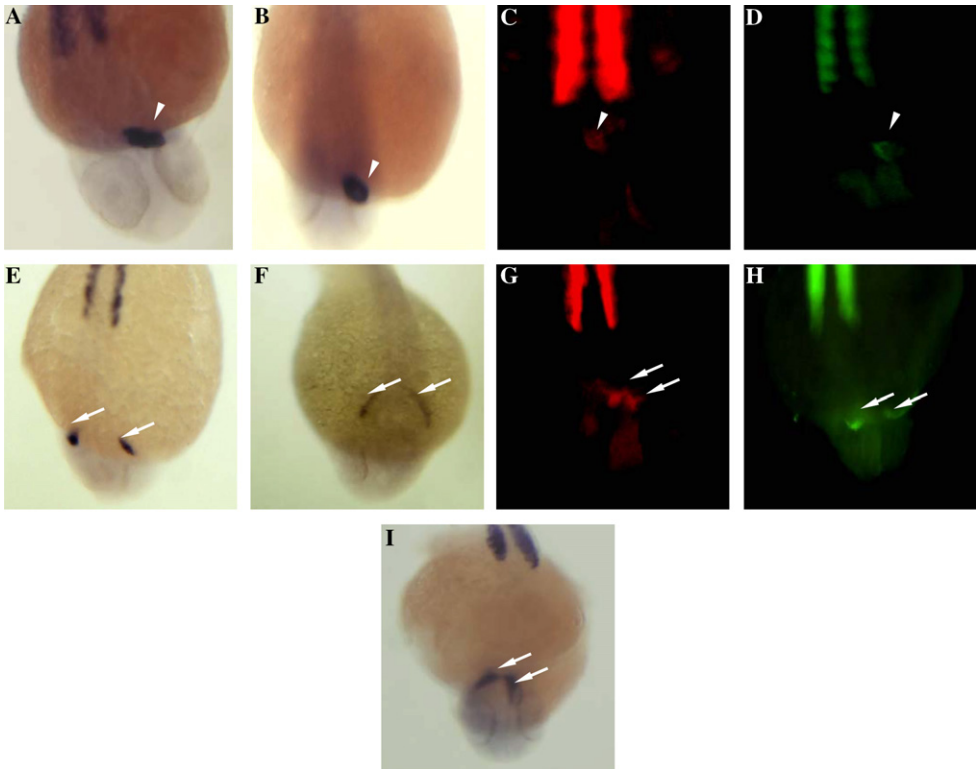

Fig. 1. Cardia bifida in wortmannin-treated zebrafish embryos. Wortmannin treatment of live zebrafish embryos specifically results in cardia bifida. Zebrafish embryos were treated at shield stage (6 hpf) with wortmannin (E–I) and compared with untreated embryos (A–D) at the same stage. Dorsal views with anterior at the bottom. (A,E,I) Expression of *vmhc* in control embryo and wortmannin-treated embryo. (B,F) Expression of *amhc* in control embryo and wortmannin-treated embryo. (C,G) Whole-mount immunofluorescence with the monoclonal antibody MF20 (TRITC). (D,H) Whole-mount immunofluorescence with the anti-tropomyosin antibody CH1 (FITC). Arrows in E, F, G, H, and I show cardia bifida and arrowheads in A, B, C, and D show single heart.

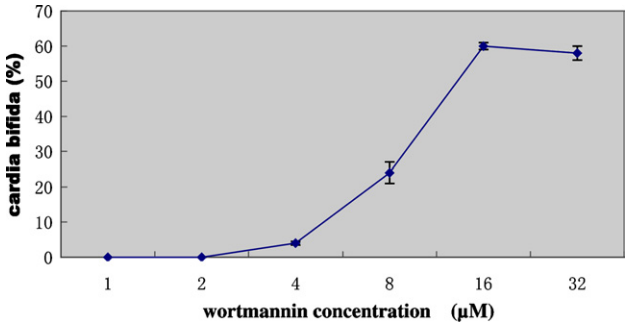

Fig. 2. Dose–response curve for induction of cardia bifida phenotype by wortmannin. Embryos were exposed to the indicated concentrations of wortmannin at 6 hpf and were transferred to embryo media without wortmannin at 24 hpf. The percentage of cardiac bifida was scored at 48 hpf. Symbols and vertical bars represent the means and SD of three experiments.

