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Disruption of blastomeric F-actin: a potential early biomarker of developmental toxicity in zebrafish

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Abstract

The expression of at least some biomarkers of toxicity is generally thought to precede the appearance of frank pathology. In the context of developmental toxicity, certain early indicators may be predictive of later drastic outcome. The search for predictive biomarkers of toxicity in the cells (blastomeres) of an early embryo can benefit from the fact that for normal development to proceed, the maintenance of blastomere cellular integrity during the process of transition from an embryo to a fully functional organism is paramount. Actin microfilaments are integral parts of blastomeres in the developing zebrafish embryo and contribute toward the proper progression of early development (cleavage and epiboly). In early embryos, the filamentous actin (F-actin) is present and helps to define the boundary of each blastomere as they remain adhered to each other. In our studies, we observed that when blastomeric F-actin is depolymerized by agents like gelsolin, the blastomeres lose cellular integrity, which results in abnormal larvae later in development. There are a variety of toxicants that depolymerize F-actin in early mammalian embryos, the later consequences of which are, at present, not known. We propose that very early zebrafish embryos (~5-h old) exposed to such toxicants will also respond in a like manner. In this review, we discuss the potential use of F-actin disruption as a predictive biomarker of developmental toxicity in zebrafish.

Keywords

Zebrafish; Biomarker; F-actin; Toxicity; Gastrulation

Introduction

During vertebrate gastrulation, an embryo body plan is established by the rearrangement of blastomeres. Early embryonic development is marked by cellular movements that ultimately generate the patterning of the embryo. This process is known as morphogenesis, and one of the earliest morphogenetic events in many animals including zebrafish is the process of epiboly. During epiboly, embryonic tissues spread and become thin [1–5]. In the zebrafish, epiboly starts at the late blastula stage approximately 1 h after the mid-blastula transition, which occurs approximately 4 h after fertilization [6]. At this stage, the embryo is composed

of four cell layers: the enveloping layer (EVL); deep cells; the yolk syncytial layer (YSL); and the yolk cell.

During early epiboly, the deep blastomeres radially intercalate, while the underlying yolk moves toward the animal pole in a process called “doming.” Consequent to this initial phase of epiboly, an inverted bowl-shaped blastoderm covers ~50% of the yolk surface, and this is referred to as the 50% epiboly stage. During the second phase of epiboly, the deep cells begin gastrulation cell movements converging dorsally and undergoing involution/ingression movements to form the germ layers [4]. At the same time, epiboly continues with all the three cell layers spreading over the yolk to the vegetal pole of the embryo, ultimately resulting in the complete internalization of the yolk [6]. The morphogenetic process of epiboly is conserved among other vertebrates and invertebrates, including amphibians, sea urchins, and *C. elegans* [1–3, 5].

As development proceeds, the blastoderm cells migrate vegetally in uniform fashion causing the blastopore circumference to continuously decrease in a purse-string-like fashion until the blastopore is completely closed at 100% epiboly. Studies in *Fundulus* (Killifish) reveal an actin ring in the YSL adjacent to the blastoderm margin that is postulated to act as the “purse strings” during blastopore closure [7]. Three distinct actin structures become prevalent during late epiboly stages: two rings at the margin of the deep cells and the EVL; and a punctate band of actin accumulation in the external YSL adjacent to the EVL margin [8]. In zebrafish, a filamentous actin (F-actin) band is first evident at 50% epiboly in a similar location in the YSL [8]. This actin band is associated with an active form of myosin, phosphorylated-myosin 2 [9], indicating the presence of an actin–myosin contractile activity in the YSL margin. This actin band is also involved in endocytosis [8], which removes the yolk membrane as the advancing blastoderm/EVL cells move vegetally over the yolk during epiboly. During early development, actin microfilaments throughout the embryo contribute to epiboly [8–10]. Pharmacological inhibitors of actin or myosin can slow the later stages of epiboly in zebrafish, implicating a direct involvement of actin in the progression of epiboly [8, 9]. Zebrafish gastrulae treated with the myosin-2 inhibitor blebbistatin show partially impaired actin accumulation in the YSL and show epiboly defects [9]. The fate of these early embryos during later development is not known. Higher doses of these inhibitors can arrest epiboly, but they also cause either a dissociation of the EVL and blastoderm cells or yolk herniation due to loss of the vegetal actin net that maintains yolk integrity [8, 10].

