Development 140, 4323-4334 (2013) doi:10.1242/dev.095992

Regeneration of breeding tubercles on zebrafish pectoral fins requires androgens and two waves of revascularization

Stephanie C. McMillan[1,2], Zhe T. Xu[2], Jing Zhang[2], Cathleen Teh[3], Vladimir Korzh[3], Vance L. Trudeau[2,4] and Marie-Andrée Akimenko[2,4,*]

SUMMARY

Sexually dimorphic breeding tubercles (BTs) are keratinized epidermal structures that form clusters on the dorsal surface of the anterior rays of zebrafish male pectoral fins. BTs appear during sexual maturation and are maintained through regular shedding and renewal of the keratinized surface. Following pectoral fin amputation, BT clusters regenerate after the initiation of revascularization, but concomitantly with a second wave of angiogenesis. This second wave of regeneration forms a web-like blood vessel network that penetrates the supportive epidermis of BTs. Upon analyzing the effects of sex steroids and their inhibitors, we show that androgens induce and estrogens inhibit BT cluster formation in intact and regenerating pectoral fins. Androgen-induced BT formation in females is accompanied by the formation of a male-like blood vessel network. Treatment of females with both androgens and an angiogenesis inhibitor results in the formation of undersized BT clusters when compared with females treated with androgens alone. Overall, the growth and regeneration of large BTs requires a hormonal stimulus and the presence of an additional blood vessel network that is naturally found in males.

KEY WORDS: Breeding tubercles, Fin regeneration, Blood vessels

INTRODUCTION

Breeding tubercles (BTs) are multicellular epidermal structures that often support a conical keratin cap (Wiley and Collette, 1970). In most teleosts, BTs are present only in males or are more developed in males than in females (Wiley and Collette, 1970). Although all BTs share the same structure, their size, shape, number and location can vary within and between species (Wiley and Collette, 1970). In *Phoxinus*, different morphotypes of BTs have been identified that, depending on location, appear isolated or grouped together to form clusters. In some species of *Phoxinus*, large BTs have been found to form clusters on the dorsal surface of the central to more anterior pectoral fin rays (Chen and Arratia, 1996). In addition, isolated BTs have been observed in various areas, including the head, body and fins (Chen and Arratia, 1996).

Since BTs are present on multiple areas of contact between males and females, they are believed to serve various roles, such as maintaining body contact during spawning or as a fitness indicator during sexual selection (Wiley and Collette, 1970; Kortet et al., 2003). Other suggested roles include defense against mechanical injury, microorganisms and parasites. BTs have also been implicated in the protection of nests and territories (Ahnelt and Keckeis, 1994; Chen and Arratia, 1996; Kortet et al., 2003). In some species, an increase in the number and size of BTs is correlated with a higher level of aggressive behavior. It has been speculated that an increase in dominance occurs along with the presence of larger and more numerous BTs, both of which are correlated with high circulating concentrations of androgens (Kortet et al., 2004; Borg, 1994; Kortet et al., 2003). Androgens have been shown to induce the formation of BTs (Ramaswami and Hasler, 1955; Egami, 1954; Arai, 1967; Wiley and Collette, 1970). In addition, a reduction in testosterone following castration results in the regression of BTs in medaka (Nagata, 1934; Yamamoto and Egami, 1974). Overall, there appears to be a correlation between the presence of androgens and the development of BTs in many fish species.

Vascularization of large BTs has been observed in some fish species (Wiley and Collette, 1970). Although not experimentally tested, blood vessels are suggested to play a role in the maintenance of BTs in these species during long periods of spawning (Wiley and Collette, 1970). Recently, studies performed in human endothelial cell culture and in mice suggested that androgens have sex-specific pro-angiogenic effects (Sieveking et al., 2010). More specifically, androgens, acting through the androgen receptor (AR), increase the downstream expression of genes that promote processes related to angiogenesis, such as migration and proliferation (Death et al., 2004; Ng et al., 2003; Ruohola et al., 1999; Sieveking et al., 2010).

These studies indicate a correlation between the presence of BTs and androgens, and raise the interesting possibility that androgens in male teleosts stimulate angiogenesis, which is necessary for BT formation and maintenance. However, such a relationship has not yet been explored. In this report, we use two zebrafish transgenic lines, *Tg(fli1a:EGFP)* and *Tg(KR21)*, to investigate the relationship between sex hormones, vascularization and the presence of BT clusters on the intact and regenerating pectoral fins of zebrafish. Using these transgenic lines, a second wave of blood vessel growth is observed in regenerating BTs. Furthermore, we show that androgens promote the formation of BTs and additional blood vessels in intact and regenerating fins. Inhibiting additional blood vessel formation reduces the ability of androgens to induce BT formation in females. We conclude that the growth of large BT clusters in intact and regenerating pectoral fins depends on the presence of androgens and the formation of a unique pattern of blood vessels.

[1]Department of Cellular and Molecular Medicine, University of Ottawa, ON K1N 6N5, Canada. [2]CAREG, 30 Marie Curie, University of Ottawa, ON K1N 6N5, Canada. [3]Department of Biological Sciences, National University of Singapore, 117543 Singapore. [4]Department of Biology, 30 Marie Curie, University of Ottawa, ON K1N 6N5, Canada.

*Author for correspondence (makimen@uottawa.ca)

Accepted 8 August 2013

MATERIALS AND METHODS

Animals

Fish were maintained at 28.5°C with a photoperiod of 14 hours of light and 10 hours of darkness, and fed regularly (Westerfield, 1995). *Et(krt4:GAP-KillerRed)sqKR21* [or *Tg(KR21)*] is an enhancer trap line similar to other killer red (KR) lines (Korzh et al., 2011). KR is a membrane-tagged dimeric red fluorescent protein that acts as a photosensitizer (Korzh et al., 2011; Bulina et al., 2006; Serebrovskaya et al., 2009). Photobleaching of *Tg(KR21)* adults on confocal and widefield microscopes has not been determined. However, our exposure times (<500 milliseconds) are far below that required to photobleach embryos using similar membrane-tagged KR lines (Teh et al., 2010). The KR21 insertion site is unknown (see http://plover.imcb.a-star.edu.sg/webpages/memKR1.html). The *Tg(fli1a-EGFP)* line was a gift from Brant M. Weinstein (Lawson and Weinstein, 2002).

Fin amputation

Zebrafish were anesthetized by immersion in system water containing 0.17 mg/ml tricaine (ethyl-aminobenzoate) (Westerfield, 1995). Caudal and pectoral fins were amputated using a scalpel and a small pair of scissors, respectively.

Imaging

For brightfield and fluorescence imaging, fish were anesthetized and placed ventral side up on an agarose plate with the pectoral fins spread out. The plate was then inverted and images were taken using a Leica MZ FLIII dissection microscope, an AxioCam HSm digital camera and AxioVision AC software (Carl Zeiss). For confocal imaging, samples were mounted using Aqua-Poly/Mount (Polysciences). Images were captured using a Zeiss LSM 510/AxioVert 200 confocal and zen2009 software. All images were processed using ImageJ (NIH) and Adobe Photoshop.

Histochemical procedures

Mallory staining was performed as described (Cason, 1950). DAPI staining was performed by adding one drop of Vectashield Mounting Medium containing DAPI onto each slide. PAS staining was performed as per the manufacturer's protocol (Sigma-Aldrich). Picrosirius Red staining was performed as previously described (Smith et al., 2008).

Chemical treatments

17β-estradiol (E2), 5α-dihydrotestosterone (DHT) and testosterone (T) (all Sigma-Aldrich) were dissolved in ethanol and added to system water at 1 μg/l; this concentration was chosen based on our preliminary studies (DHT and T) and on published data (Miles-Richardson et al., 1999) (E2). An end point of 3 weeks for E2 treatments was chosen to ensure that BT absence was not due to a delay in regeneration. Flutamide (Sigma-Aldrich), in our hands, is effective at 2 mg/l when dissolved in ethanol and added to system water. Fadrozole was dissolved in system water at 50 μg/l (Ankley et al., 2002; Zhang et al., 2009). PTK787 (Selleck Chemicals) was dissolved in system water at an effective final concentration of 500 nM (Bayliss et al., 2006). Zebrafish were kept in glass tanks throughout treatments. Control tanks contained either the same percentage of ethanol or system water alone.

Immunohistochemistry

Samples were fixed in 4% paraformaldehyde overnight at 4°C and cryosectioned (Smith et al., 2008). For Pcna immunohistochemistry, sections were submerged in 10 mM sodium citrate pH 6.0, 0.05% Tween 20, at 98°C for 20 minutes, and cooled to room temperature. A mouse anti-Pcna antibody (clone PC10, Dako M0879) was used at 1:250 along with a rabbit anti-GFP (Molecular Probes) at 1:500. Fluorescently labeled secondary antibodies Alexa Fluor 488 goat anti-mouse IgG (H+L) and Alexa Fluor 594 goat anti-rabbit IgG (H+L) (Invitrogen A11001 and A11012) were used at 1:500. Slides were counterstained with DAPI and mounted.

Quantitative RT-PCR (qRT-PCR)

Total RNA was extracted from ten caudal fins using Trizol according to the manufacturer's protocol (Invitrogen). Total RNA was checked for purity (Nanodrop) and integrity (agarose gel analysis). Total RNA (2 μg) was reverse transcribed with SuperScript II reverse transcriptase (Invitrogen) using oligo(dT). Quantitative PCR was performed using the Eco Real-Time PCR System (Illumina) and an SYBR Green labeling system (Bio-Rad, 170-8880). Primers flanking a single intron of *ar* were used: forward, 5′-ATGACCCTGGGAGCCCGCAA-3′; reverse, 5′-GTGGTGAACGCC-GGCCATGA-3′. Primers used to amplify the *ef1α* (*eef1a1l1*) control also flank a single intron: forward, 5′-CAAACATGGGCTGGTTCAAG-3′; reverse, 5′-AGTGGTTACATTGGCAGGG-3′. Four biological samples were run in triplicate per experiment, with no-template controls. Ct values, extracted from a standard curve, were taken from the Eco Real-Time PCR System program. A two-tailed Student's *t*-test at a 95% confidence level ($P<0.05$) was performed to determine the significance of gene expression values in female versus male pectoral fins.