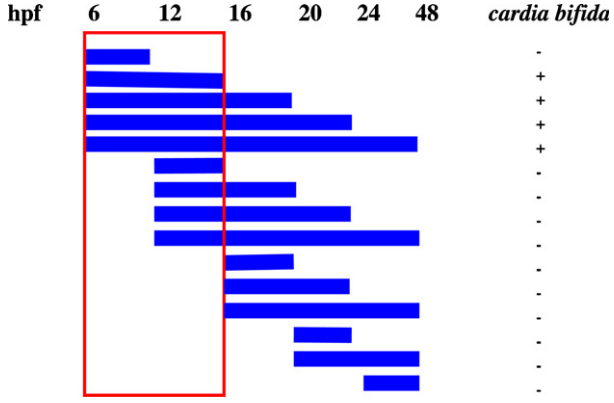

Fig. 3. Wortmannin alters cardiac development between 6 and 16 hpf. Embryos were treated with wortmannin at a concentration of 16 μM during the times indicated by blue bars, after which the embryos were transferred to embryo media. The presence of cardiac bifida was scored at 48 hpf. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

which wortmannin affects cardiac development. By adding or washing away wortmannin at various time points during development, we demonstrated that a critical stage for induction of cardia bifida occurs between 6 and 16 hpf (Fig. 3). When wortmannin is added at the initiation of the experiment but washed away before 16 hpf, no cardia bifida occurs. Similarly, adding wortmannin after 6 hpf, only one heart tube forms. Thus, the critical event affected by wortmannin that allows initiation of cardia bifida formation seems to occur between 6 and 16 hpf.

Wortmannin induces zebrafish cardia bifida through a mechanism independent of phosphatidylinositol 3-kinase and myosin light chain kinase

Wortmannin is a fungal metabolite that specifically inhibits PI3 kinase and/or MLCK. Therefore, wortmannin may induce cardia bifida by its ability to inhibit PI3 kinase and/or MLCK. To test whether inhibition of PI3 kinase or MLCK was sufficient to trigger cardia bifida, we then investigated whether inhibition of PI3 kinase or MLCK by alternative, unrelated compounds such as LY294002 [2,4], ML-7, and ML-9 [38–40] was also able to induce cardia bifida at concentrations that drastically inhibit PI3 kinase or MLCK. LY294002 is a synthetic compound derived from the broad-spectrum kinase inhibitor quercetin and inhibits PI3 kinase by competing with ATP for the active site of catalytic subunit p110 [2]. It has been widely used in cell biology because it is much more stable in solution than wortmannin [2,4]. ML-9, ML-7, and wortmannin are commonly used to block MLCK activity, but are chemically unrelated [36–40].

Zebrafish embryos were treated with the indicated concentrations of wortmannin (4, 8, 16, and 32 μM), LY294002 (2, 4, 8, 16, 32, and 64 μM), ML-7 (4, 8, 16, and 32 μM) or ML-9 (4, 8, 16, and 32 μM) beginning 6 hpf and were transferred to embryo media without kinase inhibitors at 24 hpf. As shown in Fig. 4, LY294002 was unable to induce cardia bifida at concentrations of up to 64 μM, previously shown to completely inhibit PI3 kinase in *Xenopus laevis* oocytes. It has been shown