Calcium positively regulates actin contraction [11]. In zebrafish, calcium signaling is a requirement for the progression of epiboly [8]. Calcium levels are low during early epiboly [12–14], but increase and become dynamic during the last half. Spikes in calcium concentrations are evident in the yolk cell beginning at 50% epiboly, followed by waves of calcium that traverse the blastoderm margin from 65% epiboly to blastopore closure [8, 12]. Loss of calcium causes a loss of the yolk cell actin band and an inhibition of the progression of epiboly suggesting that abnormal calcium regulation may cause death through an unregulated F-actin constrictive force [8]. Here, based on our results and other reports, we discuss the potential use of the actin cytoskeletal disruption phenotype to be an early biomarker of developmental toxicity in drug toxicity assays using zebrafish embryos.

Proteins affecting blastomeric actin cytostructure

Remodeling of the actin cytoskeleton is critical for mediating changes in cell shape, migration, and adhesion. Actin filament architecture is regulated by a large group of actin-binding proteins that modulate actin assembly, disassembly, branching, and bundling [15]. Several proteins with diverse functions have been noted to affect actin cytoskeletal architecture during epiboly. Association of the F-actin band with the Misshapen kinase at the leading edge of the EVL cells is implicated in regulating the constriction of the marginal edge of EVL cells as they advance vegetally [9]. Actin organization is also regulated by growth factor signals that stimulate the activity of Rho family GTPases, which mediate actin remodeling and formation of stress fibers, filopodia, and membrane ruffles [16].

Loss of Mtx2, a predicted transcription factor in zebrafish, results in reduction of the YSL punctate actin band and arrest of the vegetal movement of cells at the 50% epiboly point [17, 18]. Similarly, an abnormal cytoskeleton contributes to epibolic delay in Pou5fl-deficient zebrafish embryos [19]. Membrane proteins, $G\alpha_{12}$ and $G\alpha_{13}$, also regulate epiboly by promoting actin microfilament assembly through a Rho guanine nucleotide exchange factor (GEF)-dependent signaling pathway that appears to modulate epiboly during zebrafish gastrulation [20]. Eps8 (EGF receptor pathway substrate 8) [21], which directly binds actin has been hypothesized to regulate actin filament dynamics in vitro by promoting the capping of actin [22]. In *Xenopus* embryos, mis-expression of Eps8 results in defects in gastrulation by impairing blastomere movement which causes developmental abnormalities, suggesting that Eps8 might play additional roles in regulating actin organization in vivo, e.g., by recruiting Dsh (disheveled) to actin filaments and the cell membrane [23]. Dsh activity during gastrulation is dependent on both RhoA and Rac [24]. In zebrafish, the MAPK family member p38 kinase, by modulating the activity of F-actin at the yolk cell margin circumference, allows the gradual closure of the blastopore during gastrulation [25].

Gelsolin, an actin-severing cytoskeleton regulatory protein modulated by calcium and polyphosphoinositolphospholipids [26, 27], has been implicated in multiple pathways such as those involved with cell motility and signaling, apoptosis, and cancer [27]. Scinderin, the closest homolog of the actin-severing protein, gelsolin, has two similar paralogs (scinla and scinlb) in zebrafish [28]. Scinla (C/L-gelsolin) is abundant in the adult zebrafish cornea. The role of C/L-gelsolin in zebrafish dorso-ventral pattern formation has been reported [29]. Reduction of C/L-gelsolin in embryos by injection of C/L-gelsolin antisense morpholino oligonucleotides ventralizes embryos, whereas its overexpression dorsalizes them. Both of these extremes give rise to aberrant phenotypes characterized by abnormal larval features [29, 30]. The ventralized embryos display a loss of dorsal mesoderm and neuroectodermal derivatives, associated with an expansion of ventral mesodermal and ectodermal cell fates. The dorsalized embryos show opposite phenotypes with enlarged somatic mesoderm and neuroectoderm, and loss of ventral mesoderm derivatives such as blood, pronephric, and bone precursors. Although the phenotypic changes we show in this study are focused on embryos at 28 h of development, cell fate-specific gene expression changes were detected much earlier in gastrulae [29]. However, in these prior studies, we did not test the status of the actin cytoskeleton. We sought here to determine whether the developmental anomalies imparted by overexpressing C/L-gelsolin could be foreshadowed by events occurring much