Calculation of average BT width

The average BT width was obtained by dividing a standard distance (285 μm) by the number of BTs in a single line within that standard distance using ImageJ. A one-tailed Student's *t*-test at a 95% confidence level ($P<0.05$) was performed to determine the significance of any increase in BT width.

RESULTS

BT distribution in male and female zebrafish

We first analyzed the distribution of BTs on adult (>6 months old) male and female zebrafish using *Tg(KR21)*, an enhancer trap line that ubiquitously expresses the membrane-tagged fluorescent reporter killer red (KR) in the epidermis. Fluorescence in *Tg(KR21)* fish appears more intense in BTs (Fig. 1A-I), most likely because of the increased thickness caused by epidermal aggregates and/or the differential cellular distribution of the KR protein in BTs versus the rest of the epidermis. Longitudinal sections of *Tg(KR21)* pectoral fins show that KR expression in the superficial cones of BTs is localized only at the cell membrane. In the underlying epidermis, KR appears to be distributed equally between the Golgi, where it is synthesized, and the cell membrane (data not shown). The intracellular distribution pattern of KR between the Golgi apparatus and the cell membrane is consistent with that in other transgenic lines using the same membrane-tagged KR (Korzh et al., 2011).

Using *Tg(KR21)* zebrafish, we observed that BTs are present in both sexes, but are more prominent in males. Both sexes display a small cluster and a single row of BTs on each side of the head and isolated BTs on the ventral surface of the head (supplementary material Fig. S1). The presence of BTs on the dorsal surface of the head, barbels, and along the fin rays of the dorsal, anal and pelvic fins in males is sexually dimorphic (Fig. 1; supplementary material Fig. S1). On male pectoral fins, isolated BTs are observed from the second branching point to the distal tip of the second to fifth/sixth fin rays and along the entire anteriormost ray (fin ray 1 being the most anterior and ray 11 the most posterior) (Fig. 1A-C). In addition, male zebrafish display BT clusters along the dorsal surface of the second to fifth/sixth pectoral fin rays (Fig. 1A-F; supplementary material Fig. S1A). We verified that only males, identified by the presence of testes upon dissection, had BT clusters on their pectoral fins ($n=13$). The length of these clusters is proportional to the length of the ray, with the longest clusters on the third to fifth rays and the shortest clusters on the second and sixth rays (Fig. 1A,J). *Long fin* mutants, named after their long fin rays, possess longer BT clusters than wild types (supplementary material Fig. S1C). Individual BTs within each cluster are conical, and have a single pointed tip with a broad base (Fig. 1G-I). Although these BTs are larger, their morphology is similar to isolated BTs elsewhere on zebrafish (Fig. 1A-F; supplementary material Fig. S1D-R). We focused our analysis on the pectoral fin BT clusters.

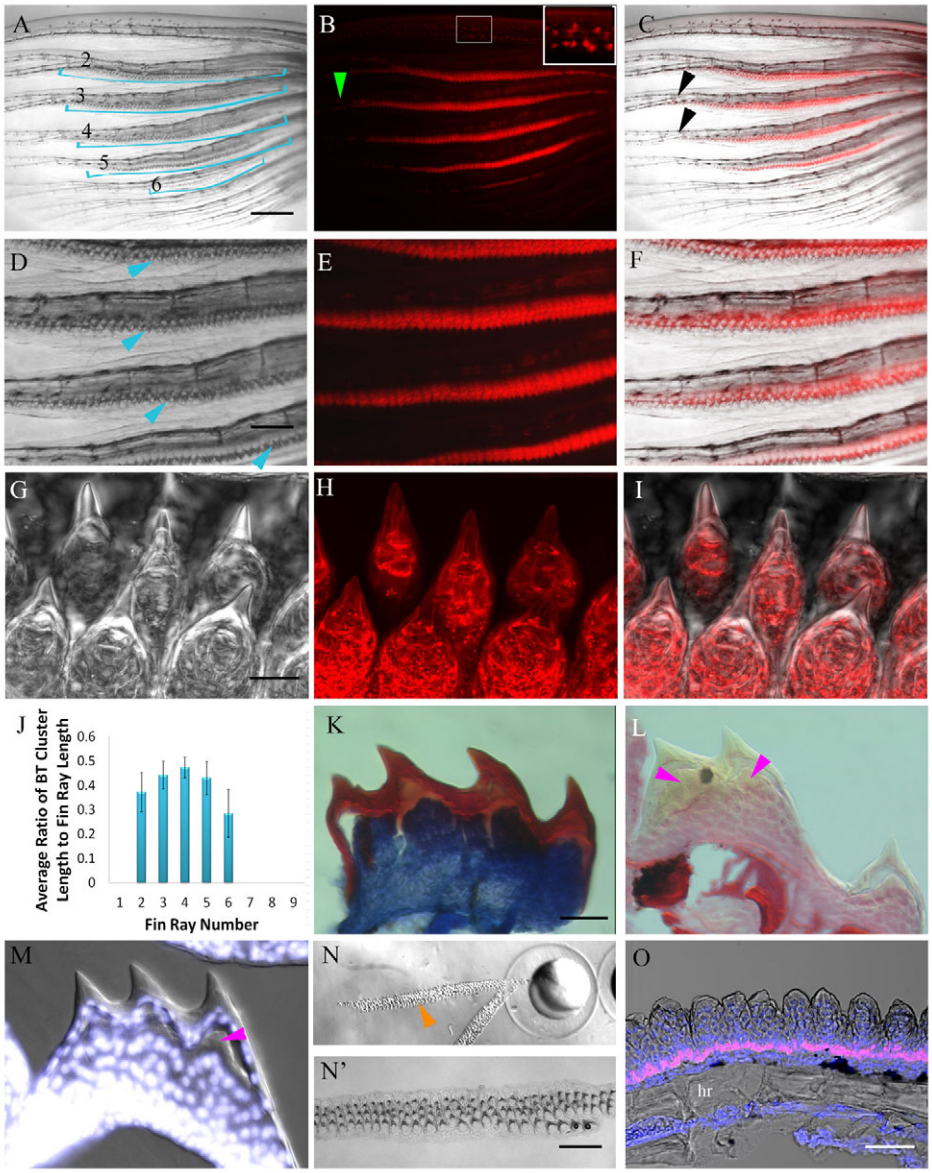

Fig. 1. Characterization of male zebrafish pectoral fin BTs. (**A-F**) Brightfield and fluorescent (red) images of BTs (indicated by blue arrowheads in D and lines in A) on *Tg(KR21)* male pectoral fins. (B,C) Isolated BTs on fin ray 1 (inset in B) and on the distal tips (green arrowhead in B, black arrowheads in C) of rays 2-6. (D-F) Higher magnifications of A-C. (**G-I**) DIC images with confocal acquisition of *Tg(KR21)* BTs. (G) DIC image of BTs. (H) Red fluorescent image of BTs. (I) Merged image of G and H. (**J**) Length of the BT cluster correlates with the length of the fin ray. Error bars indicate s.d. (**K**) Mallory stains on a transverse section of a BT keratinized cap (red) and underlying epidermis (blue). (**L**) Picrosirius Red stain indicates that there is no collagen in BTs. (**M**) Nuclei are present in all epidermal layers, including the keratin cap of BTs (DAPI stain). (L,M) A second keratin layer is observed underneath the most superficial keratin layer (pink arrowheads). (**N**,**N′**) Shed BT clusters (orange arrowhead; magnified in N′) alongside an embryo. (**O**) Pcna immunohistochemistry (red) and DAPI staining (blue/white) on longitudinal sections indicates epidermal proliferation. hr, hemiray. Scale bars: 200 μm in A-C; 100 μm in D-F; 25 μm in G-I,K-M; 500 μm in N,N′; 50 μm in O.

Pectoral fin BT cluster development in male zebrafish

The earliest developmental stage at which pectoral BT clusters appear in male zebrafish is 1.3 cm standard length and at least 2.5 months old (n=122; supplementary material Fig. S2), which correlates with sexual maturation (Westerfield, 2000). Random sampling of fish of 2.5 months to 1 year indicates that BTs develop progressively over time. In the earliest stages, a single line of small BTs is observed along the fin rays. Afterwards, a cluster of small BTs develops that progressively enlarges over time (supplementary material Fig. S2).

Pectoral fin BT clusters are epidermal structures covered with a renewable keratin cap

BT structure was analyzed on pectoral fin transverse sections. The superficial cap of BTs is conical in shape with a hollow center (Fig. 1K-M) and is made of keratin, as shown in red following Mallory staining (Fig. 1K). These caps are not stained following Picrosirius Red staining, indicating the absence of collagen (Fig. 1L). Underlying each keratin cap is a raised, supportive epidermis that consists of small aggregates of epidermal cells that are stained blue by Mallory staining (Fig. 1K). More specifically, the epidermis stains light blue along the base and progressively darkens as the keratinization process continues upwards. Progressive darkening in Mallory stains has been suggested to reflect epidermal differentiation during the process of keratinization (Wiley and Collette, 1970). Furthermore, the last two layers of epidermal cells are hypertrophied, probably owing to the large keratin content. In addition, nuclei in the epidermis, as shown by DAPI staining, are displaced towards the basal surface of each cell (Fig. 1M).

Interestingly, the outer keratin cap of each cluster is periodically shed, but males in our fish facility always have BT clusters on their pectoral fins (Fig. 1N,N′). Thus, it is likely that the second keratin-containing cell layer replaces the outer keratin cap once it is shed. Cell proliferation analysis by proliferating cell nuclear antigen (Pcna) immunohistochemistry indicates that only cells in the deepest layers of the epidermis are proliferating (Fig. 1O). The more superficial cells do not proliferate (Fig. 1O). This profile suggests that BT formation involves the proliferation of progenitors within the deepest layers of the epidermis. As these cells progress towards

the upper layers, they exit the cell cycle and differentiate into keratin-producing cells.

BT clusters possess a secondary blood vessel network

Examination of blood vessel networks in *Tg(fli1a:EGFP)* fish, which express EGFP in all endothelial cells (Lawson and Weinstein, 2002), reveals a qualitative increase in vascularization in regions of pectoral fins containing BTs in males as compared with females (Fig. 2).

