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Connexin43 (*GJA1*) is required in the population of dividing cells during fin regeneration

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ABSTRACT

In zebrafish, mutations in the gap junction gene *connexin43* lead to short bony fin ray segments that give rise to the *short fin* phenotype. The *sof*^{b123} mutant exhibits fins that are half the length of wild-type fins and have reduced levels of *cx43* mRNA. We find that *sof*^{b123} regenerating fins exhibit reduced levels of cell proliferation. Interestingly, the number of dividing cells per unit length of fin growth is similar between wild-type and mutant fins, suggesting that the number of cells that enter the cell cycle is specifically affected in *sof*^{b123}. Expression of *cx43* is identified in mitotic cells, which further suggests that Cx43 may contribute to establishing or maintaining the population of dividing cells. Indeed, missense alleles exhibiting high or low levels of gap junctional communication reveal a correlation between defects in direct cell–cell communication, cell proliferation, and segment length. Finally, targeted gene knockdown of *cx43* in adult regenerating fins recapitulates the *sof*^{b123} phenotype, revealing that the loss of Cx43 is sufficient to reduce both cell proliferation and segment length. We hypothesize that the level of gap junctional intercellular communication among dividing cells regulates the level of cell proliferation and ultimately regulates bone growth.

Introduction

The underlying mechanisms regulating the size and shape of bony structures are largely unknown. We utilize growth of the zebrafish fin to reveal such mechanisms. The fin grows throughout the lifetime of the fish, providing extended opportunities to monitor growth. The zebrafish fin is comprised of skeletal elements called fin rays (or lepidotrichia) which grow by the addition of new bony segments to their distal ends (Goss and Stagg, 1957; Haas, 1962). Each ray is made of two concave hemirays surrounding undifferentiated mesenchymal cells, vasculature, and nerves (Santamaria et al., 1992). Growth of the bony segments occurs in the absence of a cartilaginous precursor, by the process of intramembraneous ossification (Landis and Geraudie, 1990).

Fins have the capacity for regeneration, enabling more precisely timed experiments and evaluation of a more rapid growth process. Following wound healing (12–24 h post-amputation, hpa), a specialized structure called a blastema forms in the distal mesenchyme (Poss et al., 2000). The blastema is required for outgrowth and replacement of lost tissue. Organization of the blastema into two morphologically indistinct compartments occurs by 48–72 hpa (Nechiporuk and Keating, 2002). The distal-most blastema (distal most 10–50 μm) contains non-proliferative, *msxb* positive cells that may provide directional growth information. Rapidly dividing cells are located in the proximal blastema or proliferation zone, 100–200 μm proximal to the distal-most blastema. During outgrowth (72 hpa and beyond), cells migrate proximally and laterally (Nechiporuk and Keating, 2002; Poleo et al., 2001) prior to differentiation outside of the proliferation zone.

Mechanisms regulating the growth and size of fin ray segments remain unknown. Fin length mutants with defects in segment length will provide insights into the molecular and cellular requirements for bone growth. To date, the *short fin*^{b123} (*sof*^{b123}) mutant is the only fin mutant affecting segment length (Iovine and Johnson, 2000). The *sof*^{b123} phenotype was recently found to be caused by mutations in the *connexin43* (*cx43*) gene (Iovine et al., 2005). Three non-complementing ENU-induced missense mutations were identified in the coding region of *cx43* (*sof*^{j7e1} codes for Cx43-F30V, *sof*^{j7e2} codes for Cx43-P191S, *sof*^{j7e3} codes for Cx43-F209I). The original allele, *sof*^{b123}, lacks mutations in the coding sequence, although it exhibits reduced *cx43* mRNA levels. All four alleles are recessive adult viable mutations and appear to be hypomorphs (Iovine et al., 2005). The Cx43-F30V and Cx43-P191S alleles behave similarly to *sof*^{b123}, causing segment length and fin length to be the most severely affected. In contrast, the Cx43-F209I allele is relatively mild, resulting in segment length and fin length more similar to wild-type animals (but measurably smaller, Iovine et al., 2005).

Connexins are the subunits of gap junctions, proteinaceous channels required for direct cell–cell communication among adjacent cells. A single connexin is a four-pass transmembrane protein containing two extracellular domains and one intracellular loop. Six

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connexin proteins make a connexon and two connexons (one from each neighboring cell) form a gap junction channel. Gap junctional intracellular communication (GJIC) occurs by the exchange of small molecules (<1000 Da) via these channels. GJIC is important for development, homeostasis, and tissue function as evidenced by the association of mutations in connexin genes with human disease phenotypes (reviewed in Laird, 2006). For example, (primarily) missense mutations in human *CX43* cause oculodentodigital dysplasia (ODDD), a syndrome characterized by malformations of the craniofacial and distal limb skeleton among other pleiotropic phenotypes (Paznekas et al., 2003). It is not immediately clear how mutations in connexin genes result in defects in bone growth and morphology. Correlations between particular missense mutations and disease phenotypes are not apparent (Paznekas et al., 2003), nor is there a correlation between channel activity of missense alleles and the severity or type of symptoms (Roscoe et al., 2005; Seki et al., 2004; Shibayama et al., 2005). The zebrafish *sof* mutant may provide opportunities to evaluate Cx43 function with respect to a single, relatively simple structure.