earlier during gastrulation. Compared to control blastulae, F-actin was reduced in C/L-gelsolin mRNA-injected (two different doses of 25 and 50 pg mRNA/egg) embryos (Fig. 1). In the control blastulae (Fig. 1a and d), all blastomeres retained the cellular integrity demarcated by F-actin, whereas in the C/L-gelsolin mRNA-injected blastulae the cellular boundaries of the blastomeres were lost as the F-actin polymerized at different rates in a dose-dependent manner (Fig. 1b, c, e, and f at higher magnification). The “actin ring” that acts as a purse string in the blastopore closing is obvious in the control embryo (Fig. 1a, arrows), whereas in the C/L-gelsolin-injected embryos, the structure appears missing (Fig. 1b and c, arrows). After 28 h, the control blastulae developed into normal embryos (25 of 25) (Fig. 1g), but the C/L-gelsolin mRNA-injected blastulae (19 of 25, 76%) developed into malformed embryos even though they had beating hearts (Fig. 1h and i). The same percentage (~76%) showed complete F-actin depolymerization (Fig. 1c and f). The embryos (17 of 25, 68%) injected with the lower dose of 25 pg C/L-gelsolin mRNA had less severe phenotypic alterations than embryos injected with the higher dose of 50 pg. Reduced fluorescence of the actin ring (Fig. 1b) and the blastomeres (Fig. 1b and e) indicated the absence of comparable F-actin levels as in the control (Fig. 1a and d). The boundaries of the blastomeres (indicated by asterisks) by F-actin were more definitive in the control (Fig. 1d) than in the 25 ng C/L-gelsolin-injected embryos (Fig. 1e), while they were lost at the higher dose (Fig. 1f). At the lower dose, the embryos were characterized by a curved axis and underdeveloped head structures (Fig. 1h), whereas in the high-dose embryos, the axis was completely missing (Fig. 1i). Major developmental anomalies observed in both lower and higher doses compared to the normal embryos are summarized in Table 1. The morphological assessment of a number of endpoints revealed the severity of effects in the higher dose group in which F-actin was completely depolymerized. The loss of a well-defined body axis in these embryos was due to the over-development of dorsal mesoderm at the expense of the ventral mesoderm thus disrupting normal axial pattern formation. In both the low- and high-dose groups, there was a reduction or loss of yolk extension (YE) and blood island (BI) that are the derivatives of ventral mesoderm. Although morphological assessments revealed beating hearts in both the groups, blood volume in these groups (monitored by observation through the transparent embryo) were reduced, another indication that the development of ventral mesoderm was compromised. These results indicate that F-actin cytoskeletal architecture will dictate the outcome of the developmental process and may potentially serve as a biomarker of embryotoxicity/teratogenicity.

Toxicant-induced actin cytoskeletal modulation in embryos

Damage to the actin cytoskeleton in mammalian cells can be induced by many factors such as exposure to xenobiotic chemicals, oxidative stress, and radiation [31–37]. In culture, cancerous and undifferentiated mammalian cells have weaker actin cytoskeletons than normal cells [38, 39]. Cellular F-actin and monomeric G-actin (depolymerized F-actin) levels have been used as biomarkers of bladder cancer risk, breast cancer, prostate adenocarcinoma and cholestasis [37, 40–42], hepatotoxicity caused by pectenotoxins [43–46], and nanoparticle-induced toxicity [47]. Moreover, actin remodeling has been contemplated as a potential target for cancer drug development [48]. Trivalent methylated arsenicals (DMAIII), toxicants in arsenic-polluted water, disturb cytokinesis thus inducing aneuploids and tetraploids [49] by inhibiting actin assembly in Chinese hamster lung cells

