Hapln1a Is Required for Connexin43-Dependent Growth and Patterning in the Regenerating Fin Skeleton

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Abstract

Cell–cell communication, facilitating the exchange of small metabolites, ions and second messengers, takes place via aqueous proteinaceous channels called gap junctions. Connexins (Cx) are the subunits of a gap junction channel. Mutations in zebrafish cx43 produces the short fin (sof b123) phenotype and is characterized by short fins due to reduced segment length of the bony fin rays and reduced cell proliferation. Previously established results from our lab demonstrate that Cx43 plays a dual role regulating both cell proliferation (growth) and joint formation (patterning) during the process of skeletal morphogenesis. In this study, we show that Hapln1a (Hyaluronan and Proteoglycan Link Protein 1a) functions downstream of cx43. Hapln1a belongs to the family of link proteins that play an important role in stabilizing the ECM by linking the aggregates of hyaluronan and proteoglycans. We validated that hapln1a is expressed downstream of cx43 by in situ hybridization and quantitative RT-PCR methods. Moreover, in situ hybridization at different time points revealed that hapln1a expression peaks at 3 days post amputation. Expression of hapln1a is located in the medial mesenchyme and the in the lateral skeletal precursor cells. Furthermore, morpholino mediated knock-down of hapln1a resulted in reduced fin regenerate length, reduced bony segment length and reduced cell proliferation, recapitulating all the phenotypes of cx43 knock-down. Moreover, Hyaluronic Acid (HA) levels are dramatically reduced in hapln1a knock-down fins, attesting the importance of Hapln1a in stabilizing the ECM. Attempts to place cx43–sof b123 pathway suggest that hapln1a functions in a parallel genetic pathway. Collectively, our data suggest that Cx43 mediates independent Sema3d and Hapln1a pathways in order to coordinate skeletal growth and patterning.

Introduction

Gap junctions play a critical role in coupling tissue function and they have long been hypothesized to play a role in the maintenance of homeostasis, morphogenesis, cell differentiation, growth control and the process of skeletogenesis in multicellular organisms [1]. Gap junctions are proteinaceous channels formed by the docking of two connexons between neighboring cells, and they mediate the exchange of low molecular weight metabolites (<1000 Da), ions and second messengers between the contacting cells [2]. Each connexon or hemichannel is made up of six connexins, each protein containing a four pass transmembrane domain. The syndrome oculodentodigital dysplasia (ODDD), characterized by abnormalities in craniofacial elements, limbs and dentition, has been linked to missense mutations in the GJA1 gene locus in humans [3]. At least 24 separate point mutations in GJA1, which codes for Connexin43 (Cx43), have been identified in patients with ODDD [3–5]. The Cx43 knock out (Cx43−/−) mouse dies perinatally because of cardiac malformations [6,7]. Similarly, targeted gene knock-down of cx43 results in embryonic heart defects in zebrafish, signifying the essential role of cx43 during development [8]. The skeletal defects seen in the Cx43−/− KO mouse model exhibited hypomineralization of craniofacial bones and severely delayed ossification of the appendicular skeleton [9]. Moreover, the ODDD phenotype is similar to a set of craniofacial abnormalities observed in the targeted Cx43 knock-down chick model [10,11]. In zebrafish, a homozygous mutation in cx43 causes the short fin phenotype, characterized by shorter tail fins due to defects in the fin skeleton. The mechanism by which CX43 based mutations cause skeletal defect phenotypes is largely unknown. However, it is apparent that the function of Cx43 in the vertebrate skeleton is conserved.

We utilize the zebrafish short fin mutant (sof b123) to address the role of Cx43 during skeletal development. Our lab has found that the mutation in zebrafish cx43 gene causes the short fin (sof b123) phenotypes characterized by short bony fin ray segments, short fins, and reduced cell proliferation. The sof b123 mutant exhibits reduced levels of cx43 mRNA and Cx43 protein, without a lesion in the coding sequence [8]. Moreover, three additional alleles that cause missense mutations also resulted in reduced gap junctional intercellular communication (GJIC), short segments, and reduced cell proliferation [12]. Furthermore, morpholino mediated knock-down of Cx43 in wild type zebrafish completely recapitulate all the phenotypes produced by sof alleles [13]. In contrast to sof b123, the another long fin (alf dty86) mutant exhibits fin overgrowth and overlong segments due to stochastic joint failure [14]. These phenotypes are opposite those of sof, and we have shown that alf dty86 exhibits increased levels of cx43 mRNA [15]. Indeed, cx43 knock-down in alf dty86 rescues the segment length phenotype, suggesting that Cx43 over expression contributes to the alf dty86 phenotypes. (note

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that the mutation causing alf\(^{dy06}\) phenotypes is not located in the cx43 gene [15]. We interpret the cx43-dependent loss of joints in alf\(^{dy06}\) and the premature joint formation (i.e. short segment) phenotype in sof to indicate that Cx43 suppresses joint formation. At the same time, Cx43 is positively associated with cell proliferation. Thus, Cx43 functions in more than one way, both positively influencing cell proliferation and negatively influencing joint formation, thereby concomitantly regulating bone growth and skeletal patterning during the process of fin regeneration.