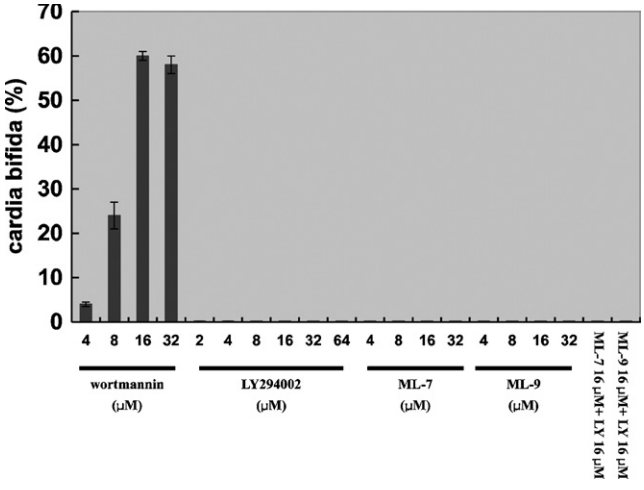

Fig. 4. Wortmannin induces zebrafish cardia bifida through a mechanism independent of phosphatidylinositol 3-kinase and myosin light chain kinase. Embryos were treated with the indicated concentrations of wortmannin (4, 8, 16, and 32 μM), LY294002 (2, 4, 8, 16, 32, and 64 μM), ML-7 (4, 8, 16, and 32 μM) or ML-9 (4, 8, 16, and 32 μM) at 6 hpf and were transferred to embryo media without kinase inhibitor at 24 hpf. The percentage of cardiac bifida was scored at 48 hpf. Symbols and vertical bars represent the means and SD of three experiments.

that wortmannin can block *Xenopus* oocyte PI3 kinase activity completely in vivo at a concentration of 100 nM [9,24]. Neither ML-7 nor ML-9 was able to induce cardia bifida at concentrations of up to 32 μM. At concentrations above 32 μM, either ML-7 or ML-9 treatment caused nonspecific defects. To block PI3 kinase and MLCK activity at the same time, we treated zebrafish embryos either with 16 μM LY294002 and 16 μM ML-7 or with 16 μM LY294002 and 16 μM ML-9. Neither treatment resulted in cardia bifida. Thus, the cardia bifida phenotype induced by wortmannin was not a consequence of inhibition of PI3 kinase and/or MLCK.

Discussion

Kinase inhibitors have been widely used to establish the signal transduction pathways regulating a broad range of fundamental cellular processes including cell growth, differentiation, survival, and migration in response to extracellular stimuli [25–27]. During the development of the vertebrate organism, the exact timing of developmental events is crucial to the formation of tissues and organ systems. A chemical genetic approach permits both temporal and dosage control over the modulation of gene function, as we show here, allowing precise initiation and termination of the functional blockade [28,29]. In the beginning, we screened 20 kinase inhibitors for their effects on the cardiovascular development employing zebrafish as a model. Only wortmannin-treated embryos display cardia bifida. The specific results prompted us to focus our attention on investigating the detailed cardiovascular defects in wortmannin-treated animals.

Wortmannin is known to be a potent inhibitor of PI3 kinase and MLCK, and the IC_{50} values for PI3 kinase and MLCK are 3.0 and 200 nM, respectively, in vitro [2,4,7]. Here, we used a relatively high concentration of wortmannin, enough to inhibit PI3 kinase and/or MLCK activity. At 100 nM, *Xenopus* PI3 kinase is fully inhibited in vivo [9]. We report here that wortmannin itself is able to induce zebrafish cardia bifida, an effect that is not mediated by its ability to inhibit PI3 kinase and/or MLCK, since another inhibitor of PI3 kinase and/or MLCK was not able to induce the same phenotype [2]. Our study suggests that wortmannin may have other molecular targets besides PI3 kinase and MLCK. Recently, it has been reported that polo-like kinase can be also significantly inhibited at the concentrations of wortmannin commonly used to inhibit PI3-kinases [41]. We predict that wortmannin induces zebrafish cardia bifida through a mechanism independent of polo-like kinase based on our results and the fact that wortmannin and LY294002 are inhibitors of polo-like kinase [41].

Currently, the zebrafish provides an excellent vertebrate model system to investigate molecular mechanisms regulating development. The simplest method for generating a loss-of-function in a particular gene in the zebrafish is the use of morpholino modified antisense or small interfering RNA (siRNA) knock-down technology to down-regulate certain gene expression [30,31]. Although important information can be obtained by morpholino or siRNA injections, this method, similar to the use of knock-out mutations, blocks gene function at the earliest stage. We set out to use a novel strategy to study signal transduction in the zebrafish embryo using a chemical genetic approach to achieve a rheostatic control of signal strength [32,28]. By binding to specific proteins, small molecules also can modulate gene product functions and result in changes in an organism's phenotype in a nonheritable manner. The ability to modulate function specifically and rapidly makes small molecules especially useful tools for studying processes like development in which the timing of protein function is critical. Moreover, the reversibility of small molecule-mediated gene product modulation provides information that is not obtainable through the use of genetic mutants.

