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Simplet controls cell proliferation and gene transcription during zebrafish caudal fin regeneration

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ABSTRACT

Two hallmarks of vertebrate epimorphic regeneration are a significant increase in the proliferation of normally quiescent cells and a re-activation of genes that are active during embryonic development. It is unclear what the molecular determinants are that regulate these events and how they are coordinated. Zebrafish have the ability to regenerate several compound structures by regulating cell proliferation and gene transcription. We report that *fam53b/simplet* (*smp*) regulates both cell proliferation and the transcription of specific genes. In situ hybridization and quantitative RT-PCR experiments showed that amputation of zebrafish hearts and fins resulted in strong up-regulation of the *smp* gene. In regenerating adult fin, *smp* expression remained strong in the distal mesenchyme which later expanded to the basal layers of the distal epidermis and distal tip epithelium. Morpholino knockdown of *smp* reduced regenerative outgrowth by decreasing cell proliferation as measured by BrdU incorporation and histone H3 phosphorylation. In addition, *smp* knockdown increased the expression of *msxb*, *msxc*, and *shh*, as well as the later formation of ectopic bone. Taken together, these data indicate a requirement for *smp* in fin regeneration through control of cell proliferation, the regulation of specific genes and proper bone patterning.

Introduction

Several species of urodeles and fish have the ability to respond to amputation injury by epimorphic regeneration (Brockes, 1997; Slack, 2003), and they do so by forming a blastema, a proliferative mass of cells that underlies a thickened epidermis (wound epithelium) (Stocum, 1984). Studies in these vertebrates show that structural regeneration requires changes in the behavior of the cells at the site of injury—the most distinct of which involves extensive proliferation of normally quiescent cells and the activation of gene expression reminiscent of developmental transcriptional programs (Akimenko et al., 2003; Poss et al., 2003; Stoick-Cooper et al., 2007; Tanaka, 2003). Understanding how tissues are reconstructed requires defining the molecular determinants that coordinate these events.

Zebrafish respond to amputation injury by completely regenerating several lost structures, including the heart and fin (Akimenko et al., 2003; Nakatani et al., 2007; Poss et al., 2003). The regeneration of compound structures is likely controlled in part by genes that are activated after amputation injury; thus, the comparison of transcriptional profiles between regenerating and non-regenerating tissues can be helpful to identify genes involved in this process. Microarray analyses using zebrafish have shown the up-regulation of several genes as part of the regeneration response (Katogi et al., 2004; Lien et al., 2006; Nishidate et al., 2007; Schebesta et al., 2006; Veldman et al., 2007), and these analyses are providing targets with which we can use zebrafish as a tool to dissect how organs and appendages regenerate. Likewise, we performed microarray profiling experiment for genes expressed in regeneration process of the heart. From our analysis, we found several genes, one of which was *fam53b/simplet* (*smp*).

The gene *smp* has been associated with the regulation of cell proliferation during medaka embryogenesis (Thermes et al., 2006). Its expression was detected in rapidly proliferating cells—in all blastomeres during the first 5 cell cleavages and by the seventh cleavage only in the central blastomeres. Subsequently, it was detected in developing somites, at the midbrain–hindbrain boundary and optic tectum, where *smp* message colocalized with the proliferating cell nuclear antigen (PCNA). These data correlated *smp* gene activity with populations of proliferating cells and indicate that *smp* functions during cell proliferation.

We show that *smp* is necessary for the regeneration process. Expression of *smp* was turned on in the zebrafish heart and fin blastema during early stages of the regeneration process. Knock-down of *smp* in regenerating fins yielded a reduction in the outgrowth of the regenerating tissue. BrdU incorporation and histone H3 phosphorylation were reduced, linking the decreased outgrowth to a reduction in

Abbreviations: BrdU, bromodeoxyuridine; dpf, days post fertilization; dpa, days post amputation; *smp*, *simplet*; Fam53b, Family with sequence similarity 53-member b; *fgf20a*, *fibroblast growth factor 20a*; H3P, histone H3 phosphorylation; *msx*, *msh-like homeobox gene*; *shh*, *sonic hedgehog*; dpa, days post amputation.

