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Dynamic remodeling of the extra cellular matrix during zebrafish fin regeneration

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ABSTRACT

Extracellular matrix plays a dynamic role during the process of wound healing, embryogenesis and tissue regeneration. Caudal fin regeneration in zebrafish is an excellent model to study tissue and skeletal regeneration. We have analyzed the expression pattern of some of the well characterized ECM proteins during the process of caudal fin regeneration in zebrafish. Our results show that a transitional matrix analogous to the one formed during newt skeletal and heart muscle regeneration is synthesized during fin regeneration. Here we demonstrate that a provisional matrix rich in hyaluronic acid, tenascin C, and fibronectin is synthesized following amputation. Additionally, we observed that the link protein Hapln1a dependent ECM, consisting of Hapln1a, hyaluronan and proteoglycan aggrecan, is upregulated during fin regeneration. Laminin, the protein characteristic of differentiated tissues, showed only modest change in the expression pattern. Our findings on zebrafish fin regeneration implicates that changes in the extracellular milieu represent an evolutionarily conserved mechanism that proceeds during tissue regeneration, yet with distinct players depending on the type of tissue that is involved.

1. Introduction

The Extra Cellular Matrix (ECM) is secreted by cells and is composed of a wide variety of components that broadly include proteins, carbohydrates and proteins modified by sugar moieties termed proteoglycans (PG). Together these components form a complex meshwork that provides both structural and functional information to the cells. Initially, the ECM was thought to play only passive roles as a space filling material between cells and tissues. More recent work suggests that in addition to providing structural stability, the ECM also acts to sequester and store growth factors, present growth factors to their receptors and sense and transduce mechanical signals (Kim et al., 2011; Hynes, 2014). Therefore, the biochemical and the mechanical cues provided by the ECM play critical roles in regulating cell behaviors including migration, shape, survival, differentiation, and proliferation. Moreover, remodeling of the ECM occurs during normal development, morphogenesis, wound healing and during the mediation of disease states such as cancer (Daley and Yamada, 2013; Tolg et al., 2014). Also, ECM remodeling has recently been shown to contribute to epimorphic regeneration of newt skeletal muscle and heart, *Xenopus* tadpole tails, and zebrafish heart (Toole and Gross, 1971; Gulati et al., 1983; Tassava et al., 1996; Calve et al., 2010; Mercer et al., 2013).

Although mounting evidence suggests the importance of the ECM on cellular functions critical for morphogenesis, development, wound healing, tissue repair and regeneration, only a handful of studies address the role of ECM and its components during epimorphic regeneration. Several studies substantiate the establishment of a common transitional matrix rich in hyaluronic acid (HA), fibronectin (FN) and tenascin C (TNC) during epimorphic regeneration (Calve et al., 2010; Mercer et al., 2012, 2013) and down regulation of ECM proteins that are characteristic of differentiated skeletal tissues like laminin (LAM) and collagen type I (Mailman and Dresden, 1976; Gulati et al., 1983). Moreover, numerous studies have identified important roles for the poly-anionic high molecular weight compound hyaluronic acid (HA). HA is

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upregulated in matrices undergoing remodeling, during regenerative repair mechanisms (Contreras et al., 2009; Calve et al., 2010; Mercer et al., 2013; Tolg et al., 2014), and has been demonstrated to modulate signal transduction pathways such as EGFR/ErbB, TGFb and BMP (Bourguignon et al., 2002; Peterson et al., 2004; Ghatak et al., 2005). HA is a linear non-sulfated glycosaminoglycan (GAG) of repeating disaccharide units of [D-glucuronic acid (1-β-3) and N-acetyl-D-glucosamine (1-β-4)] $_n$ and plays a principal role in organizing PG aggregates like aggrecan (Acan) and versican (Vcan). HA-PG aggregates are stabilized by link proteins, which bring PGs to a back bone of HA (Hardingham, 1979, 1998). The PG family is a heterogeneous group consisting of a core protein with GAG side chains attached covalently. As opposed to HA, the GAGs in PGs are frequently sulfated. Acan is distinct from Vcan in that Acan has ~100 keratin and chondroitin sulfate GAG chains attached to the core protein, whereas Vcan has only ~12–15 chondroitin sulfate GAG chains (Poole et al., 1989; Lee et al., 1998). These poly-anionic macromolecular aggregates provide the required structural organization and flexibility and bind to several cationic proteins involved in signaling that aid the progression of regeneration (Spicer and Tien, 2004).