The overall fin vasculature in females is similar to that described in caudal fins (Huang et al., 2003). One artery runs down the center of each ray and two veins run along the external border of the rays. Within and between rays, intervessel commissures and interray vessels connect the arteries to veins and veins to veins, respectively (Fig. 2A-C,G-I).

In males, regions of pectoral fins devoid of BTs present a vasculature similar to that of females (Fig. 2D). By contrast, BT clusters are highly vascularized (Fig. 2D-L,P-R). Confocal analysis of *Tg(fli1a:EGFP)* male pectoral fin longitudinal sections reveals a web of blood vessels that overlies the artery and intervessel commissures/interray vessels and penetrates into the epidermis of BT clusters (Fig. 2P-R). Longitudinal sections through the most proximal portion of the male pectoral fin suggests that these additional vessels are derived from the central blood vessels between the two hemirays (supplementary material Fig. S3). Upon reaching the epidermis, the vessel travels in parallel to the hemirays, periodically forming connections with the central blood vessels (supplementary material Fig. S3). In females, no vessels are observed in the epidermis overlying the fin rays (Fig. 2M-O).

Pectoral fin BT and blood vessel regeneration

To investigate the regenerative properties of BTs, pectoral fins of males (n=15) and females (n=15) were amputated and regeneration was observed for 15 days post-amputation (dpa) (Fig. 3). Following amputation, BTs are progressively re-established in a proximal-to-

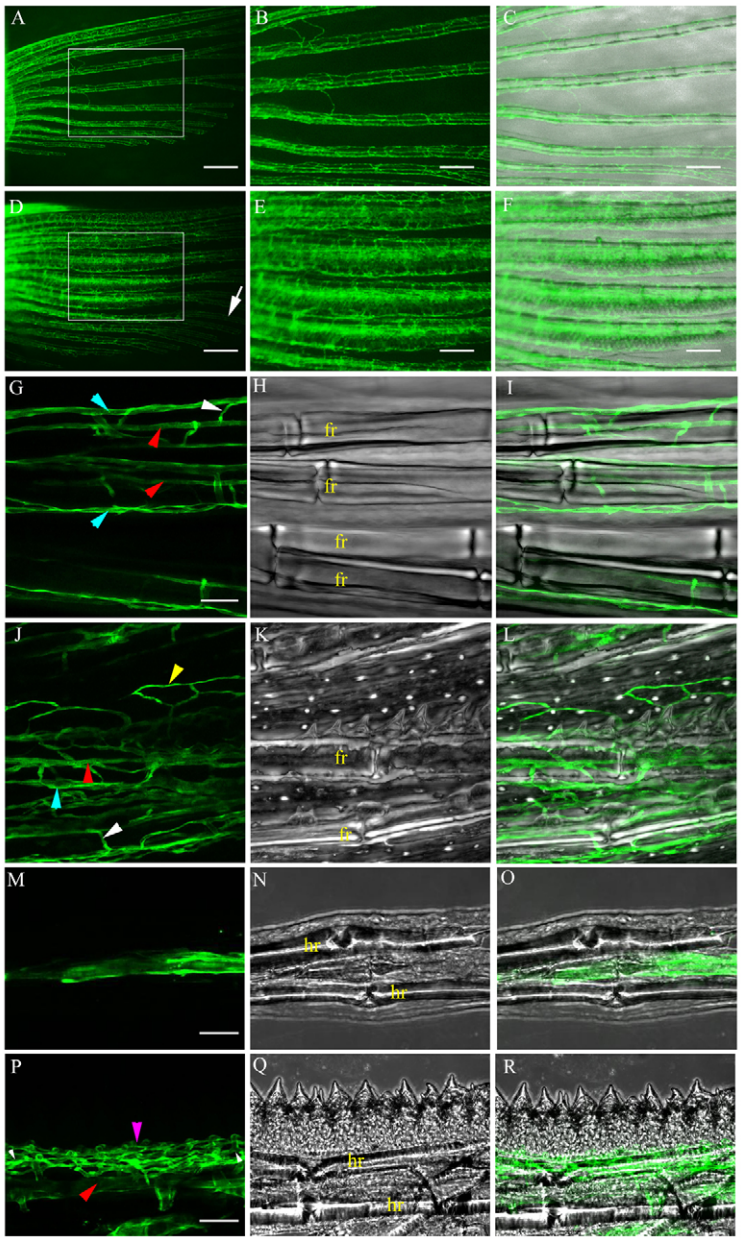

Fig. 2. Vasculature in *Tg(fli1a:EGFP)* pectoral fins. (**A-C**) Blood vessels (EGFP, green) in female zebrafish. (**D-F**) BT clusters in males are highly vascularized (boxed in D), but not in surrounding areas (arrow in D). (B,C,E,F) Higher magnification of the boxed regions in A,D. (C,F) Merged brightfield and fluorescent images. (G-R) Confocal micrographs. (**G-I**) Female with a single artery (red arrowheads), two veins (blue arrowheads) and intervessel commissures (white arrowhead). (**J-L**) Male with vascularized BTs, an artery (red arrowhead), intervessel commissures (white arrowhead), interray vessels (yellow arrowhead) and veins (blue arrowhead). (M-R) Longitudinal sections. (**M-O**) Female showing blood vessels that lie between the two hemirays. (**P-R**) Male showing blood vessels present in a web-like pattern (pink arrowhead) in the epidermis and above the artery (red arrowhead). fr, fin ray; hr, hemiray. Scale bars: 200 μm in A,D; 100 μm in B,C,E,F; 50 μm in G-L; 25 μm in M-O; 50 μm in P-R.

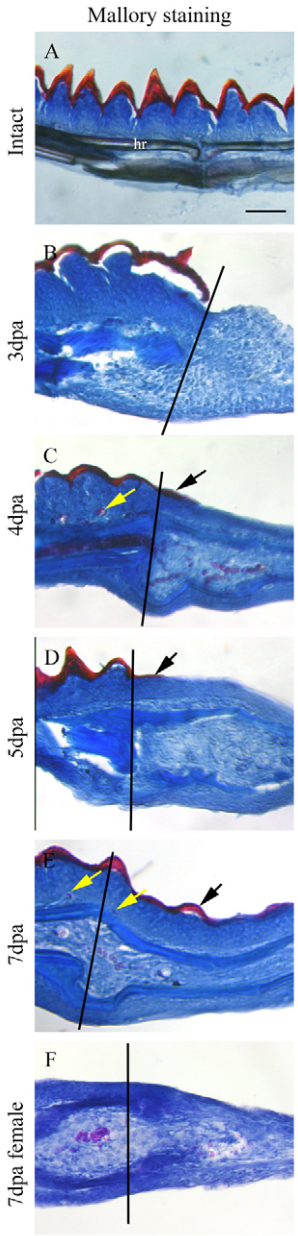

Fig. 3. Male pectoral fin BT regeneration. Mallory stain of intact (A) and regenerating (B-F) pectoral fin longitudinal sections. (**A**,**B**) Keratinized caps (red) and underlying epidermis (blue) are observed on intact fins (A) but are lost in 3-dpa regenerates (B). (**C**,**D**) At 4-5 dpa, a layer of keratin is deposited in the proximal regenerate (black arrows). (**E**) At 7 dpa, BTs (black arrow) are observed in the fin regenerate. (**F**) Females do not possess BTs. Yellow arrows indicate blood cells. Black lines (B-F) indicate the amputation plane. dpa, days post-amputation. hr, hemiray. Scale bar: 50 μm.

distal manner over the regenerating tissue. A keratin layer first appears in the regenerate adjacent to the stump between 4 and 5 dpa (Fig. 3C,D). Concomitantly, the underlying epidermis progressively thickens (Fig. 3C-E) and regenerating BTs reacquire a mature structure with keratin caps and a tooth-like shape by 7 dpa (Fig. 3E). By contrast, the female 7-dpa pectoral fin regenerate is not keratinized and the epidermis appears thinner than in males (Fig. 3F). Furthermore, PAS staining of regenerating and intact female pectoral fins reveals goblet cells along the dorsal and ventral surfaces. Goblet cells are not observed in male fins containing mature or regenerating BTs (supplementary material Fig. S4). In over half the fish examined (19 out of 30), BT clusters of 15-dpa regenerating fins appear on both the dorsal and ventral sides (Fig. 5J; supplementary material Fig. S5O,S,W). The other 11 pectoral fins reformed BTs on only the dorsal surface of the rays.

Using *Tg(fli1a:EGFP)* zebrafish, we examined BT regeneration in relation to pectoral fin revascularization. In all female fin rays (n=8) and male posterior fin rays (n=8), blood vessel regeneration follows a pattern previously observed in caudal fins (Fig. 4Aa-h,Ba-h,Da-h) (Huang et al., 2003). By contrast, initial plexus formation in the male anterior fin rays appears irregular in shape and is delayed at 3 dpa (Fig. 4Ab,Cb). From 5 dpa onwards, there is a proximal-to-distal increase in fluorescence underneath newly forming BTs (Fig. 4Cd-h). Confocal analysis of longitudinal sections of *Tg(fli1a:EGFP)* female (n=4) and male (n=4) anterior pectoral fin regenerates from 4-8 dpa revealed that the increase in fluorescence in males is due to a second wave of blood vessel regeneration (Fig. 5). In females, blood vessels regenerate between the two hemirays (Fig. 5A,C,E,G,I). The same blood vessel pattern is initially observed in males at 4 dpa (Fig. 4Cb,c, Fig. 5B). However, by 5 dpa, a second wave of blood vessel regeneration is observed along the base of newly forming BTs (Fig. 5D). As BT regeneration proceeds, blood vessels grow in a proximal-to-distal fashion along the base of the epidermis, reforming the original web of blood vessels (Fig. 2P, Fig. 5H,J). Furthermore, new vessels periodically sprout from the central blood vessels between hemirays and connect to vessels in the epidermis (Fig. 5D,F). Blood cells were often observed in the stump (Fig. 3C) and in the regenerate underneath new and forming BTs (Fig. 3E). Overall, our data suggest that BT regeneration begins following the initial wave of blood vessel regeneration, but occurs simultaneously with a second wave of neo-angiogenesis.