An important, yet poorly understood question with respect to mutations in connexin genes in general is, how does GJIC impact tangible cellular events like cell division and differentiation? One hypothesis is that Cx43 based GJIC can influence gene expression patterns [16,17]. Our lab exploited the availability of the two mutants, sof\(^{dy123}\) and alf\(^{dy06}\), in order to identify genes whose expression depends on Cx43. Thus, we utilized a novel microarray strategy to identify a set of candidate genes, which are both downregulated in sof\(^{dy123}\) and upregulated in alf\(^{dy06}\). The first gene validated from this microarray is smaghophrin3δ (sma3δ) [18]. Here, we provide molecular and functional validation of another gene identified from the microarray analysis, hapln1a (hyaluronan and proteoglycan link protein 1a). In mouse and human, the orthologous protein Hapln1 has also been referred to as either cartilage link protein (Crtl1) or link protein (LP). The function of Hapln1 is to “link” hyaluronic acid (HA) with proteins termed proteoglycans (PG) in the extracellular matrix (ECM). Remarkably, the mouse knockout for CRTL1 (aka HAPLN1) causes dwarfism, craniofacial abnormalities, and perinatal lethality in the mouse [19].

The ECM is a complex mixture of proteins and carbohydrates that forms a dense network surrounding cells. Little is known about the functional role of the ECM during zebrafish fin regeneration. Pharmacological treatments that alter the localization of PGs and collagen, as well as the expression levels of matrix metalloproteases, have been found to restrict outgrowth during fin regeneration [20,21]. Even less is known about the particular role of the Hapln1a-based ECM, including HA, in the regenerating fin. HA is a large molecular weight carbohydrate polymer with a molecular mass up to 10^6–10^7 Da [22,23]. The structure of the ECM is stabilized by Haplns by virtue of forming stable associations between HA and PGs [24–26]. Unsulfated glycosaminoglycans (such as HA) are prominent in the blastemas of late stage regenerating chilid fins, although their function has not been elucidated [27]. Apart from contributing to the physicochemical properties, ECM turnover and remodeling are critical events during tissue injury and wound repair. HA is also known to regulate cell migration, proliferation, and differentiation through activation of HA-specific cell surface receptors [28–30]. This is the first study to evaluate the effects of destabilizing the Hapln1a-based ECM during zebrafish fin regeneration. We find that reduced Hapln1a causes skeletal growth and patterning defects during fin regeneration, perhaps via destabilization of HA. Furthermore, we find that hapln1a functions downstream of cx43, providing novel insights into how skeletal morphogenesis could be influenced by Cx43.

**Materials and Methods**

**Statement on the ethical treatment of animals**

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocols used for this manuscript were approved by Lehagh’s Institutional Animal Care and Use Committee (IACUC) (protocol identification #128, approved 11/14/2012). Lehagh University’s Animal Welfare Assurance Number is A-3877-01. All experiments were performed to minimize pain and discomfort.

**Animal procedures**

The following zebrafish (Danio rerio) strains were used in this study: wild-type C32, sof\(^{dy123}\) [31] and alf\(^{dy06}\) [14]. Fish were maintained at a constant temperature of 25°C with 14 light:10 dark photoperiod [32]. Fish were anaesthetized in 0.1% tricaine and caudal-fin amputations were performed at the 50% level using razor blades. Fin regeneration was then allowed to proceed until the desired time period and the regenerated fins were harvested from anaesthetized fish. Fins were fixed overnight in 4% paraformaldehyde (PFA) in PBS and dehydrated in 100% methanol at −20°C. Knockdown and whole mount in situ hybridization experiments were performed in triplicate with 5 fins per trial. For histochemistry on sectioned tissue, a minimum of 10 sections were evaluated from each of 3 different fins. For quantitative analyses, student’s t-tests were completed to determine statistical significance (p<0.05).

**In situ hybridization on whole mount and cryosectioned fins**

RNA probes were generated from PCR amplified linear DNA in which the reverse primer contained the T7 RNA polymerase binding site. The primers used in this study for ISH are summarized in Table 1. Digoxigenin (DIG) labeled RNA probes were synthesized using DIG labeling mix (Roche) and in situ hybridization on regenerates whole fins was carried out as described [18]. Briefly, for the whole mount method, fins stored in methanol were sequentially rehydrated with methanol/PBS and then treated with 5 μg/ml proteinase K for 45 min and re-fixed in 4% PFA in PBS for 20 min followed by extensive washes with PBS and finally the pre-hybridization process was carried out with HYB (50% formamide, 5X SSC, 10 mM citric acid, 0.1% Tween20, heparin and dRNA) at 65°C for 0.5–1 h. Hybridization with DIG labeled probes suspended in HYB was carried out overnight at 65°C followed by sequential washes with HYB/PBST and finally with PBST. Anti-DIG Fab (Roche) fragments were used at 1:5000 dilution, followed by extensive PBST washes and short washes in staining buffer. Subsequently fins were transferred to a staining solution containing NBT and BCIP and allowed to develop under dark conditions until purple color was observed. The reaction was stopped by washing in PBST and fixing overnight with 4% PFA. Finally the fins were mounted on a glass slide and analyzed using a Nikon Eclipse 80i microscope. Pictures were taken using a digital Nikon camera.