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the number of proliferating cells. Unexpectedly, in situ hybridization experiments showed expanded expression of *msxb* and *shh* and the deposition of ectopic bone in *smp* morphant fins. Taken together, these data suggest roles for *smp* in the regulation of cell proliferation, gene transcription and bone formation during zebrafish organ and appendage regeneration.

Materials and methods

Fish maintenance and amputations

Fish were maintained at 28 °C as described (Brand et al., 2002). Caudal fin amputations and tissue collection were performed as described (Johnson and Weston, 1995). Five–ten percent of the ventricular apex of the heart was resected as described (Poss et al., 2002b). For the wound healing assay, incisions between 200 and 700 μm in length were made in the interray tissue. All procedures with live animals were in accordance with the Regierungspräsidium Tübingen, Department of Bioassays.

Microarray

Amputated (72 hpa) and unamputated heart samples were collected and snap-frozen in liquid N_2. Trizol reagent (Invitrogen, Carlsbad, CA, USA) was used for extraction of total RNA. Synthesis and labeling of antisense RNA were performed as recommended by array manufacturer using kits from Invitrogen (Carlsbad, CA, USA) for double-stranded cDNA synthesis, from Enzo Life Sciences (Farmingdale, NY, USA) for transcription and labeling of antisense RNA and from Affymetrix (Santa Clara, CA, USA) for probe purification and hybridization controls. Affymetrix Zebrafish GeneChips (15,617 genes) were hybridized. Computational analysis was performed with statistical language R (Team, 2007) and with packages provided by Bioconductor project (Gentleman et al., 2004). Background correction, normalization and probe set summarization were performed using multi-array algorithm with background adjustment (gc-rma) (Irizarry et al., 2003). Expression values of the replicates from the same time point were averaged and fold changes between the different time points and the unamputated control were calculated. To identify genes that are consistently upregulated at each time point, rank products were calculated (Breitling et al., 2004). Genes were selected by controlling percentage of false-positives ≤ 0.05. The raw and normalized expression data are stored at ArrayExpress (http://www.ebi.ac.uk/arrayexpress) accession number E-MEXP-1239.

RNA in situ hybridization on whole mounts and cryosections

DIG-labeled RNA probes for *smp*, *msxb* and *mmp9* were prepared from 2 dpa caudal fin total cDNA (Supplementary Table 1 for primers). The fragments were subcloned into pCRII-TOPO-TA cloning vector (Invitrogen). DIG-labeled probes were synthesized using T7 and Sp6 RNA polymerases (Roche). *shh* and *msxc* probes were kindly provided (Akimenko and Ekker, 1995). Tissue preparation and in situ hybridizations were performed as described (Barthel and Raymond, 1990; Jowett and Lettice, 1994; Xu and Wilkinson, 1992). Digital images were captured using a DIC microscope (Axiocam, Zeiss).