In zebrafish, eight cardia bifida mutations, *hands off* (*han*), *casanova* (*cas*), *bonnie and clyde* (*bon*), *faust* (*fan*), *one-eyed pinhead* (*oep*), *miles apart* (*mil*), *two-of-hearts* (*toh*), and *natter* (*nat*), have been reported, and the corresponding genes have already been isolated [33,34,13,14,17,18]. For instance, zebrafish *oep* encodes a maternally and zygotically expressed member of the EGF–CFC family essential for Nodal signaling. Embryos lacking zygotic *oep* display cardia bifida [18,33]. Zebrafish zinc finger transcription factor Gata5 is encoded by the *faust* locus. Gata5 is required for the migration of the cardiac primordia to the embryonic midline [18,19,33]. It has been reported that other known inhibitors of signalling molecules such as fibronectin or EGF–CFC families can also induce zebrafish cardia bifida [17,18,33], making it more complex to evaluate these results properly. Thus, our study suggests that wortmannin may have other molecular targets besides PI3 kinase and MLCK, which are important for signal transduction pathways regulating cardiac function.

In summary, our findings open avenues for understanding the complex molecular mechanisms of heart development and diseases. Identifying wortmannin targeted pathways governing cardia bifida represents a fascinating research for the future.

Acknowledgments

We thank several people for their contributions to this project, particularly Drs. Yang Jian and Li Guoping for critical reading of the manuscript. We are also grateful to members of Song Houyan lab for advice and support. This work was supported by grants from the "211" major discipline to H.-Y.S. and T.Z., from the National Natural Science Foundation of China to T.Z. and H.-Y.S. (30328009), and from the Fudan University Professorship Grant to T.Z.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2005.03.145.

References

[1] P.W. Brian, P.J. Curtis, H.G. Hemming, G.L.F. Norris, Wortmannin, an antibiotic produced by *Penicillium wortmanni*, Trans. Br. Mycol. 40 (1957) 365–368.
[2] E.H. Walker, M.E. Pacold, O. Perisic, L. Stephens, P.T. Hawkins, M.P. Wymann, R.L. Williams, Structural determinants of phosphoinositide 3-kinase inhibition by wortmannin, LY294002, quercetin, myricetin, and staurosporine, Mol. Cell 6 (2000) 909–919.
[3] A. Zewail, M.W. Xie, Y. Xing, L. Lin, P.F. Zhang, W. Zou, J.P. Saxe, J. Huang, Novel functions of the phosphatidylinositol metabolic pathway discovered by a chemical genomics screen with wortmannin, Proc. Natl. Acad. Sci. USA 100 (2003) 3345–3350.
[4] G.J. Brunn, J. Williams, C. Sabers, G. Wiederrecht, J.C. Lawrence Jr., R.T. Abraham, Direct inhibition of the signaling functions of the mammalian target of rapamycin by the phosphoinositide 3-kinase inhibitors, wortmannin and LY294002, EMBO J. 15 (1996) 5256–5267.
[5] M. Ui, T. Okada, K. Hazeki, O. Hazeki, Wortmannin as a unique probe for an intracellular signaling protein, phosphoinositide-3-kinase, Trends Biochem. Sci. 20 (1995) 303–307.
[6] M.P. Wymann, G. Bulgarelli-Leva, M.J. Zvelebil, L. Pirola, B. Vanhaesebroeck, M.D. Waterfield, G. Panayotou, Wortmannin inactivates phosphoinositide 3-kinase by covalent modification of Lys-802, a residue involved in the phosphate transfer reaction, Mol. Cell. Biol. 16 (1996) 1722–1733.
[7] H. Yano, S. Nakanishi, K. Kimura, N. Hanai, Y. Saitoh, Y. Fukui, Y. Nonomura, Y. Matsuda, Inhibition of histamine secretion by wortmannin through the blockade of phosphatidylinositol 3-kinase in RBL-2H3 cells, J. Biol. Chem. 268 (1993) 25846–25856.
[8] A. Arcaro, M.P. Wymann, Wortmannin is a potent phosphatidylinositol 3-kinase inhibitor: the role of phosphatidylinositol 3,4,5-trisphosphate in neutrophil responses, Biochem. J. 296 (1993) 297–301.
[9] X.J. Liu, A. Sorisky, L. Zhu, T. Pawson, Molecular cloning of an amphibian insulin receptor substrate 1-like cDNA and involvement of phosphatidylinositol 3-kinase in insulin-induced *Xenopus* oocyte maturation, Mol. Cell. Biol. 15 (1995) 3563–3570.
[10] D.J. James, C. Salaun, F.M. Brandie, J.M. Connell, L.H. Chamberlain, Neomycin prevents the wortmannin inhibition of insulin-stimulated Glut4 translocation and glucose transport in 3T3-L1 adipocytes, J. Biol. Chem. 279 (2004) 20567–20570.
[11] C. Thisse, L.I. Zon, Organogenesis—heart and blood formation from the zebrafish point of view, Science 295 (2003) 457–462.
[12] M.C. Fishman, Zebrafish—the canonical vertebrate, Science 294 (2001) 1290–1291.

