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Pten function in zebrafish: Anything but a fish story

Miriam Stumpf [a], Suma Choorapoikayil [a,b], Jeroen den Hertog [a,c,*]

[a] Hubrecht Institute – KNAW and University Medical Center Utrecht, 3584 CT Utrecht, The Netherlands
[b] CNRS, UMR 5235, Dynamique des Interactions Membranaires Normales et Pathologiques, Univ Montpellier 2, 34095 Montpellier, France
[c] Institute of Biology Leiden, Leiden University, 2300 RA Leiden, The Netherlands

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ABSTRACT

Zebrafish is an excellent model system for the analysis of gene function. We and others use zebrafish to investigate the function of the tumor suppressor, Pten, in tumorigenesis and embryonic development. Zebrafish have two *pten* genes, *ptena* and *ptenb*. The recently identified N-terminal extension of human PTEN that may facilitate cell membrane transfer, appears not to be conserved in zebrafish Ptena or Ptenb. Mutants that retain a single wild type *pten* allele develop tumors, predominantly hemangiosarcomas. Homozygous double mutants are embryonic lethal. Zebrafish embryos lacking functional Pten display enhanced proliferation of endothelial cells, resulting in hyperbranching of blood vessels. In addition, *ptena*−/−*ptenb*−/− mutant embryos display enhanced proliferation of hematopoietic stem and progenitor cells and concomitant arrest of differentiation, although Pten-deficient cells commit to all blood cell lineages. Zebrafish is an ideal model for intravital imaging and future work using *ptena*−/−*ptenb*−/− mutants will enhance our understanding of the function of Pten *in vivo*.

1. Introduction

PTEN is one of the most frequently mutated tumor suppressor genes in human cancer [1,2]. Nonsense and missense mutations in *PTEN* have been identified in many different types of cancers [3–5] and additionally, missense mutations have been identified in rare human autosomal dominant cancer syndromes known as Cowden's disease, Bannayan–Zonana and Lhermitte–Duclose disease [6–8]. Reduction of PTEN activity in mouse by as little as 20% already results in increased tumor susceptibility [9], indicating that PTEN function is crucial for homeostasis.

Whereas PTEN has many different functions in cell signaling at distinct subcellular locations – at the cell membrane or in the nucleus – and even outside the cell (this issue of Methods), PTEN is best known for its lipid phosphatase activity. PTEN dephosphorylates phosphatidylinositol(3,4,5)trisphosphate (PI(3,4,5)P3) and is selective for the 3-position [10,11]. Hence, PTEN counteracts catalytic activity of phosphatidylinositol-3kinase (PI3K) and PTEN is an antagonist of PI3K signaling (Fig. 1). Inactivation of PTEN by mutation results in activation of downstream signaling, which is commonly reflected by phosphorylation and thus activation of Akt, which is also known as PKB [12,13]. Further downstream signaling results in enhanced cell survival and proliferation, which is consistent with *PTEN* being a tumor suppressor gene.

PTEN is expressed ubiquitously and mouse knockouts lacking functional PTEN are embryonic lethal around day 8.5 of embryogenesis [14–16], indicating that PTEN is essential for normal development. Likewise, genetic mutants have been generated in other model organisms, including *Caenorhabditis elegans* and *Drosophila*, that have helped to delineate the function of Pten in development and disease [17,18]. We use zebrafish, *Danio rerio*, to investigate the function of Pten. Zebrafish is a model that facilitates experimental approaches to investigate gene function, because of intrinsic properties, including relatively short generation time, high fecundity, transparency of the embryos, rapid embryonic development in aqueous medium, easy delivery of chemical compounds and circumstantial advantages, including availability of the genome sequence and availability of transgenic indicator lines expressing fluorescent proteins under the control of tissue-specific promoters [19]. Here, we will review the role of Pten in zebrafish cancer and development and we will provide an outlook on what we can expect to learn about the function of Pten from the zebrafish model system.