Androgen treatment induces BT formation in intact and regenerating fins

BT development on male pectoral fins coincides with sexual maturation, indicating a potential hormonal contribution. We investigated the role of steroid hormones in BT cluster formation in intact and regenerating adult pectoral fins. In the following experiments, the right pectoral fin of males and females is amputated at the center of the BT cluster and at the corresponding level, respectively. The left pectoral fin is left intact.

To investigate the role of androgens in BT formation, adult males and females (>4 months old) were treated for 15 days [days of treatment (dot)] with testosterone (T) (1 μg/l) (n=6 males and 6 females). T-treated males possess BTs that are larger in intact and regenerating fins than in untreated males (Fig. 6A,B; supplementary material Fig. S6). In addition, four out of six regenerating fins developed BTs on both sides of the ray by 15 dpa (supplementary material Fig. S6B). In females, T induces BT cluster formation on both intact fins and stumps of regenerating fins after 4 dot (supplementary material Fig. S7A,B). By 6 dot, immature BTs are observed on the regenerating tissue (n=6; supplementary material Fig. S7F). These BT clusters continue to enlarge until treatments are suspended after 15 days, when BTs appear similar to those of untreated males (Fig. 6C,D; supplementary material Fig. S7). Ethanol (0.000033%) treatments alone do not affect BT formation or regeneration (n=6 males and 6 females; Fig. 6E-H).

Treatments for 23 days with flutamide (2 mg/l), an AR antagonist (Peets et al., 1974), does not affect intact and regenerating female fins (n=6; Fig. 6K,L). Treating males with flutamide reduces the size of BT clusters in intact fins and the stump after 14 dot (n=6; supplementary material Fig. S5B,F,J). Since no BTs are observed in

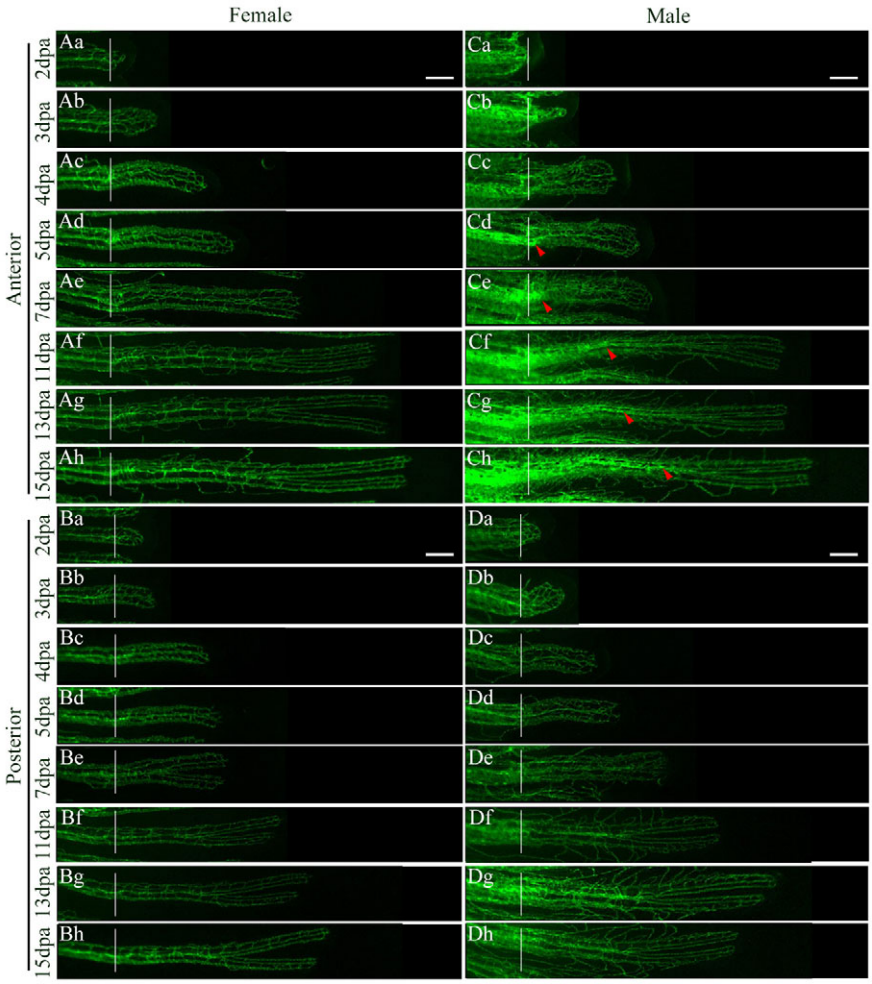

Fig. 4. Male and female *Tg(fli1a:EGFP)* blood vessel regeneration. (**Aa-h**,**Ba-h**,**Da-h**) Blood vessel regeneration follows similar patterns in the female (A,B) and posterior (D) male rays. (**Ca-h**) Plexus formation (Cb) is delayed in the male anterior ray compared with females (Ab) and appears irregular in shape. (Cd-h) At 5 dpa, a second wave of neo-angiogenesis occurs underneath new BT formation in the male anterior ray and proceeds in a proximal-to-distal manner (red arrowheads). Anterior, fin ray 4; posterior, fin ray 7. White lines indicate the plane of amputation. Scale bars: 100 μm.

regenerates after 14 days (supplementary material Fig. S5D,H,L), treatments were continued to ensure that the lack of BTs was not the result of a delay in regeneration (Fig. 6I-L). At 23 dot, only a few, small BTs are observed in the regenerate close to the stump in only one of two males examined (Fig. 6I) and BTs on the intact fin appear reduced (Fig. 6J). Ethanol treatments alone (n=6 males and 6 females treated with 0.000042% ethanol) appear similar to system water controls (Fig. 6E-H).

To exclude the possibility that the above effects are the result of aromatization of T into 17β-estradiol (E2), females (n=12) were treated with 5α-dihydrotestosterone (DHT) (1 μg/l), a non-aromatizable androgen that binds to ARs (Goldstein and Wilson, 1972; Hillier et al., 1980). Similar to T, DHT induces BT formation in both intact and regenerate stumps of female pectoral fins after 4 dot (data not shown). BTs are initially observed on fin regenerates after 6 dot (data not shown). These BTs continue to enlarge until the cessation of treatments after 15 days (Fig. 6O,P). Similar to T treatment, BTs in DHT-treated males are slightly larger than untreated BTs and all regenerating fins possess clusters that cover both sides of the ray (Fig. 6M,N; supplementary material Fig. S6). In two out of six males examined, the anterior rays in the pectoral fins failed to regenerate. This regeneration failure is similar to that described recently (Nachtrab et al., 2011). Mallory staining of 15-day T- and DHT-treated fins indicates that keratinized BTs are present on all fins except the caudal fin (data not shown).

To further test the effect of T aromatization on BT formation, males and females were treated with fadrozole (50 μg/l), an aromatase inhibitor. As expected, fadrozole does not affect males (n=6; Fig. 6Q,R). However, BTs are observed in fadrozole-treated females (n=7) after 7 (3/7), 10 (4/7) and 14 (7/7) dot in the intact fin (Fig. 6T). Similarly, BTs are observed at 7 (3/7) and 10 (7/7) dot in fadrozole-treated female fin regenerates (Fig. 6S).

Following cessation of treatments with fadrozole, females were kept in system water to determine whether BT clusters disappear. Fifty-eight days after the cessation of treatments [days post-treatment (dpt)], BT clusters are completely absent in all females (4/4; Fig. 7A-F). Similarly, DHT-treated females display highly reduced BTs that are barely visible at 88 dpt (4/4; Fig. 7G-L). Altogether, our data suggest that pectoral fin BT cluster development and maintenance depend on the presence of androgens acting through the AR. Interestingly, qRT-PCR data indicate approximately equal levels of *ar* mRNA in untreated male and female pectoral fins (supplementary material Fig. S8).

We next investigated the effects of estrogens on BT formation. Males and females were treated with E2 (1 μg/l), which binds to all zebrafish estrogen receptors (Menuet et al., 2004). After 16 dot, no changes are observed in the intact and regenerating female pectoral fins (n=6; Fig. 6W,X). By contrast, after 16 dot BTs do not form in the male pectoral fin regenerates (n=6; Fig. 6U). Additionally, BT clusters on the male intact fin and stump appear smaller (Fig. 6U,V). After 18 and 21 dot BTs are still absent in the male fin regenerate (supplementary material Fig. S5N,P,R,T,V,X). Although there is a variable delay in regeneration, these fins are able to regenerate to the point at which