[13] J.N. Chen, P. Haffter, J. Odenthal, E. Vogelsang, M. Brand, F.J. van Eeden, M. Furutani-Seiki, M. Granato, M. Hammerschmidt, C.P. Heisenberg, Y.J. Jiang, D.A. Kane, R.N. Kelsh, M.C. Mullins, C. Nusslein-Volhard, Mutations affecting the cardiovascular system and other internal organs in zebrafish, Development 123 (1996) 293–302.
[14] D.Y. Stainier, B. Fouquet, J.N. Chen, K.S. Warren, B.M. Weinstein, S.E. Meiler, M.A. Mohideen, S.C. Neuhauss, L. Solnica-Krezel, A.F. Schier, F. Zwartkruis, D.L. Stemple, J. Malicki, W. Driever, M.C. Fishman, Mutations affecting the formation and function of the cardiovascular system in the zebrafish embryo, Development 123 (1996) 285–292.
[15] R.T. Peterson, S.Y. Shaw, T.A. Peterson, D.J. Milan, T.P. Zhong, S.L. Schreiber, C.A. MacRae, M.C. Fishman, Chemical suppression of a genetic mutation in a zebrafish model of aortic coarctation, Nat. Biotechnol. 22 (2004) 595–599.
[16] L.A. Trinh, D.Y. Stainier, Cardiac development, Methods Cell Biol. 76 (2004) 455–473.
[17] L.A. Trinh, D.Y. Stainier, Fibronectin regulates epithelial organization during myocardial migration in zebrafish, Dev. Cell 6 (2004) 371–382.
[18] D.Y. Stainier, Zebrafish genetics and vertebrate heart formation, Nat. Rev. Genet. 2 (2001) 39–48.
[19] J.F. Reiter, J. Alexander, A. Rodaway, D. Yelon, R. Patient, N. Holder, D.Y. Stainier, Gata5 is required for the development of the heart and endoderm in zebrafish, Genes Dev. 13 (1999) 2983–2995.
[20] M. Westerfield, The Zebrafish Book: A Guide for the Laboratory Use of Zebrafish (*Danio rerio*), University of Oregon Press, Oregon, 1995.
[21] C.B. Kimmel, W.W. Ballard, S.R. Kimmel, B. Ullmann, T.F. Schilling, Stages of embryonic development of the zebrafish, Dev. Dyn. 203 (1995) 253–310.
[22] D. Yelon, S.A. Home, D.Y. Stainier, Restricted expression of cardiac myosin genes reveals regulated aspects of heart tube assembly in zebrafish, Dev. Biol. 214 (1999) 23–37.
[23] E. Berdougo, H. Coleman, D.H. Lee, D.Y. Stainier, D. Yelon, Mutation of weak atrium/atrial myosin heavy chain disrupts atrial function and influences ventricular morphogenesis in zebrafish, Development 130 (2003) 6121–6129.
[24] F.J. Thomson, C. Moyes, P.H. Scott, R. Plevin, G.W. Gould, Lysophosphatidic acid stimulates glucose transport in *Xenopus* oocytes via a phosphatidylinositol 3′-kinase with distinct properties, Biochem. J. 316 (1996) 161–166.
[25] T.M. Loftus, D.E. Jaworsky, G.L. Frehywot, C.A. Townsend, G.V. Ronnett, M.D. Lane, F.P. Kuhajda, Reduced food intake and body weight in mice treated with fatty acid synthase inhibitors, Science 288 (2000) 2379–2381.
[26] J. Chan, P.E. Bayliss, J.M. Wood, T.M. Roberts, Dissection of angiogenic signaling in zebrafish using a chemical genetic approach, Cancer Cell 1 (2002) 257–267.