2. Pten gene and Pten protein

The zebrafish genome encodes two *pten* genes, *ptena* and *ptenb*, that are both highly homologous to human *PTEN* [20,21]. The

* Corresponding author at: Hubrecht Institute, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands.
E-mail address: j.denhertog@hubrecht.eu (J. den Hertog).

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alignment of Ptena and Ptenb with human PTEN illustrates that the three protein sequences are highly homologous throughout [21]. The phosphatase domain and the tensin homology domain contain only a few amino acid substitutions. The tensin homology domain in Ptena contains an additional loop that is 21 and 20 residues longer than in human PTEN and Ptenb, respectively, in an unstructured region of the protein [22]. Moreover, splice variants of both genes have been reported, giving rise to an additional 23 residues in the tensin homology domain of Ptena and Ptenb, which appears not to interfere with Pten function [23]. Importantly, Ptena and Ptenb display catalytic activity with a preference for the 3-position of PI(3,4,5)P3, like human PTEN [21]. Recently, it was discovered that human PTEN dimerizes [24]. Which regions of PTEN and which amino acid residues are essential for dimerization remain to be determined. Given the high sequence conservation between Ptena and Ptenb, it is highly likely that Ptena and Ptenb homo- and heterodimerize. Future research will have to establish whether or not homo- or heterodimerization occurs and whether this affects Pten function in zebrafish.

A long form of human PTEN was identified with a 173 amino acid N-terminal extension, resulting from an alternate translation initiation site [25–27]. This long form of PTEN was recently dubbed PTEN-L to distinguish it from the PTEN protein that has already been studied for more than two decades [28]. The N-terminal extension of PTEN-L bestows it with the capacity to traverse the cell membrane [25] and/or to regulate mitochondrial function [27]. The N-terminal extension of PTEN-L is encoded by sequences immediately 5′ to the start ATG in exon 1 of the *PTEN* transcript and makes use of an alternative CUG translation initiation site. This sequence is conserved in mammals [25]. Translation of genomic sequences to the 5′-side of the start ATG of *PTEN* of a selection of species until the first in-frame stop codon confirmed that mammalian sequences are highly conserved. The first in-frame stop is localized at 196–237 amino acids from the start ATG and all mammalian species contain the alternative CUG translation initiation site in frame with the start ATG (Fig. 2). The 5′ sequence of the lizard *Anolis carolinensis pten* encodes a stretch of 203 amino acids until the first stop codon, which is somewhat conserved, albeit sequence conservation is not as high as between mammalian sequences. The N-terminal extension of *Xenopus laevis* Pten contains 73 amino acids in-frame upstream of the start ATG, which is poorly conserved. Whether reptiles and amphibians actually encode an N-terminal extension that functions like the extension in human PTEN-L remains to be determined. Sequences to the 5′-side of the start ATG of chicken (*Gallus gallus*) are missing in ENSEMBL (data not shown) and therefore it remains to be determined whether birds encode PTEN-L. Zebrafish *ptena* and *ptenb* encode short stretches of 5 and 35 amino acids, respectively, which are not conserved at all. Likewise, the *pten* genes of other fish species do not encode conserved sequences to the 5′ side of the start ATG (Fig. 2). Hence, we conclude that fish species do not express Pten-L. The lack of endogenous Pten-L may put the zebrafish model in a unique position to study the function of the N-terminal extension. For instance, comparison of ectopic expression of PTEN-L and PTEN-S in zebrafish may provide insight into functional differences that result from the presence or absence of the N-terminal extension.