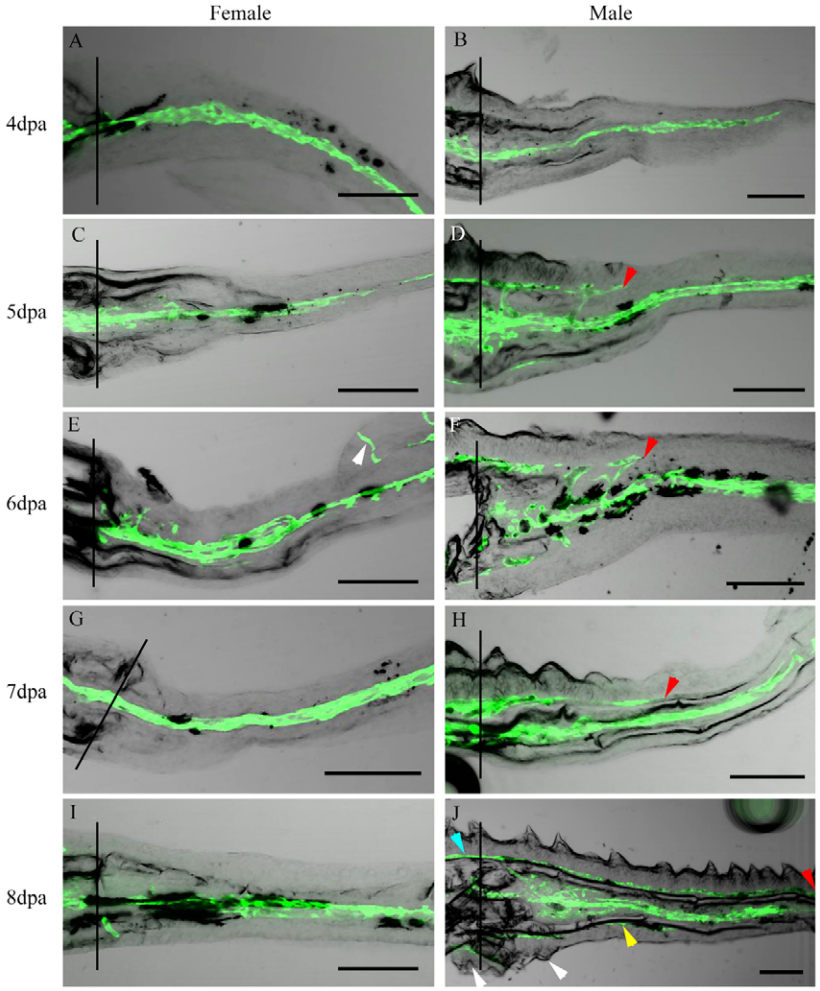

Fig. 5. BT cluster regeneration occurs alongside a second wave of neo-angiogenesis. Confocal images of longitudinal sections of regenerating male and female pectoral fins. (**A**,**C**,**E**,**G**,**I**) Female blood vessels regenerate along the center of the ray. (E) Arrowhead indicates the distal ray folded back on itself. (**B**) The first wave of male blood vessel regeneration occurs down the center of the ray. (**D**,**F**,**H**,**J**) At 5-8 dpa, a second wave travels in a proximal-to-distal fashion along the base of the epidermis (red arrowhead). (J) New BTs (white arrowheads) and blood vessels (yellow arrowhead) are observed in the ventral fin regenerate. Blue arrowhead indicates blood vessels along the base of BTs located in the stump. Black vertical lines indicate the amputation plane. Scale bars: 100 µm.

BTs should be observed. These treatments are also lethal in 50% of male fish by 7 dot (n=6). Ethanol (0.000033%) treatments alone (n=6 males and 6 females) appear similar to system water controls (Fig. 6E-H; supplementary material Fig. S5M,O,Q,S,U,W). Overall, our data suggest that androgens induce whereas estrogens inhibit BT formation in zebrafish.

Androgen treatment induces blood vessel growth and BT cluster formation in the anterior rays of female pectoral fins

To investigate the relationship between blood vessels and BT formation, *Tg(fli1a:EGFP)* female pectoral fin vascularization was examined during androgen treatments. Following 14 dot with T (1

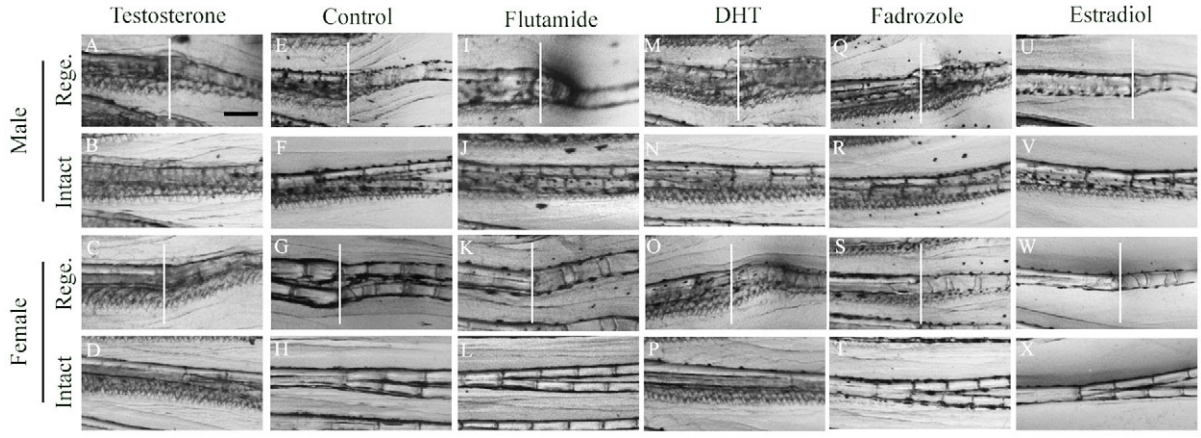

Fig. 6. Androgens induce and estrogens inhibit pectoral fin BT formation. BTs are observed in male regenerate and intact fins treated with testosterone (T) (1 µg/l) (**A**,**B**), 5α-dihydrotestosterone (DHT) (1 µg/l) (**M**,**N**) and fadrozole (50 µg/l) (**Q**,**R**). Treatment with testosterone (T) (1 µg/l) (**C**,**D**), 5α-dihydrotestosterone (DHT) (1 µg/l) (**O**,**P**) and fadrozole (50 µg/l) (**S**,**T**) for 15 days induces BT formation in the regenerating and intact female fin. Flutamide treatment (2 mg/l) (23 dot) (**I**,**J**) and 17β-estradiol (E2) (1 µg/l) (16 dot) (**U**,**V**) inhibit and reduce BT formation in the male regenerate and intact fin, respectively. Flutamide (**K**,**L**) and E2 (**W**,**X**) treatments do not affect females. Controls (**E-H**). dot, days of treatment. Scale bar: 100 µm.

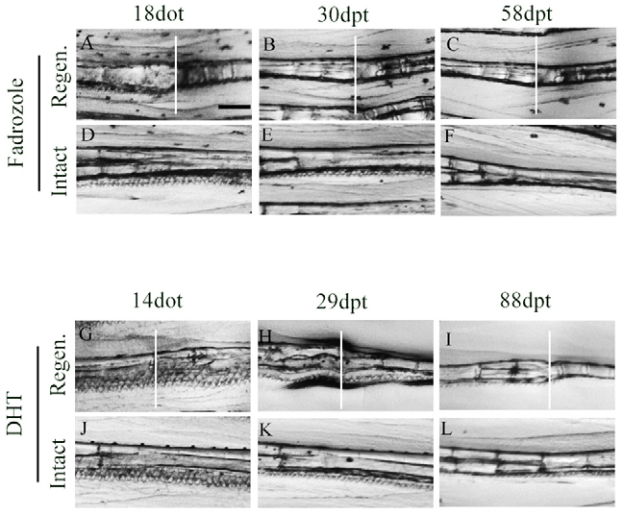

Fig. 7. Pectoral fin BTs regress following cessation of chemical treatments in females. (**A-F**) Regenerating (A) and intact (D) fins treated for 18 days with fadrozole. (B,E) At 30 dpt, BTs appear reduced. (C,F) At 58 dpt, BTs have disappeared. (**G**,**J**) Female regenerate (G) and intact fins (J) following 14 dot with DHT. (**H**,**K**) At 29 dpt, BTs appear smaller. (**I**,**L**) At 88 dpt, BTs are barely visible. White vertical line indicates the amputation plane. dpt, days post-treatment. Scale bar: 100 μm.

μg/l) or DHT (1 μg/l), all female intact (n=6) and regenerating (n=6) rays develop a male-like blood vessel pattern in the region of BT formation (Fig. 8A-F,I,J,P,T). Further analysis of longitudinal sections (n=12) of *Tg(fli1a:EGFP)* T-treated female intact and regenerating pectoral fins confirmed that T-induced BT formation occurs in conjunction with neo-angiogenesis (Fig. 8B′,D′; supplementary material Fig. S7). More specifically, in T-treated females, BT cluster formation occurs after 4 dot and vascularization is observed along the epidermis by 5 dot (supplementary material Fig. S7A,C). In T-treated female fin regenerates, blood vessel regeneration occurs in two waves, as observed in males: the central blood vessels regenerate first, followed by the vascularization of maturing BTs (supplementary material Fig. S7B,D,F,H,J). Interestingly, male pectoral fin regenerates (n=6) treated for 14 days with flutamide (2 mg/l) are devoid of BTs (supplementary material Fig. S5D,H,L), have a female-like blood vessel network (Fig. 8G,H) and no second wave of neo-angiogenesis.

Endothelial and epidermal cells proliferate upon testosterone treatment

To examine changes in cell proliferation that are associated with BT formation in T-treated females, double fluorescence immunohistochemistry for Pcna and EGFP was performed on intact pectoral fins of 5-day T-treated *Tg(fli1a:EGFP)* females. Anterior rays of untreated female pectoral fins show a few proliferating cells in the epidermis (Fig. 9A). However, similar to untreated males, cells in the deepest layers of the epidermis are proliferating in 5-day T-treated females (Fig. 1L, Fig. 9B,C). Furthermore, endothelial cells between the hemirays of 5-day T-treated females are proliferating, suggesting a potential origin for the blood vessels that vascularize these BTs (Fig. 9C).

BT cluster formation depends on the development of an additional blood vessel network

To determine whether an additional blood vessel network is required for BT formation, *Tg(KR21; fli1a:EGFP)* females were treated with

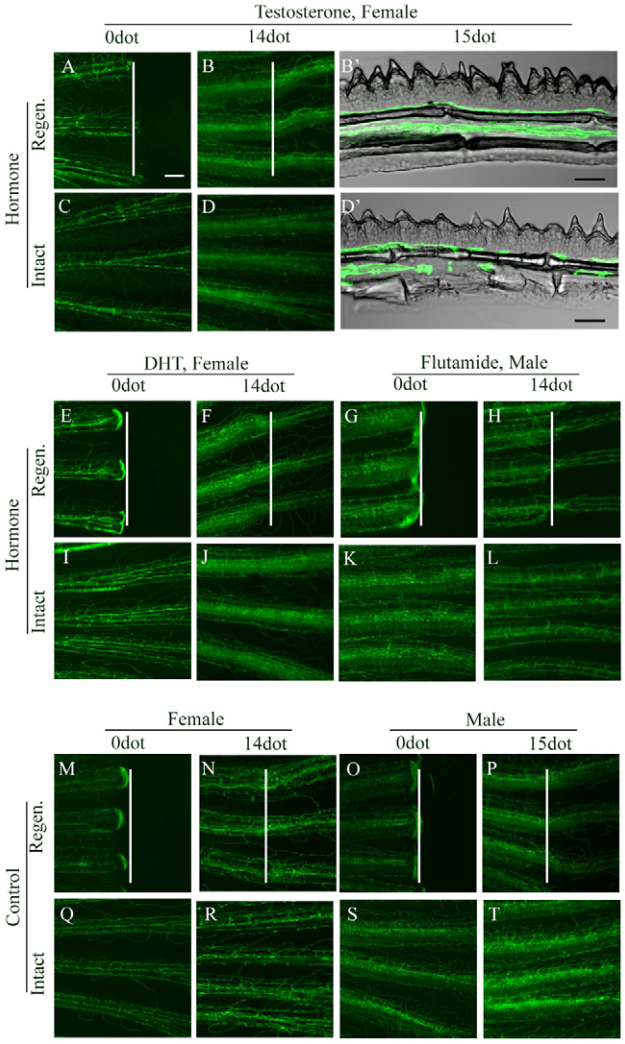

Fig. 8. Androgens induce neo-angiogenesis in intact and regenerating pectoral fins. (**A-F**,**I**,**J**) Intact and regenerating fins of T-treated (B,B′,D,D′) and DHT-treated (F,J) females (14 dot) develop vascularized BTs not observed at 0 dot (A,C,E,I). (**G**,**H**,**K**,**L**) Blood vessels of flutamide-treated (14 dot) male intact and regenerating fins acquire a female-like appearance. (**M-T**) Control untreated females (M,N,Q,R) and males (O,S,P,T). White lines indicate the amputation plane. Scale bars: 100 μm, except 50 μm in B′,D′.