[50]. Significant rearrangement of the actin cytoskeleton of hemocytes in *Drosophila* embryos (“freezing” phenotype) occurs in response to the purified insecticidal toxin Mcf1, an occurrence that resembles the outcome of bacterial infection of the embryos [51]. In cytotoxicity assays using mammalian cells, quantum dot nanoparticles induce more actin filament formation in the cell cytoplasm (actin polymerization), whereas cadmium induces actin depolymerization [52]. Studies based on 24–96 h embryos indicate that cadmium-exposed zebrafish embryos develop small eyes [53] and exhibit compromised neuronal differentiation in the retina, brain, and defects in somitogenesis and axonogenesis [54–56]. Whether these defects correlate with modulation of the actin cytoskeleton is not known.

Actin cytoskeletal modulation has rarely been a major focus of toxicant risk-assessment studies. Nonetheless, developmental toxicity of the environmental toxicant TCDD (the AhR-specific ligand, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin) has been reported using mammalian pre-implantation embryos starting from 2-cell embryos [57] to 8–16 cell morulae [58–63], in which, maternal exposure to TCDD has been shown to disrupt actin dynamics in rat 8-cell pre-implantation embryos leading to aberrant cytokinesis although not affecting survival at least to the blastocyst stage [59]. TCDD-exposed 9-cell pre-implantation embryos show enhanced F-actin cortical localization [59], and this is particularly relevant in consideration of the recent evidence suggesting long-term impacts on the health and well being of offspring following environmental perturbations and undernutrition during the periconceptional and pre-implantation period [64, 65]. Very low level TCDD exposure of 2-cell mouse pre-implantation embryos reduces the number of pre-implantation embryos that develop to the 8-cell stage. However, survivors show accelerated blastocyst formation [66]. Early embryos undergo major cytoplasmic, nuclear, and cytoskeletal remodeling that leads to the establishment of apical-basal polarity [57] in 8–16 cell morulae [58–63]. While the pre-implantation embryo may retain implantation competence after TCDD exposure, perturbations during this critical stage may compromise cellular expansion that may prove deleterious to subsequent normal development. Therefore, identifying the specific targets of TCDD and other toxicants in the pre-implantation embryo, especially those that link cell cycle control with cytoskeletal and nuclear remodeling, would be extremely valuable. Early regulation of F-actin by the p38 MAP kinase inhibitors, SB203580 and SB220025 (CSAIDTM), results in a decrease in the ability of murine pre-implantation embryos to implant [67]. TCDD has been shown to induce upregulation of adseverin, a member of the calcium-regulated gelsolin superfamily of actin-severing proteins, in mouse thymocytes [68]. TCDD-induced developmental toxicity manifested in zebrafish larvae includes edema, anemia, hemorrhage, and ischemia associated with arrested growth and development. Defects in heart and vasculature development and function and jaw malformations also occur in embryos treated with TCDD. The swim bladder fails to inflate, and the switch from embryonic to adult erythropoiesis is blocked. This profile of developmental toxicity responses, called the “blue sac syndrome” because of the blue color of the edematous yolk sac, is observed in the larval form of all freshwater fish species exposed to TCDD at the embryonic stage of development (reviewed by [69]). Zebrafish adults exposed to TCDD produce progeny with a 25% reduced survival rate [70]. Although the mechanism of such maternal transmission of TCDD toxicity to progeny is not known, the cellular integrity of the eggs derived from TCDD-treated females are likely targets. Analogous to mouse pre-

implantation embryos, these eggs could have lost some important component of their cellular architecture, thus allowing fertilization to proceed but blunting further developmental processes.

