Co-activation of hedgehog and AKT pathways promote tumorigenesis in zebrafish

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Abstract

The zebrafish has become an important model for cancer research. Several cancer models have been established by transgenic expression of human or mouse oncogenes in zebrafish. Since it is amenable to efficient transgenesis, zebrafish have immense potential to be used for studying interaction of oncogenes and pathways at the organismal level. Using the Gal4VP16-UAS binary transgenic expression approach, we established stable transgenic lines expressing an EGFP fusion protein of an activated zebrafish Smoothened (Smoa1-EGFP). Expression of the zebrafish Smoa1-EGFP itself did not lead to tumor formation either in founder fish or subsequent generations, however, co-expressing a constitutively active human AKT1 resulted in several tumor types, including spindle cell sarcoma, rhabdomyoma, ocular melanoma, astrocytoma, and myoxma. All tumor types showed GFP expression and increased Patched 1 levels, suggesting involvement of zebrafish Smo1 in tumorigenesis. Immunofluorescence studies showed that tumors also expressed elevated levels of phosphorylated AKT, indicating activation of the PI3K-AKT pathway. These results suggest that co-activation of the hedgehog and AKT pathways promote tumorigenesis, and that the binary transgenic approach is a useful tool for studying interaction of oncogenes and oncogenic pathways in zebrafish.

Findings

The Hedgehog (Hh) pathway is involved in cell fate determination and embryonic patterning during early vertebrate development, and is also implicated in tumorigenesis [1]. Activation of the Hh pathway underlies the majority of sporadic human basal cell carcinoma (BCC) [2]. Expression of sonic Hedgehog, constitutively active Smoothened, and transcription factors Gli1 and Gli2 in keratinocytes results in BCC in transgenic frog, mice, and human skin [3-6]. Since zebrafish have emerged as a promising vertebrate system to model human cancers [7-9], we decided to determine whether activation of the Hh pathway in transgenic zebrafish could render them prone to developing BCC. We generated the zebrafish version of activated Smoothened using site-specific mutagenesis of wild type smoothened cDNA [SmoW514L, referred as Smoa1 hereafter, see Additional file 1]. To facilitate observation of the tumorigenesis proc-
In contrast, expression of either wild-type or constitutively active human AKT1 alone, driven by the same promoter, did not develop tumors at one and a half years of age, we decided to test whether other oncogenes could collaborate to promote tumorigenesis. We chose to use transgenic line A as it showed rather specific expression in epithelia in comparison to line B. A recent report has shown that PI3K and AKT are essential for SHH signalling [14]. As a first test of our co-expression strategy, we expressed the wild-type and the constitutively active human AKT1 by injecting Tg(UAS:myrAKT1) or Tg(UAS:myrhAKT1) plasmid DNA into progeny of F1 siblings of the Tg(krt4:Gal4V16;14 × UAS-smoa1:EGFP) line A fish. Both the smo1 and AKT1 genes were under control of the 14 × UAS promoter, so their expression was driven by the same krt4 promoter through Gal4VP16. While no tumor was found in the resultant F2 fish injected with Tg(UAS:myrAKT1), various tumor types in the trunk, the eye, and the head region were observed in the F2 fish injected with Tg(UAS:myrhAKT1). The tumor types identified include a case of spindle cell sarcoma, rhabdomyoma, ocular melanoma, myxoma, and several cases of astrocytoma and glioblastoma (Fig. 2 and Table 1). These tumor types were diagnosed according to histological criteria used in mammals [15]. GFP expression was found exclusively in the tumors, due to either the additive effect of a large number of weakly EGFP-positive cells or the loss of mechanisms that suppress Smoa1-EGFP levels, or both. In any case, the detection of EGFP expression in tumors indicated the involvement of the zebrafish Smoa1 in tumorigenesis (Fig. 2B, 2E). Overall, nearly 10% of injected fish developed tumors before reaching 3 months of age. Surprisingly, the rest of the fish were tumor-free at 18 months of age, at which we stopped the experiment. We also identified fish that carried both zebrafish smo1 and the oncogenic human AKT1 in their germline. We crossed two of these fish to each other, and found 3 out of 15 of the offspring developed tumors at around 2 months of age, including one case of astrocytoma in the lower-trunk region (Fig. 2H), the other two cases were not diagnosed. In contrast, expression of either wild-type or constitutively active human AKT1 alone, driven by the same krt4 pro-
moter, did not induce tumors. In fact, even when we expressed the constitutively active human AKT1 driven by a strong ubiquitous actin promoter, no tumor was found in more than 50 fish over an 18 months period. Thus, the tumors likely resulted from collaboration between the constitutively active hAKT1 and Smoa1.