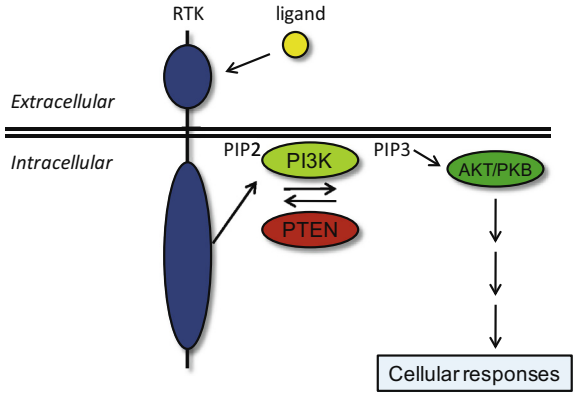

Fig. 1. PTEN antagonizes PI3K/Akt signaling. Simplified scheme of PTEN signaling. Ligand binding to its receptor leads to receptor activation and subsequently to PI3K activation, resulting in enhanced levels of phosphatidylinositol(3,4,5)trisphosphate (PIP3) and activation of downstream signaling via Akt. PTEN dephosphorylates PIP3 and loss of PTEN in cancer results in hyperactivation of the signaling pathway.

3. Modulation of Pten expression

One of the great advantages of the zebrafish as a model system is the rapid modulation of target protein expression by morpholino-mediated knockdown [29]. Antisense morpholinos are designed, aimed at either the start ATG or splice sites. Morpholinos are extremely stable in cells due to their backbone, which contains a morpholine ring, making morpholinos resistant to cellular nucleases. Morpholinos are routinely microinjected into zebrafish embryos at the one-cell stage, resulting in protein knockdown for up to 4 days. Usually, protein knockdown is transient and incomplete, resulting in expression of some residual target protein. Moreover, morpholinos do not target existing protein, such as maternally deposited target protein.

Two non-overlapping morpholinos each have been designed to target the 5′UTR of Ptena and Ptenb [20]. Antisense-morpholino-mediated knockdown of Ptena or Ptenb led to enhanced Akt phosphorylation. Distinct developmental defects were observed in response to Ptena or Ptenb knockdown [20], which is surprising, given the largely overlapping expression pattern and the similarity in enzymatic activity of Ptena and Ptenb [21].

We decided to isolate genetic mutants lacking functional Ptena and Ptenb. Target-selected gene inactivation [30] was used to identify *ptena* and *ptenb* nonsense mutations well upstream of the catalytic site cysteine. The mutants that were identified contain a mutation in exon 2 of *ptena* (Arg43 → stop) or in exon 3 of *ptenb* (Tyr65 → stop) [21]. Given their locations in the *pten* genes, these are considered to be null mutations. These mutants were bred to homozygosity. Homozygous *ptena*−/− mutants or *ptenb*−/− mutants are viable and fertile and the embryos do not display developmental defects, suggesting Ptena and Ptenb have redundant functions, which is in stark contrast to the morpholino data.

More and more evidence is emerging that morpholinos may have off-target effects [31]. The standard in the field is that two non-overlapping morpholinos are used that induce similar phenotypes. The knockdown efficacy is monitored by assessment of splicing at the morpholino target site and/or target protein expression is assessed. By now, compelling evidence is available that despite all controls, the morpholino-induced developmental defects are not always reproduced in genetic mutants. Likewise, zebrafish lacking the function of a single *pten* gene, i.e. *ptena*−/− or *ptenb*−/− zebrafish are viable and fertile. We have maintained zebrafish lines with only a single wild type *pten* gene for at least ten generations without any noticeable developmental defects. Yet, morpholino-mediated knockdown of Ptena or Ptenb induced massive developmental defects [20]. Moreover, injection of the same Ptenb morpholino, targeting the 5′UTR, by itself induced gastrulation cell movement defects, which could be rescued by a PI3K inhibitor as well as by dominant negative Cdc42. Surprisingly, the Ptenb morpholino had the same effect in homozygous *ptenb*−/− embryos from an incross of homozygous *ptenb*−/− fish, lacking functional Ptenb [32]. The most logical explanation is that the