Zebrafish embryos exposed to a lethal concentration of TCDD shortly after fertilization initially develop a normal circulation and a functional heart; when at 48 hpf the first endpoints of cardiac toxicity emerge with accumulation of edema fluid in the pericardial sac and a significant numbers die at around 144 hpf [69, 71–73]. Here, we determined whether TCDD induces actin depolymerization in early zebrafish embryo blastomeres. Zebrafish embryos (2 hpf) with intact chorions were exposed for 4 h to either 0.1% DMSO (vehicle) or an LC₁₀₀ concentration of TCDD (0.5 µg/l) dissolved in DMSO. Rhodamine-phalloidin staining of the embryos showed normal levels F-actin in the control (0.1% DMSO-treated) group (Fig. 2a) when compared to the TCDD-treated group (Fig. 2b). In higher magnification, the control blastomeres (*) were with definitive cellular boundaries of F-actin (Fig. 2c). However, TCDD-treated embryos showed less F-actin (Fig. 1d) suggesting that TCDD induced F-actin depolymerization. At 78 hpf, the control larvae (DMSO-treated) developed normally with a functional beating heart (Fig. 2e and g), whereas the TCDD-treated larvae exhibited severe pericardial sac edema with a large malformed heart and blunted growth (Fig. 2f and g). A direct correlation of the degree and pattern of actin depolymerization to the specific types of toxicities would prove difficult to predict due to the developmental complexity with simultaneous interplay of many compensatory mechanisms. Nevertheless, actin depolymerization may be an early indication of overall developmental toxicity.

Conclusion

Based on signaling pathways involved in actin cytoskeleton remodeling and their impact on biological endpoints, a schematic presentation summarizes the proposed relationship between actin cytoskeleton in the zebrafish blastulae/gastrulae and developmental toxicity (Fig. 3). Drugs and chemical compounds that can change small GTPases-like Rac and Rho in cells would result in a change in actin dynamics. The change in F-actin or G-actin contents in a cell would translate to a number of biological endpoints such as change in cell shape, disrupted cell division, altered cell–cell adhesion, cell survival, and cell migration or motility (Fig. 3).

As modern developmental toxicology research focuses more and more on how mechanistic understanding can contribute to risk-assessment processes and modeling, identification and validation of biomarkers that can be detected very early during development will become increasingly valuable. In parallel with this effort are those to develop bioassay systems that are fast, inexpensive, and can be carried out in a high-throughput manner. Actin depolymerization by cytochalasin B [8] and blebbistatin, a myosin 2 inhibitor [9], and alteration of Gα_{12/13} signaling [20] have been shown to disrupt zebrafish gastrulation by interfering with epiboly. However, the consequences of such embryonic disruption and correlation to subsequent development have not been detailed. Our studies and others' show that early depolymerization of F-actin leads to frank embryo-toxicity indicating that, in zebrafish, early disruption of F-actin cytoskeletal architecture may be one of the earliest

biomarkers of embryotoxicity/teratogenicity, being detectable within 5 h of development. We suggest that the use of the zebrafish actin cytoskeletal system can be a novel model system for the assessment of developmental toxicity in a rapid, inexpensive, and relatively high-throughput fashion. Detection of toxicity at the very early stages of vertebrate development when primitive germ layers are beginning to form and tissue differentiation has barely begun could prove to be a valuable screening procedure.

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Fig. 1.