For in situ sections, fins were fixed overnight with 4% PFA in PBS after harvest. After a brief methanol wash, fins were dehydrated in 100% methanol and stored at −20°C until use. Before sectioning, fins were sequentially rehydrated in a methanol-PBS series of washes and then were embedded in 1.5% agarose/5% sucrose dissolved in PBS and equilibrated overnight in 30% sucrose. Fins were mounted in OCT and cryosectioned (15 μm sections) using a Reichert™ Jung 2800 Frigocut cryostat. Sections were collected on Superfrost Plus slides (Fisher) and allowed to air dry overnight at room temperature. Sections can be stored at −20°C for up to a year. Before use, the slides were brought to room temperature for at least an hour. Using a marking pen (ImmEdge Pen H-4000; PAP pen, VWR Laboratories), sections were circled. An appropriate amount of probe was pre-hybridized with a mixture of 1X salt solution (NaCl, Tris HCl, Tris Base, Na2HPO4,7H2O, NaH2PO4, and 0.5 M EDTA) containing 50% deionized formamide (Sigma), 10% dextran sulfate, 1 mg/ml tRNA, and 1X Denhart’s (Fisher) at 70°C for 5 mins. Hybridiza-
tion with DIG–labeled antisense probes was carried out at 65°C overnight. The following day, slides were taken through a series of washes in a solution of 1X SSC, 50% formamide and 0.1% Tween-20 at 65°C. Slides were brought to room temperature and washed extensively in MABT (100 mM Maleic acid, 150 mM NaCl, and 0.1% Tween-20) and incubated in a blocking solution (MABT, Goat serum and 10% milk) for at least 2 hours. Anti-DIG Fab fragments (pre-absorbed against zebrafish fin tissue) were used at 1:5000 dilution and incubated overnight at 4°C. Slides were then transferred to 10% polyvinyl alcohol (PVA; MW: 86,000) solution with NBT/BCIP stock solution (Roche) and then washed 4X in MABT (30 min each) followed by 2X short washes (5–10 min) with staining buffer (100 mM Tris, 9.5, 50 mM MgCl2, 100 mM NaCl, and 0.1% Tween-20). Slides were then transferred to 10% polyvinyl alcohol (PVA; MW: 86,000) staining solution with NBT/BCIP stock solution (Roche) and allowed to develop overnight at 37°C. When a purple color started to appear on the sections, the reaction was stopped by washing the slides with PBST for at least 3 h and then stored in PBST at 4°C until imaging. Sections were mounted in 100% glycerol and examined on a Nikon Eclipse 80i microscope.

### Morpholino mediated gene knock-down in regenerating fins

All morpholinos (MOs) used in this study were fluorescein tagged, obtained from Gene Tools, LLC and used at a 1 mM concentration for injection. The sequences for MOs used in this study can be found in Table 1. Injection and electroporation were carried out as described previously [18]. Briefly, 3 dpa fish were anesthetized and approximately 50 nl of MO (targeting or mismatch) was injected into either the dorsal or ventral tail. MOs were harvested 1 dpe, after MO mediated knock-down and the experiment was carried out as described [16]. H3P was detected by biotinylated-HABP (US Biological, 1:300) followed by fluorescein labeled streptavidin-Alexa-546 conjugate, (Invitrogen, 1:200). For detection of Hapln1a or HA after MO injection (targeting or MM control), the fins were harvested 1 dpe, fixed in 4% PFA overnight and then stored in Methanol. Cryosectioning was done as described [18] and the sections were allowed to dry overnight. The sections were then rehydrated in PBS, twice for 10 min followed by two washes with ImmunoStaining block (2% BSA, 0.1% Tween in PBS). Then, sections were blocked for another 1 h at room temperature with the immunoStaining block and then incubated in primary antibody or biotin-HABP overnight at 4°C. The sections were washed with block (3x, 15 min each), incubated at room temperature for 1 hr with secondary antibody or streptavidin-Alexa-546 (pre-absorbed for 1 hr. at room temperature with fixed zebrafish fins to reduce background staining), washed (3x, 15 min each), incubated with HOECHST stain for 10 min at room temperature, followed by a quick wash with distilled water. They were blotted dry and then mounted for imaging. To test for specificity of HABP, WT fin sections were treated with bovine hyaluronidase (Sigma-H3506, final concentration 200 U/ml) in 10 mM phosphate buffer containing 100 mM NaCl (pH 6.0) for 2 hours at 37°C [33,34]. Untreated sections were incubated in buffer without enzyme. Following treatment the sections were washed twice in ImmunoStaining block before staining for HABP as described above.

### Lysate preparation and Immunoblotting

For WT and suf [32], 5 dpa regenerating fins were harvested from 10–15 fish. For MO injected fins, the injections were performed at 3 dpa fish (20–25 fish) and harvested the next day. Regenerating fins were harvested into 300–500 μl of RIPA lysis buffer, supplemented with protease inhibitor (Thermo scientific, Hal™ Protease and Phosphatase Inhibitor Cocktail) and homogenized using a tissue homogenizer (Bio-Gen, PRO 200) at high speed (5X) for 5 seconds with 10 second cooling intervals. Homogenized samples were centrifuged at 200 g for 10 min at 4°C. The supernatant was collected by centrifugation at 15,000 g for 10 min. The protein concentration was determined using the Bio-Rad Protein Assay according to the manufacturer’s instructions.