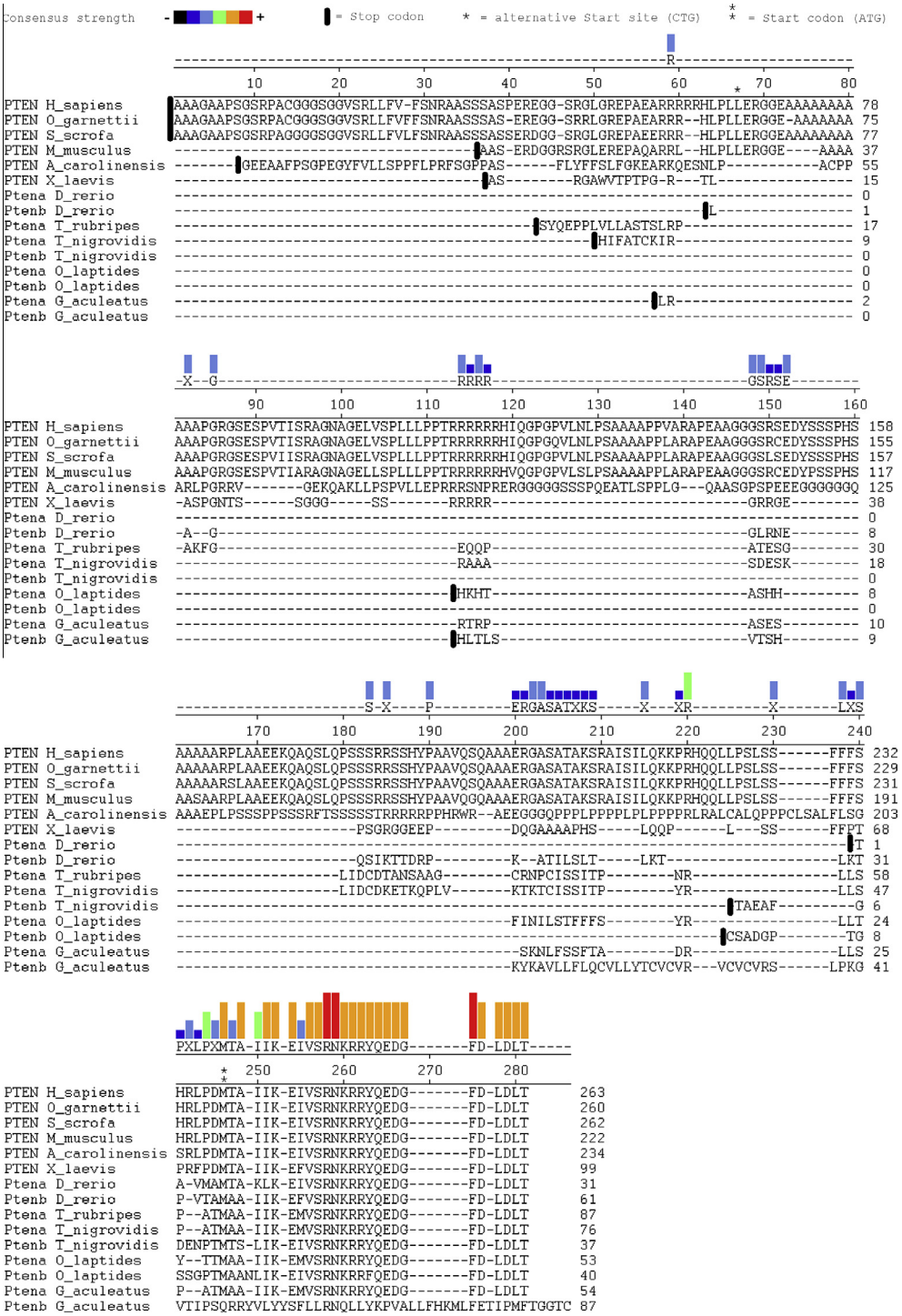

Fig. 2. PTEN-Long is evolutionarily conserved among mammals but not teleosts. Clustal-W alignment of the 5′ N-Terminal sequences until the first in-frame Stop codon (position in the sequence indicated by **I**) of Human PTEN (NM_000314.4) with genomic sequences from the Northern greater galago (*O. garnettii*), Pig (*S. scrofa*), Mouse (*M. musculus*), Lizard (*A. carolinensis*), Frog (*X. laevis*), Zebrafish (*D. rerio*), Fugu (*T. rubripes*), Green spotted puffer (*T. nigroviridis*), Mekada (*O. laptides*) and Stickleback (*G. aculeatus*). The consensus strength is represented by a color code as indicated in the figure (black for low homology, red for highest homology). All genomic sequences were obtained from the ENSEMBL database (www.ensembl.org). For translation into amino acid sequences, the Expasy Translate tool was used (http://web.expasy.org/translate) and the sequences were aligned with the MegAlign software from Lasergene. For Human PTEN, the mRNA sequence NM_000314.4 was used because the genomic sequence from the ENSEMBL database contains a frameshift at position 1592. The alternative CUG start codon is indicated with an asterisk (position 65 in human PTEN) and the ATG proper is indicated with two asterisks (position 238 in human PTEN).

Ptenb morpholinos target Ptenb as well as one or more unknown additional targets. Yet, formally it cannot be excluded that a truncated Ptenb protein is produced in *ptenb*−/− embryos that is knocked down by Ptenb morpholino, as concluded by Yeh et al. [32]. Ptenb knockdown using the same Ptenb morpholino was also reported to cause abnormal myeloid development [33]. Knockdown of Ptena, but not Ptenb, using distinct morpholinos targeting the start ATG resulted in enhanced thrombocyte count [34]. The reports about off-target effects of morpholinos and the lack of developmental defects in single *ptena*−/− or *ptenb*−/− mutants

cast considerable doubt on the Pten morpholino results described above, and these knockdown results should be confirmed in *ptena−/−* or *ptenb−/−* mutants.

Whereas homozygous single *pten* mutants do not display developmental defects, homozygous *ptena−/−ptenb−/−* double mutants display pleiotropic defects that are associated with enhanced cell proliferation and survival. *Ptena−/−ptenb−/−* embryos die around 6 days post fertilization, indicating that Ptena and Ptenb indeed have redundant functions. In the single mutants, Ptena compensates for the loss of Ptenb and *vice versa*, but embryos cannot survive without