Correlation between blastomeric F-actin integrity and embryonic development in zebrafish. Zebrafish gelsolin (scinla or C/L-gelsolin) cDNA was constructed in pCS2 vectors (*Bam*HI/*Eco*RI sites) [29]. Capped synthetic mRNAs were prepared using the SP6 mMESSAGE mMACHINE kit (Ambion, Austin, TX) after linearizing the plasmid with Not I that was used as the template to synthesize the mRNA for C/L-gelsolin in vitro. C/L-gelsolin mRNA that was transcribed in vitro was purified using RNA purification columns (Ambion, TX) (Fig. 1) and diluted in Danieau buffer immediately before microinjection. Synthesized C/L-gelsolin mRNA was injected into the yolk mass of one- to two-cell embryos. Post injection (4 h), damaged embryos were discarded and the rest were allowed to grow at 28°C for up to 28 h. Embryos were fixed at different stages in 4% paraformaldehyde for further processing. Zebrafish embryos (5-h old, late blastulae/gastrulae) were fixed overnight in 4% paraformaldehyde in phosphate buffer saline (PBS) at 4°C. For actin immunostaining, embryos were washed three times for 5 min each in 0.5% Triton in PBS; followed by 30 min incubation in blocking solution (10% normal goat serum, 1% DMSO, and 0.1% Triton in PBS). Embryos were then incubated for 30 min in blocking solution containing Rhodamine-Phalloidin (Molecular Probes, Eugene, OR). They were then washed three times for 5 min with PBS containing 0.1% Triton (PBT). Zebrafish embryos were mounted on agarose-coated dishes in PBT medium. Images acquired using a Leica stereomicroscope with UV illumination show **a** control embryo, **b** embryo injected with 25 pg C/L-gelsolin mRNA, and **c** embryo injected with 50 pg C/L-gelsolin mRNA. The dorsal side (*upper hemisphere*) shows the blastomeres and the ventral side (*lower hemisphere*) contains the yolk. Arrows indicate the actin ring (purse string). Enlarged views of a region of the dorsal side of the embryos in **a**, **b**, and **c** are presented in **d**, **e**, and **f**, respectively, showing the F-actin

architecture. *Asterisks* indicate some blastomeres. Bright-field images of representative embryos at 28 hpf are shown in **g** control embryo, **h** embryo injected with 25 pg C/L-gelsolin mRNA, and **i** embryo injected with 50 pg C/L-gelsolin mRNA

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Fig. 2.

TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin) induces blastomeric F-actin depolymerization in zebrafish embryos. Embryos at 2 hpf (with intact chorions) were treated with 0.1% dimethylsulfoxide (DMSO) ($n = 50$) or 5 ng/ml TCDD ($n = 50$) for 4 h. Half of the embryos ($n = 25$) from each group were then washed three times with phosphate buffer saline (PBS) and fixed in 4% paraformaldehyde. The rest were allowed to develop further. Embryos (6 hpf) fixed in 4% paraformaldehyde were processed for F-actin staining with rhodamine-phalloidin as described in Fig. 1 legend. Images show **a** control embryo and **b** embryo treated with TCDD. The dorsal side (*upper Hemisphere*) of the embryos shows the blastomeres and the ventral side (*lower hemisphere*) contains the yolk. *Arrows* indicate the actin ring (purse string). Enlarged views of a region of the dorsal side of the embryos in **a** and **b** are presented in **c** and **d**, respectively, showing the F-actin architecture. *Asterisks* indicate some blastomeres. Bright-field images of representative embryos at 78 hpf are shown in **e** control embryo and **f** TCDD-treated embryo. Magnified views of the images in **e** and **f** are presented in **g** and **h**, respectively, showing the pericardium (*large arrow*) and the heart (*small arrow*)

Fig. 3.

Actin cytoskeleton is modulated by a variety of signaling pathways. Both polymerization (formation of filamentous actin or F-actin) and depolymerization (formation of monomeric globular actin or G-actin) of actin are modulated by Rho and Rac GTPases downstream of known signaling pathways, as well as by the expression of actin-binding proteins. A number of drugs can also directly bind to the actin cytoskeleton and modulate its architecture. Modulation of actin dynamics in turn regulates a number of cellular physiological processes, e.g., cell shape, cell division (cytokinesis), cell–cell adhesion/interaction, cell survival, and cell motility or migration. Any alteration in these processes in the early embryos can contribute to developmental toxicity

Table 1

Assessment of morphological endpoints of C/L-gelsolin-injected embryos

Structure	C/L-gelsolin mRNA (25 pg/embryo)	C/L-gelsolin mRNA (50 pg/embryo)
Size	Small	Completely blunted
Somites	Not well defined	Poorly defined/cloudy
Body shape	Bent/curved body axis	Disrupted body axis
Notochord	Poorly defined	No cellular differentiation
Tail	Bent/curved	Absent
Heart	Beating heart	Beating heart
Blood volume	Reduced	Completely reduced
Yolk extension	Reduced	Absent
Blood island	Absent	Absent