### Results

#### Hapln1a influences Skeletal Morphogenesis

The following primary antibodies were used: Rabbit anti-histone-3-phosphate (anti-H3P, Millipore, 1:200); Mouse anti-Hapln1a antibody (MD Bioproducts, 1:500). The following secondary antibodies were used: anti-mouse Alexa 488 or 546 (1:200); anti-rabbit Alex 546 (1:200). For H3P staining, whole fins were harvested 1 dpe, after MO mediated knock-down and the experiment was carried out as described [16]. H3P staining was done as described [18] and the sections were allowed to dry overnight. The sections were then rehydrated in PBS, twice for 10 min followed by two washes with ImmunoStaining block (2% BSA, 0.1% Tween in PBS). Then, sections were blocked for another 1 h at room temperature with the immunoStaining block and then incubated in primary antibody or biotin-HABP overnight at 4°C. The sections were washed with block (3x, 15 min each), incubated at room temperature for 1 hr with secondary antibody or streptavidin-Alexa-546 (pre-absorbed for 1 hr. at room temperature with fixed zebrafish fins to reduce background staining), washed (3x, 15 min each), incubated with HOECHST stain for 10 min at room temperature, followed by a quick wash with distilled water. They were blotted dry and then mounted for imaging. To test for specificity of HABP, WT fin sections were treated with bovine hyaluronidase (Sigma-H3506, final concentration 200 U/ml) in 10 mM phosphate buffer containing 100 mM NaCl (pH 6.0) for 2 hours at 37°C [33,34]. Untreated sections were incubated in buffer without enzyme. Following treatment the sections were washed twice in ImmunoStaining block before staining for HABP as described above.

#### Primers for ISH and Primers for qRT-PCR

| Gene     | Primers for ISH | Primers for qRT-PCR | Morpholinos |
|----------|-----------------|---------------------|-------------|
| hapln1a  | F = GGTTCTCCGCTT-GCCAGCG\ R = AAGGTTGTACCAGCCCCAA | F = AAAGCATAATTGCCATACCGG | Splice MO = ATCAAAGTGTGAT-TCTTACCTTGGC GC ATG MO = GCCAC-AGAAAAACAGAGCATCCT 5MM MO = GCCAGACAAGAGACCAATGATC |
| sema3d   | F = CGAAGTGTAGTACCATTTACG RT7 = TAAAAGCACTAAGAAGGTGAGATAAACACAC | F = TGGATGAGGAGAAGACCGAT | MO = TGTCGGCTCTCGTCCACTCCCATC |
| actin    | F = TTTTGTGGAGAGATGAGGCC R = CCAACCATCTCCTTGTG | |

Table 1. Primers and Morpholinos.
4°C and supernatant was used for further analysis. The protein samples were concentrated using a lyophilizer (Edwards-Freeze dryer super modul) and the pellet was resuspended in a minimal volume of PBS containing (Thermo scientific, Halt Protease and Phosphatase Inhibitor Cocktail, 100X) and the protein levels estimated by Bradford’s assay.

To release Hapln1a from HA for detection by immunoblotting, equal concentrations of Hapln1a MO and MM lysates were treated with 100 U/ml of bovine hyaluronidase (Sigma) for 2 hours at 37°C as described [35]. Immunoblotting was performed as described [13] using mouse anti-Hapln1a (MD Bioproducts, 1:500) and anti-α-tubulin (Sigma, 1:3000) in combination with peroxidase-conjugated goat anti-mouse IgG (1:10,000). Signal detection was performed using ECL Prime western blotting detection reagent (AmershamTM - GE Healthcare).

**Measurements**

The regenerate length, segment length, and the number of dividing cells were estimated/calculated as described [18]. For each experiment, at least 5 fish were evaluated in triplicate and a student’s t-test was performed to evaluate statistical significance. Image Pro software was used to measure the regenerate length from the plane of amputation to the tip of the fin and segment length between two joints under bright field. Cell number was calculated by counting the number of histone-3-phosphate (H3P) positive cells from the distal-most 250 μm² of the 3rd fin ray.

**Quantitative real-time PCR**

For qRT-PCR analysis, TRIZOL RNA extraction was made from the 5 dpa regenerating fins of wild-type, sof b123, and alf dty86 and 1 dpe for MO injected fins (targeting or MM). A minimum of 10 fins was used for total RNA extraction. For each sample, 1 μg of total RNA were reverse transcribed with SuperScript III reverse transcriptase (Invitrogen) using oligo-dT primers. Primers for qPCR analysis of hapln1a, sema3d and actin were designed using Primer express software (Table 1). Three independent RNA samples were used for the experimental comparison and qPCR for each gene was done in duplicates. The samples were analyzed using Rotor-Gene 6000 series software (Corbett Research) and the average cycle number (CT) was determined for each amplicon. The samples were analyzed using Rotor-Gene 6000 series software (Corbett Research) and the average cycle number (CT) was determined for each amplicon.