functional Pten [21]. Moreover, expression of Ptena or Ptenb by microinjection of synthetic mRNA encoding Ptena or Ptenb at the one-cell stage in *ptena−/−ptenb−/−* double mutants both rescue the developmental defects, indicating that Ptena and Ptenb have redundant functions ([35] and unpublished results). The observed developmental defects in *ptena−/−ptenb−/−* embryos are largely due to enhanced PI3K signaling, because inhibition of PI3K using LY294002 rescues the developmental defects to a large extent [21].

4. Tumorigenesis in Pten mutant zebrafish – hemangiosarcoma

Given the role of PTEN as a tumor suppressor in human cancer [1,2], and given that reduction of PTEN expression levels by as little as 20% enhances tumor susceptibility in mouse [9], it is not surprising that *pten* mutant zebrafish are more prone to cancer. However, enhanced tumor susceptibility only becomes evident upon loss of three of the four *pten* alleles, i.e. in *ptena+/−ptenb−/−* and *ptena−/−ptenb+/−* fish [36]. A cohort of 294 *ptena+/−ptenb−/−* fish were observed and 10% of these zebrafish with a single wild type *pten* allele developed a tumor within the first year of their life. These tumors occurred often, but not exclusively, close to the eye. The tumor-bearing fish were fixed, sectioned, mounted and stained using standard histopathological techniques. Pathological analysis and immunohistochemistry of sections using an endothelial marker (CD31) and a cell proliferation marker (PCNA) demonstrated that the tumors consist of an overgrowth of endothelial cells that form proper blood-filled vessels that are connected to blood circulation. Pathologically, these tumors were diagnosed as hemangiosarcomas, which is consistent with an enhanced incidence of hamartomas in human patients with germline *PTEN* mutations. Some tumors were excised and surprisingly, a single wild type *pten* allele was still detected in these tumors, indicating that tumor formation was not associated with loss-of-heterozygosity, but rather was due to haploinsufficiency. Not all *ptena+/−ptenb−/−* fish develop tumors. Apparently, additional factors are involved in tumor development. It is noteworthy that tumors often occur close to the eye, where the *rete mirabile* is localized, a meshwork of blood vessels. At this particular location, as a result of the abundance of blood vessels, it is not unlikely that substantial (vascular endothelial) growth factor signaling is occurring that may drive tumor formation in the event that three of the four *pten* alleles are lost.