Activation of the PI3K-AKT pathway leads to phosphorylation of AKT1 [14]. To determine whether phospho-AKT1 levels were increased in the tumors, we performed immunofluorescence studies on a 3-week-old fish with a trunk tumor showing strong GFP expression and on a 12-week-old fish with an ocular tumor showing weak GFP expression. Immunofluorescence analysis indicated that both tumors had significantly higher levels of phosphorylated AKT (Fig. 3B, 3D) when compared to age-matched, tumor-free transgenic fish (Fig. 3A, 3C). To demonstrate that both the SHH and AKT pathways were activated in a same tumor, paraffin sections of the astrocytoma as shown in Fig. 2G was used for immunofluorescence study against Patched 1 and phosphorylated AKT. As shown in Fig. 3F and 3G, the tumor had elevated levels of both Patched 1 and phosphorylated AKT, indicating that both pathways were activated in this tumor. These results suggest that the tumors resulted from collaborative expression of both the zebrafish Smoa1 and the constitutively active human AKT1.

Here we demonstrated that several tumor types in zebrafish can be induced by transgenic expression of oncogenes, most likely due to co-expression of an oncogenic zebrafish Smoa1 and the constitutively active human AKT1. It was surprising that we did not find any skin tumor, as activation of either the Hh or AKT pathway [16,17] leads to skin hyperplasia and skin tumors in mouse. We also generated a transgenic line using the Tet-Off system that expressed the Smoa1-EGFP driven by a 5 kb zebrafish cytokeratin 5 (krt5) gene promoter. This transgenic line expressed higher levels of zebrafish Smoa1, but we did not find any skin tumor in fish that reached two years of age (Data not shown). It is possible that either: 1) the level of Smoa1 expression was below the threshold for tumorigenesis; 2) the Smoa1-EGFP fusion protein was not potent enough; 3) the krt4 and krt5 promoters were not

**Table 1: Tumors induced by co-expression of zebrafish smoal and constitutively active human AKT1**

| Tumor location (cases) | Fish age (weeks) | GFP expression | Elevated phospho-AKT1 | Diagnoses     |
|------------------------|------------------|----------------|-----------------------|---------------|
| Trunk (4)              | 3                | Yes            | ND                    | Rhabdomyoma   |
|                        | 4                | Yes            | Yes                   | ND            |
|                        | 6                | Yes            | ND                    | ND            |
|                        | 12               | Yes            | ND                    | Rhabdomyoma   |
| Eye (3)                | 4                | Yes            | ND                    | Ocular Melanoma|
|                        | 12               | Yes            | Yes                   | Astrocytoma   |
| Head (4)               | 6                | Yes            | ND                    | Glioblastoma  |
|                        | 6                | Yes            | ND                    | Glioblastoma  |
|                        | 8                | Yes            | ND                    | ND            |
|                        | 12               | Yes            | ND                    | Astrocytoma   |
| Others (2)             | 4                | Yes            | ND                    | Myxoma        |
|                        | 8                | Yes            | ND                    | Spindle cell sarcoma |

*The tumors were found from 4 batches of injection involving 147 injected F2 fish that had survived beyond 2-week-old. Fish were observed for one and a half years, no other tumors were found in fish more than 3 months of age. ND: Not determined.*
active in keratinocyte progenitors; or 4) the combinations thereof. Alternatively, zebrafish skin may be quite refractory to developing basal cell carcinoma in comparison to frog and mammals, due to differences either in structure or in tumorigenic mechanisms. As for the other tumor types, especially the neuronal tumors, aberrant activation of the Hh pathway has been implicated in medulloblastoma and glioma [18]. Studies on AKT pathway implicate it not only in skin tumors, but also in glioblastoma [19]. Furthermore, the Akt signalling pathway contributes to SHH-induced medulloblastoma formation [20]. Therefore, it is conceivable that tumor types of neuronal origin may be induced in fish. But how exactly these tumors were induced in our transgenic fish is unknown. Immunofluorescence study showed that transgenic fish expressed the fusion protein not only in skin epithelia, but also in retina, which was not found in a previous study using the same promoter. An enhancer-trapping event might have occurred that led to ectopic expression of low levels of zebrafish Smoa1 in neuronal progenitor cells, which made the cells prone to developing tumors. Expression of a second oncogene, the constitutively active human AKT1, in the same cell types eventually led to tumorigenesis. It is also possible that integration of the transgenic construct into the zebrafish genome somehow activated an endogenous oncogene or inactivated a certain tumor suppressor gene, therefore contributed to tumor formation. We tried to determine the insertion copy numbers and the insertion sites in Line A fish through linker-mediated PCR and inverse PCR. We only detected one insertion, which was integrated at an intergenic region on zebrafish chromosome 11 (Data not shown). We do not know the effects of this integration on tumorigenesis in this particular transgenic line.