The correlation between the zebrafish *cx43* mutant fin phenotypes in vivo and the defects of gap junctional coupling in vitro suggests the possibility that the level of GJIC via Cx43 gap junctions contributes to bone growth (Hoptak-Solga et al., 2007). The goal of this study is to identify the underlying cellular defect in the *sof* mutants. Previous studies revealed that *cx43* is expressed both in the distal mesenchymal compartment of growing fin rays (i.e. in the same compartment as dividing cells) and more proximally in differentiated osteoblasts flanking joints (Iovine et al., 2005). Here we examine cell proliferation in wild-type and *sof* regenerating fins. We find that the number of dividing cells is reduced in all four *sof* alleles and that *cx43* is expressed in mitotic cells, suggesting a cell autonomous role for *cx43* in proliferating cells. Furthermore, we find that targeted gene knockdown of *cx43* recapitulates both the segment length and cell proliferation defects of *sof* mutants, indicating that reduced Cx43 function is sufficient to cause both phenotypes. We suggest that GJIC via Cx43 gap junctions coordinates cell proliferation with joint formation to regulate normal segment growth.

Materials and methods

Fish maintenance

Both wild-type and *sof*^{b123} stocks were from the C32 strain (Rawls et al., 2003) and were kept at a constant 25 °C. Fish were exposed to a 14 light:10 dark photoperiod (Westerfield, 1993).

Immunoblots and Cx43 antibody

The final 16 amino acids of zebrafish Cx43 was chosen as the antigen in conjunction with Quality Controlled Biochemicals (www.qcb.com). QCB completed peptide synthesis, immunization, rabbit maintenance, and affinity purification of bleeds.

Escherichia coli lysates expressing the GST–Cx43CT fusion protein were grown to confluency before adding 0.3 mm IPTG to induce protein expression. Lysates were prepared using 50 mg/ml lysozyme in lysis buffer (50 mM glucose, 20 mM Tris–Cl pH 8.0, 10 mM EDTA pH 8.0). In addition, 2.2N NaOH and 8%BME was added to the mixture. Total protein was precipitated using TCA and pellets were resuspended in SDS buffer. Samples were diluted 1:100 in SDS buffer and increasing volumes were loaded (0.6 μl to 1.6 μl, all in a total volume of 10 μl). For the antibody competition identical gels were prepared. The anti-Cx43 antibody was either used directly (1:2000) or following pre-incubation with the peptide made as the Cx43 antigen. Band densities were determined using Scion imaging software (Scion Corp., Frederick, MD) and the percent reduction was calculated.

Wild-type and *sof* fin (5 dpa regenerates) and whole body (21 days post fertilization) tissue was dried and homogenized using a mortar and pestle stored on dry ice and resuspended in homogenization buffer (20 mM Tris–HCl pH 7.4, 10 mM $MgCl_2$, 0.6 mM $CaCl_2$, 0.5 mM EGTA, 0.005% Triton-X 100, 0.1 mM PEFABLOC SC). Protein concentration was determined using a spectrophotometer (OD 280). Samples were prepared in SDS sample buffer. Protein was first separated using 12% SDS-PAGE at 26 mA and then transferred onto nitrocellulose membranes. Following transfer, blots were rinsed in 40% isopropanol, rinsed in dH_2O and blocked in 5% milk in SuperTBST for 30 min at room temperature. Blots were then incubated with either anti-Cx43 (1:2000) or anti-α-tubulin (Sigma, clone B512, 1:1000) for 1 h at room temperature, rinsed for 1 h in TBST, and incubated with peroxidase-conjugated Goat anti-rabbit IgG or Goat anti-mouse IgG (1:250,000, pre-absorbed with fin tissue, Pierce, Rockford, IL) for 1 h at room temperature. Following incubation, blots were rinsed in TBST for 1 h at room temperature. Using ECL chemiluminescent reagents (SuperSignal® West Femto Maximum Sensitivity Substrate, Pierce, Rockford, IL), blots were developed and exposed to X-ray film (XPosure™ film, Pierce, Rockford, IL). Band densities were determined as above.