**Results**

**Hapln1a is expressed downstream of cx43**

Recently, we described a microarray strategy to identify genes that function downstream of Cx43. We identified ~50 genes that were both downregulated in sof b123, and alf dty86 [18]. One of the candidate genes identified was hapln1a. To validate that hapln1a is expressed downstream of cx43, we compared the expression levels of hapln1a in WT, sof b123, and alf dty86 regenerating fins by whole mount in situ hybridization (Figure 1). As expected, hapln1a mRNA expression appeared to be downregulated in sof b123. We noticed some variability among alf dty86 regenerating fins, where some fins exhibited upregulation and some appeared more similar to WT regenerating fins (two representative images are shown in Figure 1). Therefore, we performed quantitative RT-PCR (qPCR) to evaluate the level of hapln1a expression in sof b123 and alf dty86 regenerating fins compared with WT (Table 2). As expected, hapln1a is downregulated in sof b123. However, we did not observe an upregulation of hapln1a in alf dty86, suggesting that among a population of alf dty86 regenerating fins, there is little difference in the expression level of hapln1a between WT and alf dty86. To demonstrate the tissue-specific expression of hapln1a, we performed in situ hybridization on cryosections. The longitudinal section of a regenerating fin ray reveals several outer epidermal layers, including the basal layer of epidermis, which is adjacent to the mesenchyme. The mesenchyme is located medially. Within the mesenchymal compartment, a blastema is established at the distal, or growing end, of each ray. Blastemas are comprised largely of dividing cells that contribute to new tissue growth (reviewed in [36,37]). The hapln1a mRNA is expressed throughout the mesenchyme with slightly higher expression in the blastema, and to a lesser extent in the skeletal precursor cells (Figure 1).

In order to provide a secondary test to demonstrate cx43-dependence of hapln1a, we examined its expression in fins treated for cx43 knock-down [13]. Indeed, the expression of hapln1a is reduced in WT fins treated for cx43 knock-down compared to cx43 mismatch-treated fins by both in situ hybridization (Figure S1) and by qPCR (Table 2). In contrast, cx43 expression is not reduced in WT fins treated for hapln1a knock-down (Table 2), indicating that cx43 expression does not depend on hapln1a. In summary, we find that hapln1a is expressed both in sof b123 and in cx43 knock-down fins, providing independent confirmation that hapln1a is expressed in a cx43-dependent manner. Together, these data support the conclusion that hapln1a is molecularly downstream of cx43.

To evaluate when during fin growth hapln1a may be required in WT regenerating fins, we examined the expression pattern of hapln1a in non-regenerating fins, and at 2 dpa, 3 dpa, 5 dpa and 8 dpa (Figure 2). In non-regenerating fins hapln1a expression was not detectable. By 2 dpa, the expression of hapln1a was slightly upregulated, and by 3 dpa hapln1a was expressed strongly within each fin ray. At 5 dpa, there was a clear decrease in expression, and its expression was decreased even more by 8 dpa. Thus, hapln1a expression is expressed at 2 dpa and is maximally expressed at 3 dpa. The maximum expression at 3 dpa corresponds with the time point at which maximum rate of regeneration has been observed [38].

**Hapln1a knock-down is functionally downstream of Cx43**

In order to determine if hapln1a is functionally downstream of cx43, we completed morpholino-mediated gene knock-down of cx43.
hapln1a in WT regenerating fins [13,15]. Two targeting morpholinos (MOs) were generated, an ATG blocker (ATG MO) that inhibits protein translation and a splice blocker (Splice MO) that was designed to inhibit the splicing of intron 1. As a control, we used a mismatch MO (MM MO), which includes five mismatches to the target sequence for the ATG-blocking MO (Table 1). All MOs are conjugated to fluorescein, permitting validation of cellular uptake. Following microinjection and electroporation, MOs are conjugated to fluorescein, permitting validation of the target sequence for the ATG-blocking MO (Table 1). All MOs are conjugated to fluorescein, permitting validation of cellular uptake. Following microinjection and electroporation, MO-positive fish were selected for fluorescein-positive cells at 24 hours post electroporation (hpe). Positive fins were either harvested for analysis of cell proliferation or permitted to regenerate for 4 additional days for evaluation of segment length and regenerate length. The effect of hapln1a knock-down on cell proliferation was evaluated by counting the number of mitotic cells detected by H3P immunostaining. Regenerate length was measured as the distance between the amputation plane and the distal end of the fin. Segment length was measured as the distance between the first two joints in the regenerate. Interestingly, we found that hapln1a knock-down with both the ATG MO and Splice MO exhibited the same phenotypes as cx43 knock-down. Thus, knock-down fins exhibited reduced fin length (Figure 3A), reduced segment length (Figure 3B), and reduced cell proliferation (Figure 3C). The specificity of these knock-downs is demonstrated by the use of two independent gene-targeting MOs and also by the mismatch control. Collectively, these data reveal that cx43 and hapln1a act in a common pathway to promote cell proliferation and to inhibit joint formation.