Cancers result from progressive accumulation of mutations in multiple cancer genes [21,22]. How different cancer genes interact with each other leading to different types of cancer is still a challenging subject. So far, oncogenic interactions have largely been studied in cultured cells or in mouse models. As a complement to these studies, the zebrafish provides a powerful model system to study interactions between different cancer genes and pathways at the organismal level because it is amenable to highly-efficient and cost-effective transgenic strategies [23,24]. Binary transgenic technology, such as the Gal4VP16-UAS system, the Tet-On and Tet-Off system [25], and the mifepristone inducible LexPR system [26] work efficiently in zebrafish, making it possible to express multiple oncogenes in the same tissue or cell type under the control of the same non-endogenous transcription factor. A limitation for using these binary transgenic approaches, as we have learned from our experience, is that stable lines carrying oncogenes may have diminished

Figure 3
Elevated phospho-AKT1 and Patched 1 levels in zebrafish tumors. A, B, a 3-week-old transgenic fish with trunk tumor (B) and its age-matched tumor-free fish (A). C, D, a 12-week-old fish with an eye tumor (D) and its age-matched tumor free fish (C). Immunofluorescence was done on cryosections for the above fish. E-H, an astrocytoma from a double transgenic fish showed elevated levels of both Patched 1 (F) and phosphorylated AKT (H). Immunofluorescence was done on paraffin sections for this tumor. Negative controls for Patched 1 (E) and phosphorylated AKT (G) were treated the same way except no primary antibodies were added. N, Notochord; AKT-P, phosphorylated AKT. Scale bars, 100 μm for A-D, 50 μm for E-H.
survivability. Therefore, their potential to be used for collaborative studies with other onco genes may be somewhat compromised. Another challenge will be to untangle the role of individual oncogenes in the tumor formation and progression processes.

In conclusion, we provide in vivo evidence that co-expression of the zebrafish Smoa1 and the constitutively active human AKT1 lead to tumorigenesis in zebrafish, establishing that the binary transgenic approach is a useful tool for studying collaboration between oncogenes and oncogenic pathways in the zebrafish model.

Materials and methods
Zebrafish and maintenance
Zebrafish (Danio rerio) were maintained in Aquatic Habits systems (Apopka, FL) on a 14 to 10 light dark cycle using a protocol approved by OHSU IACUC. The leopard long fin (TL), and the rose mutant fish which has a mutation in the endothelial receptor b1 gene causing light pigmentation were used. Embryos were reared in 0.3× Danieu’s solution at 28.5°C.

DNA constructs and microinjection
The transgenic DNA constructs are based on either the miniTol2 vector (denoted T2) or the I-Sce I meganuclease vector (denoted I) [see additional file 1]. The Tg(krt4:Gal4VP16;14 × UAS:smoa1-EGFP) transgenic construct contains a 2.2 kb zebrafish krt4 promoter, the Gal4VP16-UAS sequence from pEF-GVP-UG, and coding sequence for an activated zebrafish smo fused to EGFP at the C-terminus. The IU-mCherry plasmid, which contains 14 × UAS and E1b sequence from pUG and mCherry coding sequence, is the basic vector from which other UAS-driven vectors are derived, including pU-IhAKT1 and pU-myrhAKT1 (myristylated human AKT1 lacking the Pleckstrin Homology Domain sequence, referred to as constitutively active human AKT1). To deliver transgenes for transient expression or to establish stable lines using Tol2-based plasmids, about 1 nl sterile isotonic saline solution containing transgene DNA (30 ng/µl) and transposase RNA (30 ng/µl) was injected into fertilized eggs of the leopard strain. For the meganuclease-based constructs, the vector was mixed with I-SceI meganuclease and 30 pg of plasmid in 1 nl of the mixture was injected. Transgenic lines were identified by PCR using a zebrafish smoothened forward primer (5’-GGAAAGGAACAACTTTGGATG-3’) and an EGFP reverse primer (5’-CTGAACITGTGGCCGTIT- TACGTC-3’).