T (1 μg/l) and PTK787 (500 nM), an inhibitor of vascular endothelial growth factor receptor tyrosine kinases that effectively inhibits regenerative angiogenesis in the zebrafish caudal fin (Wood et al., 2000; Bayliss et al., 2006). Immediately following pectoral fin amputation, PTK787 treatments were initiated. Females were treated for 2 days with PTK787 prior to T treatment to ensure that PTK787 had time to take effect. At 6 dpa, a single row of BTs is observed on PTK787/T-treated female intact fins and the stump of regenerating pectoral fins. No BTs are visible in the regenerate (Fig. 10Aa-Ca; supplementary material Fig. S9Aa,Ba). At 8 dpa, BTs on the intact fin have grown slightly and isolated BTs are observed on PTK787/T-treated fin regenerates (Fig. 10Ea,Fa; supplementary material Fig. S9Ca,Da). At 14 dpa, a small BT cluster has formed in the proximal PTK787/T-treated fin regenerate (Fig. 10Ga,Ha; supplementary material Fig. S9Ea). In contrast to PTK787/T-treated females, T-treated females (4 dot) quickly

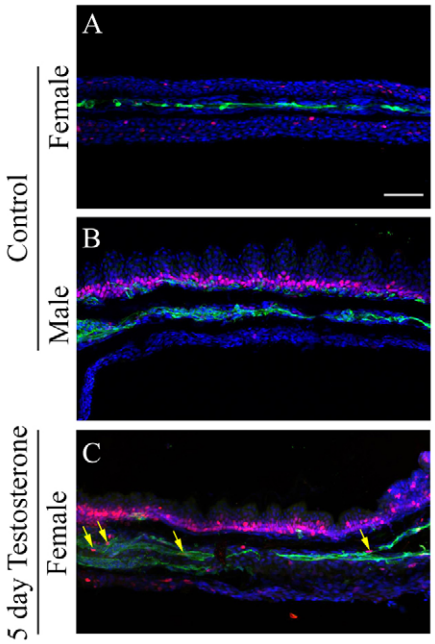

Fig. 9. Pectoral fin endothelial and epidermal cells proliferate upon testosterone treatment. Pcna (red) and DAPI (blue) staining of *Tg(fli1a:EGFP)* intact fin longitudinal sections. (**A**) Females display few proliferating cells. (**B**) Males present many proliferating cells deep in the epidermis. (**C**) T-treated females (5 dot) develop a proliferation profile similar to that of males in the epidermis. Endothelial cell proliferation is induced (arrows). Scale bar: 50 μm.

develop large BTs on intact and regenerating fins (Fig. 10Ab-Cb; supplementary material Fig. S9Ab,Bb). These clusters continue to grow until treatments are suspended 12 days later (Fig. 10Eb-Ib; supplementary material Fig. S9Cb-Fb). Finally, all PTK787 treatments, including PTK787/T treatments, efficiently inhibit neo-angiogenesis in the intact and regenerating pectoral fins, limiting the distance of tissue regeneration (Fig. 10Aa-Ja,Ac-Jc). Control females that received PTK787 alone or system water did not form BTs (Fig. 10Hc,d,Ic,d).

DISCUSSION

The investigation of large, sexually dimorphic BT clusters in intact and regenerating male pectoral fins outlines a mechanism by which these structures are formed, maintained and regenerated (Fig. 11). BT cluster regeneration occurs alongside a second wave of neo-angiogenesis, which forms the web-like blood vessel network of BT clusters. Androgen-induced BT formation occurs through ARs. Concomitantly, androgens induce angiogenesis to promote BT formation. Although the inhibition of neo-angiogenesis using PTK787 does not fully block androgen-induced BT formation, it is likely that blood vessels already present in the fin are sufficient for the growth of small BTs. We propose that the additional blood vessels in males and androgen-treated females are required to promote the formation of large BT clusters. However, we cannot exclude the possibility that BTs themselves play a role in the induction of angiogenesis. Finally, since BT formation is inhibited by E2-induced activation of estrogen receptors, we suggest that it is the balance between these two sex steroids that determines the presence or absence of BT clusters in zebrafish.

Initial comparisons between male and female zebrafish demonstrate that BTs in males are more prominent than in females. In males, sexually dimorphic BTs appear on all fins except the caudal fin, with the largest BT clusters occurring along the anterior pectoral fin rays. These BT clusters are present all year round, which is consistent with studies in *Oryzias latipes* (medaka) (Okada and Yamashita, 1944; Yamamoto and Egami, 1974). However, this observation contrasts with previous studies of BTs in other species such as *Carassius auratus* (goldfish), *Pimephales promelas* (fathead minnow), *Stenodus leucichthys* (freshwater whitefish) and *Osmeridae* (smelt), which have BTs that appear before the spawning season and gradually disappear over time (Smith, 1978; Berg, 1948; Wiley and Collette, 1970). These authors suggest that the presence of BTs depends on temperature, light and breeding season. We observed the persistent presence of BTs in zebrafish that were kept at a constant temperature of 28.5°C with a photoperiod of 14 hours of light and 10 hours of darkness (Westerfield, 1995). Since temperature, light cycles and nutrition are variable in the natural habitat of zebrafish (Spence et al., 2008), it is possible that wild zebrafish lose BTs when seasons change. Furthermore, wild zebrafish breed between April and August (Engeszer et al., 2007), whereas zebrafish in captivity mate all year round. The function of BTs in zebrafish is still unknown, but previous studies in other species have implicated these structures in the maintenance of body contact during spawning (Kortet et al., 2003; Wiley and Collette, 1970). If BTs are necessary for zebrafish mating, their constant presence on the dorsal surface of pectoral fins might be necessary for year-round breeding in laboratory facilities. However, the requirement for BTs during breeding in zebrafish has yet to be determined.

Despite the fact that pectoral fin BT clusters are consistently observed on laboratory zebrafish, the superficial keratin caps are regularly shed. The proliferation of cells in the deepest layers of the epidermis allows the continual production of new progenitors that differentiate and mature into keratin-producing cells and assures the efficient replacement of shedding keratin caps. To our knowledge, the mechanisms responsible for this renewal have not been described in any fish species.

Since BT clusters are large, it is suggested that blood vessels are required to efficiently form and maintain these structures over long periods of time. Initial examination of the male zebrafish pectoral fins revealed that BT clusters are highly vascularized. Analysis of BT regeneration in relation to blood vessel revascularization shows that BT formation correlates with a second wave of neo-angiogenesis in pectoral fin regenerates. This second wave is likely to derive from vessels set down in the first wave, which supports tissue regeneration. Thus, the delay in the first wave of blood vessel regeneration in males compared with females could be due to an added requirement of BT vascularization. Consequently, we suggest that the overall delay in male pectoral fin regeneration observed by Nachtrab et al. (Nachtrab et al., 2011) might result from BT cluster formation that limits the availability of factors (i.e. blood vessels) required for fin regeneration. Overall, these results indicate that BT cluster formation and growth may benefit from additional blood vessels, but this might be costly in terms of regeneration.

In other teleost species, the size, number and appearance of BTs correlate with circulating concentrations of androgens (Borg, 1994; Kortet et al., 2003). Furthermore, previous studies have shown that androgens induce whereas estrogens reduce the formation of BTs (Smith, 1974; Smith and Smith, 1986; Ramaswami and Hasler, 1955; Pawlowski et al., 2004). Here we show that androgens (T and DHT) and an aromatase inhibitor (fadrozole) induce the formation of BTs in both intact and regenerating pectoral fins of female zebrafish. Fadrozole can effectively decrease the serum levels of