Hapln1a knock-down destabilizes HA

To evaluate the effect of hapln1a knock-down on HA in WT regenerating fins, we completed a MO-mediated hapln1a knock-down (i.e., using the ATG MO) and evaluated both Hapln1a staining and HA staining on the treated fins. The success of hapln1a MO-mediated protein knock-down was further confirmed by immunostaining. Thus, comparison of the MM MO treated fins with the ATG MO treated fins revealed that Hapln1a protein is reduced following hapln1a knock-down (Figure 4A-B). Moreover, reduced Hapln1a protein levels in ATG MO treated fins was confirmed through immunoblotting (Figure 4C). Hapln1a protein is glycosylated and typically detected as several bands above its predicted molecular weight of 38 kD [35]. All bands are reduced following Hapln1a knock-down. Since Hapln1a is crucial for the stabilization of the HA-PG network in the ECM, we also evaluated the level of HA in hapln1a knock-down fins. We utilized biotinylated HA-binding protein (HABP-biotin) in combination with streptavidin-Alexa546 to detect HA. First, we demonstrate that HABP is specific for the detection of HA by treating fin sections with the HA-degrading enzyme hyaluronidase. Endogenous levels of HA may be observed in WT 5 dpa fins (untreated). In contrast, treatment of fin sections with hyaluronidase greatly reduced the staining by HABP (Figure 5A-B), suggesting that HA is required for detection. Importantly, we also observed a clear reduction in the amount of detectable HA following hapln1a knock-down (Figure 5C-D). These findings strongly support the hypothesis that HA is destabilized in the absence of sufficient Hapln1a.

To further demonstrate that hapln1a functions in a cx43-dependent manner, we next evaluated HA levels in both sos1Δ22Δ and hfyn1aΔ22Δ. The effect of regenerate for 4 additional days for evaluation of segment length 24 hours post electroporation (hpe). Positive fins were either harvested for analysis of cell proliferation or permitted to regenerate for 4 additional days for evaluation of segment length and regenerate length. The effect of hapln1a knock-down on cell proliferation was evaluated by counting the number of mitotic cells detected by H3P immunostaining. Regenerate length was measured as the distance between the amputation plane and the distal end of the fin. Segment length was measured as the distance between the first two joints in the regenerate. Interestingly, we found that hapln1a knock-down with both the ATG MO and Splice MO exhibited the same phenotypes as cx43 knock-down. Thus, knock-down fins exhibited reduced fin length (Figure 3A), reduced segment length (Figure 3B), and reduced cell proliferation (Figure 3C). The specificity of these knock-downs is demonstrated by the use of two independent gene-targeting MOs and also by the mismatch control. Collectively, these data reveal that cx43 and hapln1a act in a common pathway to promote cell proliferation and to inhibit joint formation.

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To further demonstrate that hapln1a functions in a cx43-dependent manner, we next evaluated HA levels in both sos1Δ22Δ and hfyn1aΔ22Δ.
Figure 3. Morpholino mediated knock-down of hapln1a recapitulates all cx43-dependent phenotypes. (Top row) Total regenerate length was measured in MO and MM treated fins. Hapln1a knock-down resulted in reduced fin length (*). The dotted line represents the amputation plane. (Middle row) Segment length was measured in MO and MM treated fins. Hapln1a knock-down resulted in reduction of segment length (*). Arrowheads identify joints. (Bottom row) Total number of H3P positive cells (red) was counted. Images of representative whole fins stained for H3P are shown for each condition. Knock-down of Hapln1a resulted in reduced cell proliferation (*) compared to the mismatch control. MO represents targeting morpholino; ATG MO targets translation initiation; Splice MO targets splicing event; MM represents control morpholino with 5 mismatches to target sequence; CS represents uninjected/untreated side. All experiments were completed in triplicate with a minimum of five fins treated with MO per trial. Statistical significance was determined by student’s t-test where p<0.05. By student’s t-test, there is no statistical difference between MM and CS. Error bars represent the standard deviation. Scale bar is 50 μm.

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regenerating fins and in fins treated for cx43-knockdown [13]. Since hapln1a levels depend upon Cx43 activity, we predicted that reduced Cx43 would similarly lead to reduced levels of HA. Indeed, this is what we found. In sof b123 regenerating fins compared with WT regenerating fins, we observed reduced levels of HA (Figure 6A-B). Similarly, in WT fins treated for MO mediated cx43 knockdown, HA levels are reduced compared with the MM control-treated fins (Figure 6C-D). Together with our previous findings, these data strongly support the conclusion that hapln1a functions in a common pathway with cx43, and that this pathway mediates cell proliferation and joint formation at least in part through influencing the Hapln1a-based ECM.

Hapln1a functions in a cx43-dependent and sema3d-independent genetic pathway

In our recent study, we found that Cx43 and Sema3d function in a common molecular pathway to promote cell proliferation and joint formation [18]. We next wished to determine how hapln1a contributes to this previously defined pathway. Our prior findings suggest that Sema3d utilizes the Nrp2a receptor to mediate cell proliferation and the PlxnA3 receptor to mediate joint formation. Indeed, in addition to Cx43 knock-down, both Sema3d- and PlxnA3 knock-down rescues joint formation in alf dty86. Therefore, we first tested if hapln1a knock-down functioned similarly. Interestingly, segment length was not rescued in alf dty86 fins by hapln1a knock-down (Figure 7). Failure of hapln1a knock-down to rescue segment length in alf dty86 was not due to a failure of the