Histological study of zebrafish tumors
Zebrafish with tumors were fixed in 4% paraformaldehyde at 4°C for 24–48 hours, washed with PBST and decalcified in 0.5 M EDTA for up to 7 days depending on size. Tissues were dehydrated in a series of graded ethanol solutions and xylene, then embedded in paraffin. Orientation of the fish depended on location of the lesions. For fish with no gross lesions, individuals were cut in half sagittally just to the left of midline and both halves of the fish were placed into the cassette for sectioning. For small fish, serial sections were prepared. Sections were 4–6 µm thick and were stained with hematoxylin and eosin. Since standardized diagnostic criteria for fish tumors is not yet available, diagnostic criteria for other species, such as human and mouse were used to identify zebrafish tumors.

Immunofluorescence study of zebrafish tumors
For cryosections, zebrafish with tumors were fixed in 4% paraformaldehyde at 4°C for 24 hours, washed with PBST. Tumor tissues were first immersed in 15% glucose for 1 hour, followed by immersion in 30% sucrose overnight. They were then embedded in O.C.T compound and sectioned at 12 µm thickness. For paraffin sections, fish were treated as mentioned in histological study of zebrafish tumors. A rabbit anti-GFP polyclonal antibody (Invitrogen, 1:500), a rabbit anti-phospho-AKT (Thr308) antibody (C31E5, Cell Signalling Technology, 1:200), and a goat anti-Patched 1 (zebrafish) antibody (Everest Biotech, 1:200) were used for immunofluorescence studies of the tumors. Images were acquired with an inverted Zeiss microscope equipped with a CCD camera and Axiovision software. All images are processed in Photoshop.

Abbreviations
PI3K: Phosphoinositide 3-kinases; AKT1: v-akt murine thymoma viral oncogene homolog 1; UAS: upstream activation sequence; CMV: Cytomegalovirus.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
BJ and WC conceived, planned the experiments. BJ made the transgenic constructs and generated transgenic fish. JS identified the tumors. CJE and MRT did immunofluorescence studies on tumors. All authors read and approved the manuscripts.

Additional material

Additional file 1
Amino acid sequence alignment of mouse and zebrafish Smoothened.

The alignment indicated that the W514 of zebrafish Smoothened is the W539 equivalent of mouse Smoothened. It was mutated to L in the active forms (highlighted in red). Accession numbers for zebrafish and mouse Smoothened are [NP_571102] and [NP_795970], respectively. Click here for file [http://www.biomedcentral.com/content supplementary/1476-4598-8-40-S1.doc]
Additional file 2
Overall strategy for co-expression of oncogenes in zebrafish. Stable transgenic lines expressing zebrafish Smo1 were generated using a Tol2-based vector (A). Constitutively active human AKT1 (myrhAKT1) was incorporated into a meganuclease-based vector (B). The zebrafish krt4 promoter could simultaneously activate smo1-EGFP and myrhAKT1 expression through Gal4VP16-UBA.
Click here for file
[http://www.biomedcentral.com/content/supplementary/1476-4598-8-40-S2.tif]

Additional file 3
Expression patterns of transgenic line A. The data indicated that the cytokeratin 4 promoter drove epithelial cells-specific expression (arrows) of smo1-EGFP as shown by in situ hybridization against EGFP in 12 hpf F1 embryos (A), and of tdTomato in a 24 hpf embryo generated by crossing the Tg(krt4:Gal4VP16:14 × UAS:smo1-EGFP) and Tg(UAS:tdTomato) transgenic fish (B). At adult stage, GFP was detected predominantly in skin epithelial cells (C, arrow) and the retinal ganglion cells (D, arrow).
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[http://www.biomedcentral.com/content/supplementary/1476-4598-8-40-S3.tif]

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