Morpholino-mediated knock down and morphometric analyses

Morpholino transfections and morphometric analyses were performed at 2 dpa as described (Thummel et al., 2006). Caudal fins were amputated and allowed to regenerate for 2 days at 28 °C. One lobe of each amputated fin was injected with 10 nl of morpholino (7.5 mM) and electroporated using tweezers with platinum electrodes (CUY615, Protech International) and a square pulse stimulator (SD9, Grass Technologies, RI, USA). The lateral area (A_0) of each lobe distal to the amputation margin was immediately measured using morphometrics software (IM500 v5.222, Leica). Fins were allowed to regenerate for an additional 24 h and the area of each lobe distal to the amputation plane was measured again (A_1). The difference (A_1–A_0) was an indication of the extent of regenerative outgrowth after morpholino transfection. Percent outgrowth was calculated by dividing the area of the transfected lobe by the area of the untransfected lobe and multiplying by 100 (Thummel et al., 2006). Results are graphed as percent regenerative outgrowth by taking the untransfected lobe as reference (100%). Two sets of antisense and mismatch morpholino oligonucleotides (Gene Tools, OR, USA) were used against *smp*: 5′ UTR morpholino; 5′-GCAACACACATCTTTGCCACGGTCC-3`; 5′ UTR mismatch control; 5′-GCAAgACAgATgTTTcCCACGcTCC-3′; Exon3–Intron3 splice antisense morpholino; 5′-GAATATCTGCACTTACCCATGATTC-3′, Exon3–Intron3 splice mismatch morpholino; 5′-GAtTATgTGCAgTTACgCATcATTC-3′). All morpholinos had a 3′ fluorescein tag. Images were taken using a DFC300-FX CCD camera on an MZFL III stereoscope (Leica). Measurements were performed using morphometrics software (IM500 v5.222, Leica). Statistical significance was analyzed using Student's *t*-test. Fifteen fins were used for each set of morpholinos.

Histology and morphometric analyses

Morphant fins were embedded in paraplast media (Sigma) and sectioned at 7 μm using a microtome (RM2165, Leica). Sections were stained with Hematoxylin and Eosin (Roth) as described (Lyon, 1998), and mounted in Permount (Fischer Chemicals). Samples were visualized under an AxioImager (Zeiss) and cells were counted on individual sections. Nine fins were used for each group. Ninety-four paraffin sections were counted for statistical analyses. Statistical significance was determined with Student's *t*-test. Alizarin red (Sigma) staining of bony rays was performed as described (Sire et al., 1997). Stained fins were embedded in cryoprotective embedding medium (TissueTek, EMS) and sectioned (14 μm) using a cryostat microtome (Leica).

BrdU incorporation and immunohistochemistry

Fish were injected intraperitoneally with 2.5 mg/ml BrdU 30 min before harvesting. The 30 min pulse labels only rapidly proliferating cells. Whole mount BrdU and Zns-5 immunohistochemistry stainings on 3 dpa caudal fin regenerates were performed as described (Poss et al., 2002b) using rat monoclonal anti-BrdU (1:50, Chemicon International Inc.) and mouse monoclonal anti-Zns-5 (1:50, Zebrafish Information and Resource Center) as primary antibodies and Goat anti-rabbit Cy3-coupled (Dianova, 1:500), Rabbit anti-rat Cy3-coupled (Dianova, 1:500) and Goat anti-Mouse Cy-3 coupled (1:500, Dianova) as secondary antibodies. H3P cell-counting was performed as described previously (Poss et al., 2002b). Stained tissues were either pictured as whole mounts or as cryosections under laser-scanning confocal microscope (Zeiss). At least 6 fins were used for each staining.

BrdU and Zns-5 double immunostaining on cryosections were performed sequentially. 12 μm-thick cryosections were fixed in 4% PFA/PBS for 15 min, washed with PBTx (PBS+0.1% Triton X-100), and DNA was denatured with 2N HCl for 30 min. Sections were washed with PBTx and blocked with PBTx/5% BSA for 1 h at 37 °C. The first primary antibody (mouse anti-Zns-5, 1:50) was applied overnight at 4 °C. The corresponding secondary antibody (Goat anti-mouse coupled to Alexa-488 1:100, Invitrogen) was applied (1 h at 37 °C) after PBTx wash steps. Samples were re-fixed with 4% PFA/PBS (15 min at room temperature) and stained with the second primary antibody (Rat anti-BrdU, 1:50) for 3 h at 37 °C. Tissues were washed with PBTx, and second secondary antibody (Goat anti-rat coupled to Cy3, 1:200) was applied for 2 h at room temperature. Samples were fixed in 4% PFA/PBS, counterstained with DAPI (Sigma) and analyzed under a structured illumination fluorescence microscope (ApoTome, Zeiss).