Our lab was the first to demonstrate the functional consequences of reduced HA during zebrafish fin regeneration (Govindan and Iovine, 2014). For example, we found that the link protein Hapln1a (Hyaluronan and Proteoglycan Link Protein 1a) is required for cell proliferation and fin ray joint formation. Hapln1a belongs to the family of link proteins that play a critical role in stabilizing the ECM by linking the aggregates of HA and PGs. We define Hapln1a and the associated HA and PGs as Hapln1a-ECM. We have shown that reduction in Hapln1a levels lead to reduction in HA levels that might contribute to the observed skeletal phenotypes in the regenerating fins (Govindan and Iovine, 2014). Together, data from our study and other studies highlight the importance of ECM components (HA and PGs) stabilized by Hapln1a during skeletal growth and patterning (Matsumoto et al., 2009; Contreras et al., 2009).

To achieve a better understanding about how the ECM is remodeled during zebrafish fin regeneration, we looked at components of the ECM over time. We focused on the expression pattern of Hapln1a-ECM components (i.e. Hapln1a, HA, Acan, Vcan), as well as the other components of the putative transitional matrix i.e. FN and TNC (Mailman and Dresden, 1976; Gulati et al., 1983; Calve et al., 2010; Mercer et al., 2013). In addition, we included LAM, which is characteristic of differentiated tissues. We find that all components of the transitional matrix (HA, FN and TNC) are also upregulated during fin regeneration. Moreover, we find that Hapln1a and Acan expression patterns change extensively over the time course, while Vcan pattern is less dynamic. In contrast, LAM expression pattern showed modest changes from ontogeny to regenerating fins. These findings provide the first examination of ECM remodeling during skeletal regeneration of zebrafish fin.

2. Materials and method

2.1. 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 Lehigh's Institutional Animal Care and Use Committee (IACUC) *(protocol identification #128, approved 11/16/2014). Lehigh University's Animal Welfare Assurance Number is A-3877-01.* All experiments were performed to minimize pain and discomfort.

2.2. Housing and husbandry

Zebrafish are housed in a re-circulating system built by Aquatic Habitats (now Pentair). Both 3 L tanks (up to 12 fish/tank) and 10 L tanks (up to 30 fish/tank) are used. The fish room has a 14:10 light:dark cycle and room temperature (RT) varies from 27 to 29 °C (Westerfield, 1993). Water quality is automatically monitored and dosed to maintain conductivity (400–600 μS) and pH (6.95–7.30). Nitrogen levels are maintained by a biofilter. A 10% water change occurs daily. Recirculating water is filtered sequentially through pad filters, bag filters, and a carbon canister before circulating over UV lights for sterilization. Fish are fed three times daily, once with brine shrimp (hatched from INVE artemia cysts) and twice with flake food (Aquatox AX5) supplemented with 7.5% micropellets (Hikari), 7.5% Golden Pearl (300–500 micron, Brine Shrimp direct), and 5% Cyclo-Peeze (Argent).

2.3. Animal procedures

The wild-type C32 zebrafish (*Danio rerio*) strain was used in this study. Fish were anaesthetized in 0.1% tricaine and caudal-fin amputations were performed at 50% level. Fin regeneration was then allowed to proceed until the desired time period (3, 5 or 7 days post amputation [dpa]) and the regenerated fins were harvested from anaesthetized fish. Fins were processed for immunohistochemistry as described below. A minimum of 5 different fins for each time point were sectioned and approximately 15–20 sections per fin were analyzed for each of the ECM component under study.