[27] L.I. Zon, R.T. Peterson, In vivo drug discovery in the zebrafish, Nat. Rev. Drug Discov. 4 (2005) 35–44.
[28] R.T. Peterson, B.A. Link, J.E. Dowling, S.L. Schreiber, Small molecule developmental screens reveal the logic and timing of vertebrate development, Proc. Natl. Acad. Sci. USA 97 (2000) 12965–12969.
[29] J.R. Yeh, C.M. Crews, Chemical genetics: adding to the developmental biology toolbox, Dev. Cell 5 (2003) 11–19.
[30] A. Nasevicius, S.C. Ekker, Effective targeted gene "knockdown" in zebrafish, Nat. Genet. 26 (2000) 216–220.
[31] S. Boonanuntanasarn, G. Yoshizaki, T. Takeuchi, Specific gene silencing using small interfering RNAs in fish embryos, Biochem. Biophys. Res. Commun. 310 (2003) 1089–1095.
[32] U. Langheinrich, Zebrafish: a new model on the pharmaceutical catwalk, Bioessays 25 (2003) 904–912.
[33] J.F. Reiter, H. Verkade, D.Y. Stainier, Bmp2b and Oep promote early myocardial differentiation through their regulation of gata5, Dev. Biol. 234 (2001) 330–338.
[34] Y. Kikuchi, L.A. Trinh, J.F. Reiter, J. Alexander, D. Yelon, D.Y. Stainier, The zebrafish bonnie and clyde gene encodes a Mix family homeodomain protein that regulates the generation of endodermal precursors, Genes Dev. 14 (2000) 1279–1289.
[35] M. Isosaki, Inhibition of wortmannin activities by amino compounds, Biochem. Biophys. Res. Commun. 324 (2004) 1406–1412.
[36] S. Nakanishi, S. Kakita, I. Takahashi, K. Kawahara, E. Tsukuda, T. Sano, K. Yamada, M. Yoshida, H. Kase, Y. Matsuda, Y. Hashimoto, Y. Nonomura, Wortmannin, a microbial product inhibitor of myosin light chain kinase, J. Biol. Chem. 267 (1992) 2157–2163.
[37] S. Watanabe, X.E. Wang, M. Hirose, N. Sato, Effect of myosin light chain kinase inhibitor wortmannin on the wound repair of cultured gastric mucosal cells, Biochem. Biophys. Res. Commun. 199 (1994) 799–806.
[38] S. Ito, H. Kume, H. Honjo, I. Kodama, H. Katoh, H. Hayashi, K. Shimokata, ML-9, a myosin light chain kinase inhibitor, reduces intracellular Ca^{2+} concentration in guinea pig trachealis, Eur. J. Pharmacol. 486 (2004) 325–333.
[39] B. Tian, L.C. Brumback, P.L. Kaufman, ML-7, chelerythrine and phorbol ester increase outflow facility in the monkey eye, Exp. Eye Res. 71 (2000) 551–566.
[40] M. Grimm, P. Haas, B. Willipinski-Stapelfeldt, W.H. Zimmermann, T. Rau, K. Pantel, M. Weyand, T. Eschenhagen, Key role of myosin light chain (MLC) kinase-mediated MLC2a phosphorylation in the alpha 1-adrenergic positive inotropic effect in human atrium, Cardiovasc. Res. 65 (2005) 211–220.
[41] Y. Liu, K.R. Shreder, W. Gai, S. Corral, D.K. Ferris, J.S. Rosenblum, Wortmannin, a widely used phosphoinositide 3-kinase inhibitor, also potently inhibits mammalian polo-like kinase, Chem. Biol. 12 (2005) 99–107.