Loss of tumor suppressor genes is often not sufficient to drive tumorigenesis. Additional events are required. Expression of oncogenic MYC in zebrafish induces T-cell acute lymphoblastic leukemia (T-ALL) [37]. Likewise, inducible expression of MYC induces T-ALL and removal of MYC leads to regression of tumors [38]. Inducible expression of MYC oncogene in *ptena+/−ptenb+/−* mutant zebrafish does not accelerate the onset of MYC-induced T-ALL. However, tumor regression upon removal of oncogenic MYC in double heterozygous *ptena+/−ptenb+/−* fish is greatly reduced. Hence, mutation of *pten* promotes loss of MYC oncogene dependence in T-ALL.

5. Zebrafish cell lines

Zebrafish constitutes an excellent model system for *in vivo* analyses. Sometimes it is desirable to assess molecular biological or biochemical traits in an *in vitro* cell culture system, rather than in a whole organism *in vivo*. For this purpose, only a few stable zebrafish cell lines are available. Most human cell lines are derived

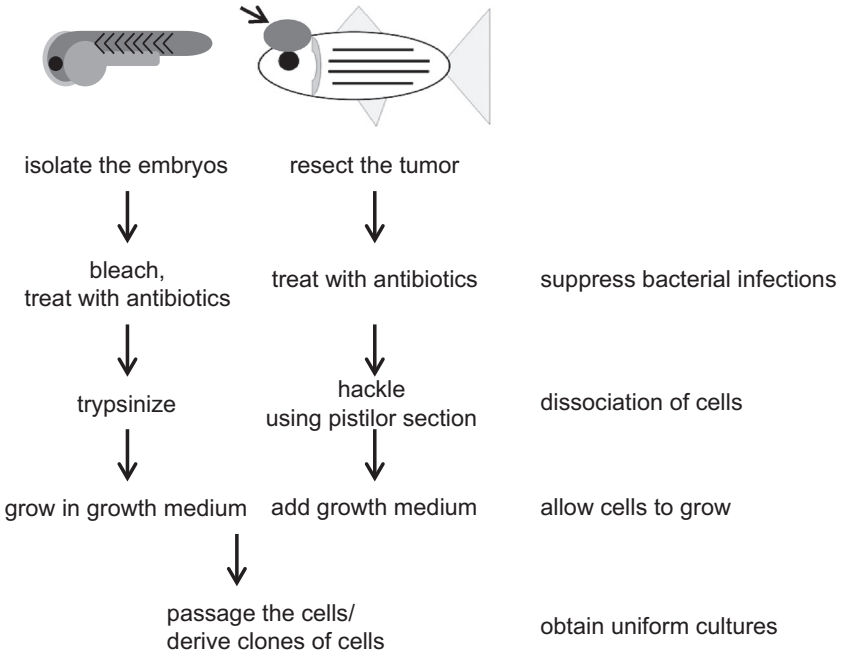

Fig. 3. Outline of the protocol to derive cell lines from zebrafish tumors or single zebrafish embryos. Crucial steps of the protocol are indicated with their purpose on the right. For details of the protocol, see [39].

from tumors. Tumorigenesis in haploinsufficient *pten* mutant zebrafish prompted us to develop a protocol for the establishment of zebrafish cell lines from tumors [39]. Crucial steps in the protocol are the isolation of the tumor, dissociation of the cells from the tumor, the composition of the medium and suppression of bacterial infection, particularly in the initial phase of establishing cell lines from zebrafish tumors. We have adapted the protocol to isolate stable cell lines from single zebrafish embryos, which will facilitate *in vitro* analysis of complex mutants (Fig. 3). Using this protocol, we established several cell lines from tumors of *pten* haploinsufficient mutants. Interestingly, we confirmed the *ptena+/−ptenb−/−* genotype in the tumor-derived cell line, indicating that indeed tumor formation was not due to loss-of-heterozygosity, but rather was due to *pten* haploinsufficiency [36].