2.4. Fixing conditions and cryosectioning

Prior to immunostaining, ontogenetic fins (i.e. unamputated) and regenerating fins (3, 5 and 7 dpa) were fixed overnight (O/N) with 4% PFA in PBS (for detection of HA, Hapln1a, Fibronectin, Tenascin-C and Laminin). 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 in PBS and equilibrated in 30% sucrose in PBS. For detection of Acan and Vcan, fins were fixed for 10 min with 2% PFA at RT followed by three 10 min washes with 1X PBS, and were next embedded in 1.5% agarose/5% sucrose in PBS and equilibrated in 30% sucrose in PBS. Following that embedded fins were mounted in OCT and cryosectioned (15 μm sections) using a Reichertâ Jung 2800 Frigocut cryostat. Sections were collected on Superfrost Plus slides (Fisher) and allowed to air dry O/N at RT. Sections can be stored at −20 °C for up to a year. The slides were stored at −20 °C for at least one day before starting the experiment.

2.5. Immunofluorescence

First, the slides were brought to RT for at least 1 h. Sections were circled using a marking pen (ImmEdge Pen H-4000; PAP pen, VWR Laboratories). For Hapln1a and Vcan immunostaining, the sections were rehydrated twice for 10 min in PBS followed by two washes with block (2% BSA, 0.1% TritonX 100 in PBS). Then, sections were blocked for another 1 h at RT and then incubated in respective primary antibodies. The following primary antibodies were used: Mouse anti-Hapln1a antibody (MD Bioproducts, 1:500) and Rabbit anti-versican (H-56) (Santa Cruz Biotechnology–SC–25831) O/N at 4 °C.

For FN, TNC and LAM the sections were rehydrated twice for 10 min in PBS followed by a brief Trypsin-EDTA treatment (1:1 diluted with PBS) (Gibco-Life Technologies #25300-054) for 3 min at RT and then washed with PBS twice for 10 min. Following that,

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slides were washed with block for 10 min at RT. Then, sections were blocked for another 1 h at RT, incubated in respective primary antibodies O/N at 4 °C. The following primary antibodies were used: Rabbit anti-human fibronectin (Sigma-F3648, 1:100); Rabbit anti-chicken tenascin (US Biological-T2550-23, 1:500) and Rabbit anti-rat laminin (Thermo scientific-RB-082-A1, 1:100).

For Acan immunostaining, the sections were rehydrated twice for 10 min in PBS at RT, followed by antigen unmasking by digestion with chondroitinase. First, the slides were incubated with the chondroitinase treatment buffer (50 mM Tris, 60 mM sodium acetate, 0.02% BSA, pH 8.0) at 37 °C for 5 min, followed by deglycosylation using chondroitinase ABC enzyme (Sigma-C2905, final concentration 0.05U in the treatment buffer) for 2 h at 37 °C. Following that, slides were washed with block for 10 min at RT. Then, sections were blocked for another 1 h at RT, incubated with Mouse anti-aggrecan (BC-3) (Thermo Scientific-MA3-16888) primary antibody O/N at 4 °C.

Following incubation with primary antibodies, sections were washed three times in block (15 min each), incubated at RT for 1 h with secondary antibody goat anti-mouse Alexa-488 (Invitrogen, 1:200, pre-absorbed for 1 h, at RT with fixed zebrafish fins to reduce background staining) and washed again three times in block (15 min each). Sections were next incubated with propidium iodide (final concentration 0.01 mg/ml in block) for 30 min at RT, followed by a quick wash with distilled water. Then the slides were blotted dry and mounted for imaging. Confocal microscopy was used to image the sections using a 40 × /1.3 numerical aperture objective on an inverted microscope (Axiovert 200 M; Carl Zeiss, Jena, Germany) equipped with an LSM510 META scan head (Carl Zeiss). Argon ion and 543 HeNe lasers were used to generate the 488 and 543 lines used for excitation, and pinholes were typically set to 1–1.5 Airy units. Images were exported as TIFF files and printed using Photoshop.