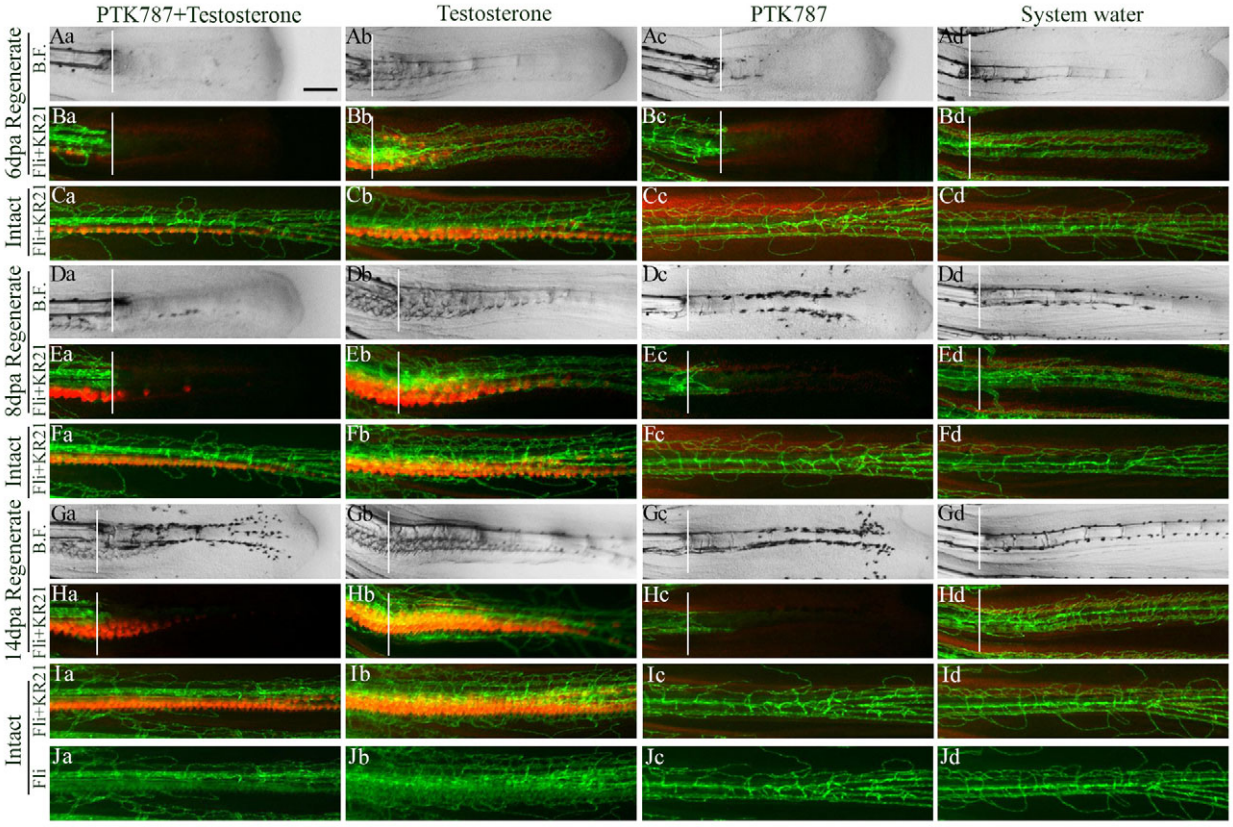

Fig. 10. Inhibition of neo-angiogenesis affects T-induced BT growth in the female pectoral fin. (**Aa-Ja**) A single row of BTs on the intact fin and stump after 6-14 dot with PTK787 and 4-12 dot with T. Small BTs appear in the fin regenerate after 8 dot with PTK787 and 6 dot with T (Da,Ea). A small BT cluster appears on the regenerate after 14 dot with PTK787 and 12 dot with T (Ga,Ha). (**Ab-Jb**) After 4 dot with T, BTs develop on the regenerate, stump and intact fin and continue to grow from 6-12 dot. (Jb) After 12 dot, the blood vessel network of T-treated females is similar to that of males. (**Ac-Jc**) PTK787-treated females do not possess BTs. (Aa-Ja,Ac-Jc) Neo-angiogenesis and regenerative outgrowth are inhibited in all PTK787-treated females. (**Ad-Jd**) System water controls regenerate normally and do not grow BTs. B.F., brightfield. Fli, *fli1a:EGFP* (green). KR21 (red) outlines the BTs. White vertical lines indicate the amputation plane. Scale bar: 100 μm.

E2, leading to differentially expressed estrogen-responsive genes and impaired ovarian development in female goldfish (Zhang et al., 2009). Similarly, fadrozole reduces the serum levels of E2 in female fathead minnows, whereas androgen serum levels remain stable (Ankley et al., 2002). Since E2 inhibits BT formation in the fin regenerate and reduces the appearance of BTs in intact fins, the presence of BTs in fadrozole-treated female zebrafish is likely to be the result of an absence of E2 inhibition, not of an increase in androgens. Although Ramaswami and Hasler (Ramaswami and Hasler, 1955) suggest that E2 does not prevent BT formation in developing male *Hyborhynchus*, it is possible that the effects of E2 treatments in the Ramaswami study are not sufficient to overcome androgen-induced BT formation in developing males. In support of our results, Miles-Richardson et al. (Miles-Richardson et al., 1999) found a significant reduction in BT size in male fathead minnows treated with E2.

Using flutamide, an AR antagonist (Peets et al., 1974), BT formation was inhibited and reduced in the fin regenerate and intact fins, respectively. A few small BTs were observed in the proximal regenerate in a single male after 21 dot. Zebrafish treated with

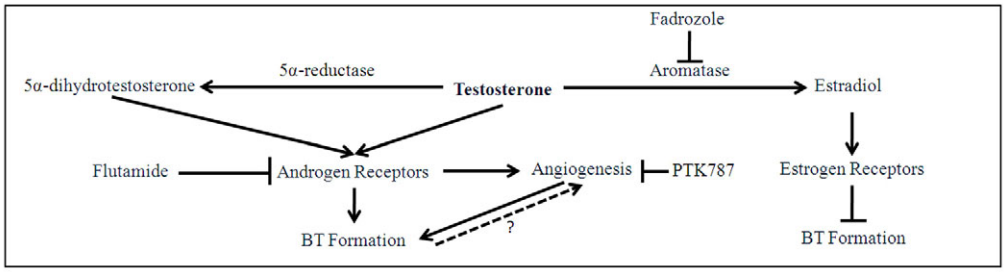

Fig. 11. A model for BT formation. BTs are induced by androgens 5α-dihydrotestosterone (DHT) and testosterone (T) through androgen receptors (ARs). Receptor blocking via flutamide inhibits BT formation. Androgen-induced neo-angiogenesis through the AR is necessary for BT formation, as PTK787 inhibits BT formation. Inhibition of T conversion to 17β-estradiol (E2) by fadrozole induces BT formation. Estradiol inhibits BT formation through the activation of estrogen receptors. BT formation itself might induce angiogenesis through an as yet unknown mechanism (dashed arrow).

vinclozolin, another competitive AR inhibitor, upregulate *ar* transcription as a compensatory mechanism (Smolinsky et al., 2010). Therefore, the inability of flutamide to completely inhibit BT regeneration in all males might result from an increase in *ar* expression in response to treatments. Initial qRT-PCR experiments indicate that *ar* mRNA is not differentially expressed between untreated male and female intact pectoral fins, suggesting that *ar* expression levels do not naturally influence BT formation. We propose that endogenous levels of circulating androgens and estrogens may determine the presence or absence of BTs.

Androgen-treated females develop a male-like blood vessel network surrounding newly formed BTs. These new vessels are likely to originate from the proliferation of endothelial cells in the central vessels between the hemirays. Overall, these data are consistent with recent *in vitro* and *in vivo* mouse studies demonstrating the sex-specific pro-angiogenic effects of androgens in males (Sieveking et al., 2010). More specifically, in cell culture and in mice, androgens increase the expression of genes (*Vegf* and *Vcam1*) and promote processes related to angiogenesis (Death et al., 2004; Ruohola et al., 1999; Sieveking et al., 2010). Our data indicate that females treated with androgens experience the same pro-angiogenic effects, resulting in male-like vascularized BTs. However, we cannot exclude the possibility that the increase in tissue thickness associated with BT growth might also play a role in the induction of angiogenesis. Furthermore, the absence of a second wave of blood vessel formation in flutamide-treated zebrafish is consistent with data that indicate that endothelial cell proliferation induced via androgens is reduced through the effects of flutamide (Williams et al., 2004). The absence of BTs in flutamide-treated males is likely to be due to the general inhibition of the AR and/or the absence of a second wave of blood vessel growth. Overall, the presence and absence of the vascularized BT clusters upon androgen and flutamide treatments, respectively, suggest that additional vascularization in untreated males and androgen-treated females is required for BT formation.

The angiogenesis inhibitor PTK787 (Wood et al., 2000) successfully inhibited neo-angiogenesis and reduced BT growth in intact and regenerating pectoral fins of T-treated females. Previous data indicate that regeneration can occur up to 1 mm from an intact blood supply (Bayliss et al., 2006). Therefore, although treatment with both PTK787 and T did not completely inhibit BT formation, we suggest that the blood vessels already present in the intact fin and stump of females are sufficient to allow the formation of undersized BT clusters. However, when exogenous androgens are provided, the rapid growth of large BT clusters in intact and regenerate fins depends on the induction of a blood vessel network similar to that observed naturally in males.

Acknowledgements
This work benefited from the assistance of Natasha Bougie, Louai Naddaf and Thinh N'Guyen. We thank Vishal Saxena for help with fish husbandry and Dr Marc Ekker for discussion and critical reading of the manuscript.

Funding
Funding from the Canadian Institute of Health Research (M.-A.A.) [89824] and Natural Sciences and Engineering Research Council (V.L.T.) [203152-2011] is acknowledged with appreciation. Deposited in PMC for immediate release.

Competing interests statement
The authors declare no competing financial interests.

Author contributions
S.C.M. designed and performed the majority of the experiments. Z.T.X. and J.Z. performed important initial studies in support of this study. J.Z. edited the final images. C.T. and V.K. generated the *Et(kr4:GAP-KillerRed)sqKR21* transgenic line. V.L.T. assisted in the design of the hormone treatments and provided critical reading of the manuscript. M.-A.A. conceived the project and directed the study. S.C.M. and M.-A.A. wrote the manuscript.