6. Pten in angiogenesis

Zebrafish embryos lacking functional Pten display pleiotropic defects that are associated with enhanced proliferation and survival [21]. Since *pten* haploinsufficient zebrafish develop hemangiosarcomas, consisting of blood-filled vessels, we investigated the vasculature in *ptena−/−ptenb−/−* embryos, because of its endothelial origin. Zebrafish are best used for their intravital imaging potential, as the embryos are transparent and develop rapidly. Transgenic lines are available that express fluorescent proteins in all endothelial cells. One of these lines, *Tg(kdrl:eGFP)* expresses Green Fluorescent Protein under the control of the *kdrl* promoter [40]. The entire vasculature is fluorescently labeled in these embryos and confocal time-lapse imaging allows assessment of vasculo- and angiogenesis in real-time. In *ptena−/−ptenb−/−* mutants, initial blood vessel formation occurs normally, but at 3 and 4 dpf, excessive sprouting and filopodia formation are observed, resulting in hyperbranching. This is particularly obvious in the trunk region where intersegmental vessels display massive overgrowth (Fig. 4) [35]. These defects are consistent with hemangiosarcomas in *pten* haploinsufficient adult zebrafish. Interestingly, hyperbranching at 4 dpf is rescued to a large extent by microinjection of synthetic mRNA encoding Ptena at the one-cell stage, by treatment of the embryos with PI3K inhibitor, LY294002, or with the VEGFR-selective inhibitor, Sunitinib. Surprisingly, *vegfaa* expression is highly upregulated in *ptena−/−ptenb−/−* embryos, which may explain why particularly endothelial cells are affected in these mutants. Moreover, *vegfaa* expression is upregulated in hemangiosarcomas in adult *ptena+/−ptenb−/−* haploinsufficient zebrafish, providing support for the hypothesis that Vegf signaling is enhanced in zebrafish with *pten* inactivating mutations, which may contribute to tumorigenesis in these fish.

7. Pten in hematopoiesis

PTEN is frequently found to be mutated in hematologic malignancies, including bone marrow failure and leukemia [41,42]. Zebrafish is an ideal system to investigate the early stages of hematopoiesis because zebrafish embryos can survive without circulating red blood cells until the larval stage (10–12 dpf). Therefore, we investigated hematopoiesis in zebrafish embryos lacking functional Pten, using molecular markers, (immuno)histological staining and transgenic lines. In zebrafish, Hematopoietic Stem and Progenitor Cells (HSPCs) emerge from the floor of the dorsal aorta and transiently colonize the caudal hematopoietic tissue (CHT) [43–45]. These HSPCs expand and commit to most blood lineages, they differentiate and supply the developing embryos with mature blood cells. Subsequently, HSPCs migrate again to colonize the thymus and kidney marrow to produce blood cells for the duration of adult life. Hematopoiesis in mammals is similar in that HSPCs emerge from the aorta [46], transiently colonize the fetal liver and migrate to the thymus and bone marrow to produce definitive blood cells [47].

Using the *Tg(CD41-eGFP)* line, which expresses low levels of GFP in HSPCs and high levels of GFP in thrombocytes [48], we demonstrated that HSPCs colonize the CHT in *ptena−/−ptenb−/−* embryos [49]. Moreover, whole mount immunohistochemistry using phosphoHistone-3 specific antibodies indicates that HSPCs hyperproliferate in *ptena−/−ptenb−/−* embryos, which accounts for the enhanced number of HSPCs that is observed at 4 dpf. A panel of markers indicated that the HSPCs engage in all blood lineages we tested, including thromboid, myeloid and lymphoid. However, *ptena−/−ptenb−/−* embryos do not contain fully mature blood cells, in that definitive blood cell markers are not expressed in these cells. Together, these results indicate that in embryos lacking Pten, HSPCs hyperproliferate and do not differentiate terminally, which is consistent with the loss of a tumor suppressor. Strikingly, inhibition of PI3K at late stages rescues the ability of *ptena−/−ptenb−/−* HSPCs to differentiate terminally.