Figure 4. Morpholino mediated knock-down of hapln1a in WT regenerating fins results in reduced Hapln1a. Immunostaining for Hapln1a and HOECHST staining for DNA (blue). The green reveals the location of the targeting and control MOs, which are fluorescein tagged. (A) Longitudinal section of a fin ray treated with Hapln1a control morpholino (MM). (B) Longitudinal section of a fin ray knocked down for Hapln1a with a targeting morpholino (KD). Compared to the control MM fins, Hapln1a knock-down (KD) fins exhibit reduced staining for Hapln1a. (C) Immunoblots confirming reduced levels of Hapln1a protein following Hapln1a knockdown. Hapln1a-MO treated fins (KD) were compared to control morpholino (MM). Tubulin was used as a loading control. Arrow identifies the basal layer of the epidermis; m, mesenchyme; e, epithelium. Scale bar is 50 μm. doi:10.1371/journal.pone.0088574.g004

Figure 5. Morpholino mediated knock-down of hapln1a in WT regenerating fins results in reduced HA levels. HA is detected by biotin-HABP with streptavidin-Alexa-546. HOECHST detects DNA (blue). The green reveals the location of the targeting and control MOs, which are fluorescein tagged. (A,B) Fin sections from 5 dpa WT regenerating fins were either untreated or treated with hyaluronidase. The reduced signal in the hyaluronidase-treated sections demonstrates that HABP detects HA. (C) Longitudinal section of a fin ray treated with Hapln1a control morpholino (MM). (D) Longitudinal section of a fin ray knocked down for Hapln1a with a targeting morpholino (KD). Compared to the control MM fins, Hapln1a knock-down (KD) fins exhibit reduced staining for HA. Arrow identifies the basal layer of the epidermis; m, mesenchyme; e, epithelium. Scale bar is 50 μm. doi:10.1371/journal.pone.0088574.g005

Figure 6. HA levels are reduced in sof b123 and in cx43-KD regenerating fins. Immunostaining for HA (Red) and HOECHST staining for DNA (blue). In the panels treated for cx43-MO or MM, the green reveals the location of the MOs, which are fluorescein tagged. HA levels were detected as described in Figure 4. (A,C) HA levels in WT 5 dpa regenerating fins and in WT fins treated for cx43-MM. (B,D) HA levels are reduced in sof b123 regenerating fins and in WT fins treated for cx43-MO. Arrow identifies the basal layer of the epidermis; m, mesenchyme; e, epithelium. Scale bar is 50 μm. doi:10.1371/journal.pone.0088574.g006
knock-down since both Hapln1a and HA were similarly reduced (i.e. evaluated by immunostaining for Hapln1a protein or for HA levels as completed above, see Figure S2). The finding that haph1a knock-down does not rescue joint formation in alfΔsof is consistent with our finding that hapln1a is not up-regulated in alfΔsof. Moreover, this suggests that hapln1a functions independently of soa3d-plexa3-dependent joint formation. We next evaluated changes in hapln1a and soa3d gene expression following reciprocal knock-down experiments. Thus, in soa3d knock-down fins hapln1a is not affected (Table 2), suggesting that hapln1a is not downstream of soa3d. Similarly, in hapln1a knock-down fins soa3d expression is not affected (Table 2), suggesting that soa3d is not downstream of hapln1a. Collectively, our findings provide evidence that hapln1a and soa3d both function downstream of cx43, but independently of each other. Therefore, these data suggest that at least two pathways contribute to cx43-dependent cell proliferation and cx43-dependent joint formation (Figure 7A). Future studies will be completed to determine if the Sema3d and Hapln1a gene products instead functionally interact in a common pathway.

Discussion

This is the first report to provide a functional analysis of hapln1a in zebrafish. Our finding that Hapln1a functions downstream of Cx43 is supported by multiple independent lines of evidence. The hapln1a gene exhibited reduced expression levels in sofΔsof and cx43 knock-down fins compared to WT regenerating fins by in situ hybridization. Additionally, qPCR analysis confirmed that hapln1a expression is dependent upon the level of cx43 expression. Knock-down of Hapln1a using two independent MOs recapitulated all of the Cx43-dependent phenotypes, namely reduced regenerate length, reduced segment length, and reduced cell proliferation. Thus, hapln1a functions downstream of Cx43. Moreover, we found that HA levels were strongly reduced in Hapln1a knock-down fins, suggesting that the loss of Hapln1a protein influences the stability of HA. The finding that HA levels are similarly reduced in sofΔsof and in cx43 knock-down fins provides additional evidence that cx43 and hapln1a function in a common pathway. Attempts to include hapln1a in the established cx43-soa3d pathway suggest that hapln1a may function independently of soa3d (Figure 8A). Therefore, Cx43 promotes cell proliferation and suppresses joint formation via the coordination of (at least) two downstream pathways. We find it interesting that Hapln1a is located in the ECM that physically connects the medial cx43-positive compart-