Whole mount Cx43 staining

Fins (5 dpa) were harvested and fixed in 2% PFA in 0.1 mM phosphate buffer (PB) for 30 min at room temperature. Fins were washed (3×10 min) in 25 mM PB, incubated in Trypsin/EDTA (Gibco) for 10 min on ice and washed in 25 mM PB+10% sucrose for 1 h at room temperature. Fins were blocked (1M Tris–HCl pH 7.4, 5 M NaCl, 0.3% Triton-X 100, 6% goat serum) for 30 min at room temperature and incubated with Cx43 antibody (1:200) overnight at 4 °C. After incubation, fins were washed in block (3×5 min) and incubated in Goat anti-rabbit Alexa 546 (1:200, Molecular Probes) antibody for 2 h at room temperature followed by 1× PBS washes (3×10 min).

For double labeling, fins (3 dpa) were processed for H3P as described (see below, beginning after the rehydration series). Fins were mounted on slides in 50% glycerol for whole mount imaging. Fins were cryosectioned to identify doubly-stained cells in the mesenchymal compartment. Sixty-two sections (intact epithelium and mesenchymal compartments, and containing at least one H3P positive cell) were sampled from five different wild-type fins.

Cryosectioning

Fins were rinsed in 1× PBS (3×10 min) and embedded in 1.5% agarose/5% sucrose blocks, and submerged in 30% sucrose overnight at 4 °C. Blocks were frozen on dry ice and mounted using O.C.T. Compound (Tissue Tek®, Sakura, the Netherlands), and 20 μm sections were cut using a cryostat (Leica 2800 Frigocut E; Cambridge Instruments, Germany). Sections were collected on Superfrost Plus slides.

Morpholino injections and electroporation

Injection and electroporation experiments were performed as described (Thummel et al., 2006). Two targeting and two control morpholinos were used in this study, and all four were modified with fluorescein to provide a charge and for detection. *cx43*-MO (Iovine et al., 2005) was designed against the translational start site of *cx43*. As a control, a related morpholino containing 5 mis-matches was used, 5mm*cx43*-MO (5′-CCT CTT ACC TCA GTT ACA ATT TAT A 3′). *cx43*-MO2 (5′-GTT CTA GCT GGA AAG AAG TAA AGA G 3′) was designed against the 5′UTR of *cx43* with its mismatch control (5mm*cx43*-MO2; 5′-GTT GTA GGT GGA AAC AAC TAA ACA G 3′). Morpholinos were purchased from Gene Tools, LLC and were diluted to 1.2 mM in dH_2O.

Adult fish were first anesthetized using Tricane-S. Fin amputation was performed under a dissecting microscope using a scalpel and ruler to precisely amputate 50% of the caudal fin. At 3 days post amputation, morpholino was injected into the blastema of the three longest fin rays in either lobe using a Narishige IM 300 Microinjector. Approximately 50 nl of morpholino was injected per ray. Immediately following injection, both dorsal and ventral halves were electroporated using a CUY21 Square Wave Electroporator (Protech International, Inc.). The following parameters were used: ten 50-ms pulses of 15 V with a 1 s pause between pulses. At 24 hpe (hours post electroporation), success was evaluated by monitoring fluorescein uptake. Fish were returned to their tanks for either H3P analyses (1 dpe) or segment length analyses (4 dpe). In all experiments, 3–4 fins were tested per data point. Cell counts or segment length measurements were completed and standard deviation was calculated. Student's *t*-tests were completed to determine statistical significance.

In situ hybridization

In situ hybridization (ISH) as well as riboprobe synthesis (using digoxigenein-labeled UTP) was performed as described by Poss et al. (2000) using a probe against full length *cx43* (Iovine et al., 2005).

Detection of proliferating cells using H3P

Fins were amputated to 50% and permitted to regenerate for 1, 3, 7, or 9 days (3–4 fins per time point per strain) before harvesting and fixation in 4% paraformaldehyde in PBS overnight at 4 °C. Fins were dehydrated in 100% methanol overnight at −20 °C and re-hydrated in a methanol/PBS series. The fins were treated with 1 mg/ml collagenase in PBS for 45 min at room temperature and blocked using a 0.5% BSA in PBS solution with 0.1% Triton-X. A rabbit antibody against anti-phosphohistone H3 (H3P, Upstate Biotechnology) was diluted 1:100 in block and fins were incubated overnight at 4 °C. Following a series of washes in block, fins were incubated with an anti-rabbit antibody conjugated to Alexa-546 (Molecular Probes) (diluted 1:200 in block) for 2.5 h at room temperature. Washes were performed in block before mounting in Vectashield. H3P positive cells were visualized using a Nikon Eclipse E80 compound microscope and photographed using ImagePro software. Labeled cells were counted within the distal-most 250 μm. In the day 1 fins (which do not have 250 μm of tissue in the regenerate)