Supplementary material
Supplementary material available online at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.095992/-/DC1

References

Ahnelt, H. and Keckeis, H. (1994). Breeding tubercles and spawning behaviour in chondrostomanasus (Teleostei: Cyprinidea): a correlation? *Ichthyol. Explor. Freshwat.* **5**, 321-330.
Ankley, G. T., Kahl, M. D., Jensen, K. M., Hornung, M. W., Korte, J. J., Makynen, E. A. and Leino, R. L. (2002). Evaluation of the aromatase inhibitor fadrozole in a short-term reproduction assay with the fathead minnow (Pimephales promelas). *Toxicol. Sci.* **67**, 121-130.
Arai, R. (1967). Androgenic effects of 11-ketotestosterone on some sexual characteristics in the teleost, Oryzias latipes. *Annot. Zool. Japon.* **40**, 1-5.
Bayliss, P. E., Bellavance, K. L., Whitehead, G. G., Abrams, J. M., Aegerter, S., Robbins, H. S., Cowan, D. B., Keating, M. T., O'Reilly, T., Wood, J. M. et al. (2006). Chemical modulation of receptor signaling inhibits regenerative angiogenesis in adult zebrafish. *Nat. Chem. Biol.* **2**, 265-273.
Berg, L. S. (1948). Freshwater fishes of the USSR and adjacent countries. *Zool. Inst. Akad. Nauk. SSSR* **3**, 1381.
Borg, B. (1994). Androgens in teleost fishes. *Comp. Biochem. Physiol.* **109C**, 219-245.
Bulina, M. E., Lukyanov, K. A., Britanova, O. V., Onichtchouk, D., Lukyanov, S. and Chudakov, D. M. (2006). Chromophore-assisted light inactivation (CALI) using the phototoxic fluorescent protein KillerRed. *Nat. Protoc.* **1**, 947-953.
Cason, J. E. (1950). A rapid one-step Mallory-Heidenhain stain for connective tissue. *Stain Technol.* **25**, 225-226.
Chen, X. and Arratia, G. (1996). Breeding tubercles of phoxinus (teleostei: cyprinidae): morphology, distribution, and phylogenetic implications. *J. Morphol.* **228**, 127-144.
Death, A. K., McGrath, K. C., Sader, M. A., Nakhla, S., Jessup, W., Handelsman, D. J. and Celermajer, D. S. (2004). Dihydrotestosterone promotes vascular cell adhesion molecule-1 expression in male human endothelial cells via a nuclear factor-kappaB-dependent pathway. *Endocrinology* **145**, 1889-1897.
Egami, N. (1954). Effects of hormonic steroids on the formation of male characteristics in females of the fish, Oryzias latipes, kept in water containing testosterone propionate. *Annot. Zool. Japon.* **27**, 122-127.
Engeszer, R. E., Patterson, L. B., Rao, A. A. and Parichy, D. M. (2007). Zebrafish in the wild: a review of natural history and new notes from the field. *Zebrafish* **4**, 21-40.
Goldstein, J. L. and Wilson, J. D. (1972). Studies on the pathogenesis of the pseudohermaphroditism in the mouse with testicular feminization. *J. Clin. Invest.* **51**, 1647-1658.
Hillier, S. G., van den Boogaard, A. M., Reichert, L. E., Jr and van Hall, E. V. (1980). Alterations in granulosa cell aromatase activity accompanying preovulatory follicular development in the rat ovary with evidence that 5alpha-reduced C19 steroids inhibit the aromatase reaction in vitro. *J. Endocrinol.* **84**, 409-419.
Huang, C. C., Lawson, N. D., Weinstein, B. M. and Johnson, S. L. (2003). reg6 is required for branching morphogenesis during blood vessel regeneration in zebrafish caudal fins. *Dev. Biol.* **264**, 263-274.
Kortet, R., Vainikka, A., Rantala, M. J., Jokinen, I. and Taskinen, J. (2003). Sexual ornamentation, androgens and papillomatosis in male roach (Rutilus rutilus). *Evol. Ecol. Res.* **5**, 411-419.
Kortet, R., Taskinen, J., Vainikka, A. and Ylönen, H. (2004). Breeding tubercles, papillomatosis and dominance behaviour of male roach (rutilus rutilus) during the spawning period. *Ethology* **110**, 591-601.
Korzh, V., Teh, C., Kondrychyn, I., Chudakov, D. M. and Lukyanov, S. (2011). Visualizing compound transgenic zebrafish in development: a tale of green fluorescent protein and KillerRed. *Zebrafish* **8**, 23-29.
Lawson, N. D. and Weinstein, B. M. (2002). In vivo imaging of embryonic vascular development using transgenic zebrafish. *Dev. Biol.* **248**, 307-318.
Menuet, A., Le Page, Y., Torres, O., Kern, L., Kah, O. and Pakdel, F. (2004). Analysis of the estrogen regulation of the zebrafish estrogen receptor (ER) reveals distinct effects of ERalpha, ERbeta1 and ERbeta2. *J. Mol. Endocrinol.* **32**, 975-986.
Miles-Richardson, S.R., Kramer, V.J., Fitzgerald S. D., Render J. A., Yamini., B., Barbee, S. J. and Giesy, J. P. (1999). Effects of waterborne exposure of 17 b-estradiol on secondary sex characteristics and gonads of fathead minnows (Pimephales promelas). *Aquat. Toxicol.* **47**, 129-145.
Nachtrab, G., Czerwinski, M. and Poss, K. D. (2011). Sexually dimorphic fin regeneration in zebrafish controlled by androgen/GSK3 signaling. *Curr. Biol.* **21**, 1912-1917.

Nagata, Y. (1934). Gonadectomy in Oryzias latipes. *Zool. Mag.* **46**, 293-294.
Ng, M. K., Quinn, C. M., McCrohon, J. A., Nakhla, S., Jessup, W., Handelsman, D. J., Celermajer, D. S. and Death, A. K. (2003). Androgens up-regulate atherosclerosis-related genes in macrophages from males but not females: molecular insights into gender differences in atherosclerosis. *J. Am. Coll. Cardiol.* **42**, 1306-1313.
Okada, Y. K. and Yamashita, H. (1944). Experimental investigation of the manifestation of secondary sexual characters in fish, using the medaka, Oryzias latipes. *J. Fac. Sci. Imp. Univ. Tokyo* **6**, 383-437.
Pawlowski, S., van Aerle, R., Tyler, C. R. and Braunbeck, T. (2004). Effects of 17alpha-ethinylestradiol in a fathead minnow (Pimephales promelas) gonadal recrudescence assay. *Ecotoxicol. Environ. Saf.* **57**, 330-345.
Peets, E. A., Henson, M. F. and Neri, R. (1974). On the mechanism of the anti-androgenic action of flutamide (alpha-alpha-alpha-trifluoro-2-methyl-4′-nitro-m-propionotoluidide) in the rat. *Endocrinology* **94**, 532-540.
Ramaswami, L. S. and Hasler, A. D. (1955). Hormones and secondary sex characters in the minnow, Hyborhynchus. *Physiol. Zool.* **28**, 62-68.
Ruohola, J. K., Valve, E. M., Karkkainen, M. J., Joukov, V., Alitalo, K. and Härkönen, P. L. (1999). Vascular endothelial growth factors are differentially regulated by steroid hormones and antiestrogens in breast cancer cells. *Mol. Cell. Endocrinol.* **149**, 29-40.
Serebrovskaya, E. O., Edelweiss, E. F., Stremovskiy, O. A., Lukyanov, K. A., Chudakov, D. M. and Deyev, S. M. (2009). Targeting cancer cells by using an antireceptor antibody-photosensitizer fusion protein. *Proc. Natl. Acad. Sci. USA* **106**, 9221-9225.
Sieveking, D. P., Chow, R. W. and Ng, M. K. (2010). Androgens, angiogenesis and cardiovascular regeneration. *Curr. Opin. Endocrinol. Diabetes Obes.* **17**, 277-283.
Smith, R. J. (1974). Effects of 17 alpha-methyltestosterone on the dorsal pad and tubercles of fathead minnows (Pimephales promelas). *Can. J. Zool.* **52**, 1031-1038.
Smith, R. J. F. (1978). Seasonal changes in the histology of the gonads and dorsal skin of the fathead minnow, Pimephales promelas. *Can. J. Zool.* **56**, 2103-2109.
Smith, J. D., Smith, R. J. F., (1986). Effect of hormone treatments on alarm substance cell counts in the pearl dace, Semotilus margarita. *Can. J. Zool.* **64**, 291-295.
Smith, A., Zhang, J., Guay, D., Quint, E., Johnson, A. and Akimenko, M. A. (2008). Gene expression analysis on sections of zebrafish regenerating fins reveals limitations in the whole-mount in situ hybridization method. *Dev. Dyn.* **237**, 417-425.
Smolinsky, A. N., Doughman, J. M., Kratzke, L. T. and Lassiter, C. S. (2010). Zebrafish (Danio rerio) androgen receptor: sequence homology and up-regulation by the fungicide vinclozolin. *Comp. Biochem. Physiol. C Toxicol. Pharmacol.* **151**, 161-166.
Spence, R., Gerlach, G., Lawrence, C. and Smith, C. (2008). The behaviour and ecology of the zebrafish, Danio rerio. *Biol. Rev. Camb. Philos. Soc.* **83**, 13-34.
Teh, C., Chudakov, D. M., Poon, K. L., Mamedov, I. Z., Sek, J. Y., Shidlovsky, K., Lukyanov, S. and Korzh, V. (2010). Optogenetic in vivo cell manipulation in KillerRed-expressing zebrafish transgenics. *BMC Dev. Biol.* **10**, 110-213X-10-110.
Westerfield, M. (1995). *The Zebrafish Book: A Guide for the Laboratory Use of Zebrafish (Brachydanio rerio)*. Eugene, OR: University of Oregon Press.
Westerfield, M. (2000). *The Zebrafish Book: A Guide for the Laboratory Use of Zebrafish (Danio Rerio)*. Eugene, OR: University of Oregon Press.
Wiley, M. L. and Collette, B. B. (1970). Breeding tubercles and contact organs in fishes: their occurrence, structure, and significance. *Bull. Am. Mus. Nat. Hist.* **143**, 147-216.
Williams, M. R., Dawood, T., Ling, S., Dai, A., Lew, R., Myles, K., Funder, J. W., Sudhir, K. and Komesaroff, P. A. (2004). Dehydroepiandrosterone increases endothelial cell proliferation in vitro and improves endothelial function in vivo by mechanisms independent of androgen and estrogen receptors. *J. Clin. Endocrinol. Metab.* **89**, 4708-4715.
Wood, J. M., Bold, G., Buchdunger, E., Cozens, R., Ferrari, S., Frei, J., Hofmann, F., Mestan, J., Mett, H., O'Reilly, T. et al. (2000). PTK787/ZK 222584, a novel and potent inhibitor of vascular endothelial growth factor receptor tyrosine kinases, impairs vascular endothelial growth factor-induced responses and tumor growth after oral administration. *Cancer Res.* **60**, 2178-2189.
Yamamoto, M. and Egami, N. (1974). Fine structure of the surface of the anal fin and the processes on its fin rays of male Oryzias latipes. *Copeia* **1974**, 262-265.
Zhang, D., Popesku, J. T., Martyniuk, C. J., Xiong, H., Duarte-Guterman, P., Yao, L., Xia, X. and Trudeau, V. L. (2009). Profiling neuroendocrine gene expression changes following fadrozole-induced estrogen decline in the female goldfish. *Physiol. Genomics* **38**, 351-361.