Figure 7. Hapln1a knock-down does not rescue segment length in alfΔsof. Compared to the uninjected control side (CS) the Hapln1a morpholino treated side (MO) does not show significant difference in the segment length. Statistical significance was determined by Student’s t-test where p<0.05. Error bars represent the standard deviation.
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Figure 8. Model depicting Cx43-Hapln1a mediated effect on skeletal patterning during fin regeneration. (A) Proposed pathway for Hapln1a mediated effects of Cx43. Hapln1a functions in a cx43-dependent, but sema3d-independent pathway, positively influencing cell proliferation and inhibiting joint formation. (B) Cartoon illustrating the compartments of gene in the regenerating fin: The cx43 mRNA is expressed throughout the mesenchyme (red) accompanied by cx43-dependent hapln1a upregulation in the same compartment (red), but primarily in the distal blastema (blue-red) and to a lesser extent in the proximal skeletal precursor cells (blue). The cx43 expression co-localized with the PG aggrecan in the pharyngeal arches and with the PG dermacan in the pectoral fins. Functional studies were not performed as part of these studies. However, reduced Hapln1 function is correlated with skeletal defects in other animals, including human. For example, single-nucleotide polymorphisms identified in the HAPLN1 gene have recently been associated with spinal osteoarthritis in aging female populations [46]. Moreover, targeted gene knockout of CRTLI (aka HAPLN1) in the mouse reveals that Crtl1/Hapln1 is indispensable during skeletal development. For example, the Crtl1−/− mice showed defects in cartilage and bone development with short limbs and craniofacial abnormalities, suggesting a prominent function for Crtl1 during chondrocyte differentiation [19]. In addition to these skeletal defects, Crtl1−/− mice die perinatally and exhibit a spectrum of myocardial defects. These defects have been attributed to the reduction in the PG versican [47], which may promote cell proliferation [48–50]. Interestingly, cartilage-specific expression of transgenic Crtl1/Hapln1 inhibits perinatal lethality and rescues skeletal abnormalities in Crtl1−/− mice [51], reinforcing the importance of the HA-PG network during heart development and skeletogenesis. These studies provide evidence that the “link” function of Hapln1 is critical for stability of components of the ECM and for regulation of cell differentiation and cell proliferation. Our findings on the function of Hapln1 in the regenerating fin are consistent with the role of Hapln1 in the developing mouse skeleton. Future studies will be...
focused on defining how Hapln1a mediates skeletal growth and patterning.

We propose that Cx43 activity in the blastema activates gene expression of hapln1a in the medial mesenchyme (Figure 8B). Secretion of Hapln1a in the ECM establishes the HA-PG network, stabilizes HA, and contributes to the regulation of cell proliferation and joint formation via unknown mechanisms. The Hapln1a-dependent ECM might be involved in providing the necessary microenvironment for the surrounding cells. Alternatively, Hapln1a may be required to maintain a stable population of HA, which in turn mediates signaling pathways through interaction with HA-specific cell surface receptors. Indeed, it is known that HA has diverse functions in skeletal biology including bone remodeling [22], bone resorption [52], and osteogenesis [53,54]. Further, there is growing evidence that the ECM can influence interactions between locally secreted growth factors and their receptors [55–59]. Continued studies are required to determine if interactions between locally secreted growth factors and their remodeling [22], bone resorption [52], and osteogenesis [53,54].

Conclusions

The identification of the Cx43-dependent Hapln1a pathway is novel and reveals tangible roles for the ECM during bone growth and skeletal patterning. We find that reduced Hapln1a levels are correlated with reduced HA levels, which may provide insights into the underlying mechanism of Hapln1a function. Combined with known skeletal defects associated with the loss of Hapln1 in the mouse, and with skeletal diseases associated with Hapln1 polymorphisms in human, these findings demonstrate that the role of Hapln1a is conserved in zebrafish. Therefore, continued studies designed to elucidate the mechanism of Hapln1a-dependent cell proliferation and joint formation will provide new and relevant insights into skeletal development in all vertebrates.

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Supporting Information

Figure S1 Cx43 knock down results in reduced expression levels of hapln1a. Whole mount in situ hybridization shows reduced expression of hapln1a in WT fins treated for cx43 knock-down (Cx43KD) fins compared to WT fins treated for cx43 mismatch control (Cx43MM) control fins. (TIF)

Figure S2 The hapln1a-KD was effective in alf dty86. Immuno staining for Hapln1a or HA (red) and HOECHST staining for DNA (blue). The green reveals the location of the targeting and control MOs, which are fluorescein tagged (A) Longitudinal section of an alf dty86 fin ray treated with Hapln1a control morpholino (MM). (B) Longitudinal section of an alf dty86 fin ray knock down for Hapln1a with a targeting morpholino (KD). Compared to the control MM fins, Hapln1a knock-down (KD) fins exhibit reduced staining for Hapln1a. HA was detected by biotinylated-HABP followed by streptavidin-Alexa 546 conjugate. (C) Longitudinal section of an alf dty86 fin ray treated with Hapln1a control morpholino (MM). (D) Longitudinal section of an alf dty86 fin ray knock down for Hapln1a with a targeting morpholino (KD). Compared to the control MM fins, Hapln1a knock-down (KD) fins exhibit reduced staining for HA. Arrow identifies the basal layer of the epidermis; m, mesenchyme; e, epithelium. Scale bar is 50 μm. (TIF)

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Author Contributions

Conceived and designed the experiments: JG MKI. Performed the experiments: JG. Analyzed the data: JG MKI. Contributed reagents/materials/analysis tools: JG MKI. Wrote the paper: JG MKI.
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