Dong et al. [50] reported similar results, in that *ptena−/−ptenb−/−* embryos exhibit an increased number of myeloid cells, which however are immune deficient. Inhibition of PI3K corrects expansive myelopoiesis in the *ptena−/−ptenb−/−* embryos.

The function of Pten in zebrafish hematopoiesis is consistent with the role of PTEN in mammalian hematopoiesis. Conditional

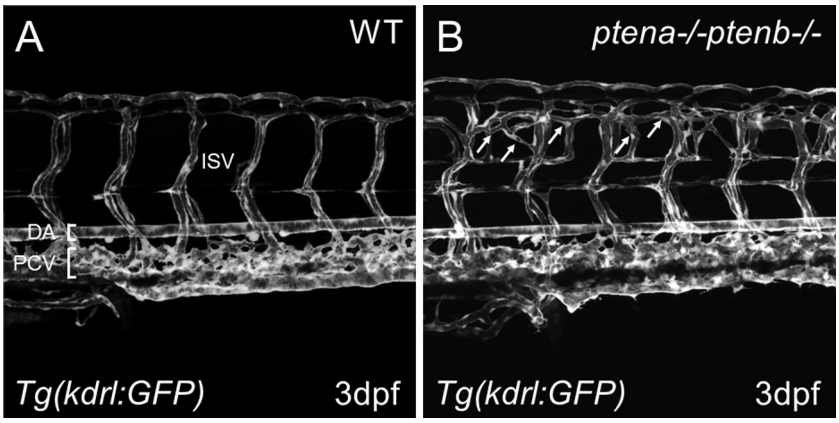

Fig. 4. Hyperbranching of the vasculature in *ptena−/−ptenb−/−* zebrafish embryos. *Tg(kdrl:GFP)* embryos expressing GFP in all endothelial cells of the vasculature were imaged at 3 dpf. An area of the trunk and tail region is shown here; anterior to the left, dorsal up. The dorsal aorta (DA), posterior cardinal vein (PCV) and one of the intersegmental vessels (ISV) are indicated. (A) Wild type and (B) *ptena−/−ptenb−/−*. Note the increase in blood vessels (hyperbranching) particularly in the dorsal side of *ptena−/−ptenb−/−* embryos (some of which are highlighted with arrows), compared to wild type, indicating hyperproliferation of endothelial cells.

mouse knockouts have been generated in which functional PTEN is deleted in adult bone marrow cells or in fetal liver. In these mice, HSCs lacking PTEN are driven into the cell cycle, which results in depletion of HSCs. These conditional PTEN-deficient mice die of a myeloproliferative disorder that resembles acute myeloid/lymphoid leukemia. Hyperproliferation of HSPCs in the CHT of Pten mutant zebrafish is reminiscent of the expansion of bone marrow HSCs in conditional mouse models.

8. Outlook

Whereas we and others have addressed several important questions regarding Pten function using zebrafish embryos as a model, many more questions remain. Specific benefits of zebrafish as a model system should be exploited in full to obtain new insights into the function of Pten. We envisage particularly that intravital imaging of zebrafish (mutants) will lead to important new findings. Many transgenic lines have been derived with tissue- or cell type-specific expression of fluorescent proteins that will facilitate analysis of the function of Pten *in vivo*. Moreover, transgenic multi-color cell-tracing lines have been generated that will allow assessment of the clonality of Pten-deficient cells and tissues [51,52]. Another area that is relatively unexplored currently is the analysis of the effect of cancer drivers in Pten-deficient background. This will undoubtedly lead to the formation of tumors other than hemangiosarcomas and all the benefits of the zebrafish as a model will then facilitate the analysis of the underlying mechanism of tumorigenesis on the one hand and the cell biology of cancer, particularly with respect to stem cells, on the other.

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