2.6. Histo-chemical analysis of HA

HA was detected as described (Govindan and Iovine, 2014). Briefly the sections were rehydrated twice for 10 min in PBS followed by two washes with block. Then, sections were blocked for another 1 h at RT, incubated with biotinylated hyaluronic acid binding protein (bHABP-Calbiochem-385911, 1:100) O/N at 4 °C. The sections were washed three times in block (15 min each), incubated at RT for 1 h with streptavidin-Alexa-546 conjugate, (Invitrogen, 1:200) and washed again three times in block (15 min each). Sections were next incubated with propidium iodide (final concentration 0.01 mg/ml in block) for 30 min at RT, followed by a quick wash with distilled water. Then the slides were blotted dry and mounted for imaging as described above.

2.7. Semi-quantitative analysis for protein expression during the time course of regeneration

Semi quantitation analyses of fluorescence staining for expression of each protein under study was performed using ImageJ software. Briefly, the tissue sections for each time point were selected using the freeform drawing tool and converted to RGB stack. Measurements were set to obtain the values for area, integrated density, and mean gray values for Alexa 488 fluorescence. To account for the background, three unstained regions surrounding the tissue were selected and the values were obtained as before. To calculate the corrected total fluorescence pixel intensity for each section the following formula was used. Corrected total fluorescence = Integrated Density − (Area of selected tissue × Mean fluorescence of the background). This was repeated for each time point and for each of the ECM components under study. The average and standard deviation were calculated and plotted as Average total pixel intensity on the Y-axis. For ontogeny Acan fin sections, since the staining is localized to the joints, the area around the joint was selected instead of the whole section and the corrected total fluorescence was calculated as before.

3. Results and discussion

Previous studies on newt skeletal muscle and heart regeneration have shown that upon tissue injury, ECM remodeling results in the synthesis of a regeneration specific transitional matrix rich in HA, TNC and FN (Toole and Gross, 1971; Repesh et al., 1982; Tassava et al., 1996; Contreras et al., 2009; Calve et al., 2010; Calve and Simon, 2012; Mercer et al., 2013). These components are considered as the fundamental ECM components during embryonic development (Chiquet et al., 1981; Kosher et al., 1981; Chiquet and Fambrough, 1984; Matsumoto et al., 2009) and wound healing (Longaker et al., 1991; Whitby and Ferguson, 1991; Ghosh et al., 2006). The spatial and temporal expression and the concentration of these components influence the type of response that is elicited upon tissue injury. Comparative gene ontology analysis has shown that the up regulation of this transitional matrix is an evolutionarily conserved response between species during tissue regeneration, as opposed to repair mechanisms that result in scarring (Mercer et al., 2013). Importantly, during early stages of repair, HA provides a highly hydrated environment and TNC generates anti-adhesive effects thereby promoting the undifferentiated state. Concomitant with the up regulation of this transitional matrix, several studies have shown that the components of differentiated tissues, like collagen type I and LAM, are suppressed by the action of matrix metallo proteases (MMP's). Together, these changes lead to significant changes in the local environment that prevent scarring and instead are conducive for regeneration to proceed (Mailman and Dresden, 1976; Gulati et al., 1983; Vinarsky et al., 2005). We have demonstrated in this study that the components of the transitional matrix are also expressed during zebrafish caudal fin regeneration. To our knowledge, this is the first study to describe the expression pattern of these ECM proteins in a time course during fin regeneration.

The zebrafish caudal fin is made up of 16–18 segmented bony fin rays (lepidotrichia) that are connected by soft inter ray tissue that is devoid of any skeleton. The lepidotrichia are made of two concave hemi-rays that are lined by bone secreting osteoblasts (Akimenko et al., 2003; Tal et al., 2009). Lepidotrichia surround blood vessels, nerves, fibroblasts and pigment cells and are covered by a multilayered epidermis. Following amputation, a wound epidermis derived from the stump epithelium closes the wound. Formation of a multilayered epidermis has been shown to be an important requirement for blastema establishment and proliferation of cells (Chablais and Jazwinska, 2010). Just beneath the wound epidermis and distal to each fin ray, a blastema is formed that consists of de-differentiated and highly proliferative cells (Knopf et al., 2011; Sousa et al., 2011; Tu and Johnson, 2011; Stewart and Stankunas, 2012). The longitudinal section of a fin ray reveals a simple architecture with a central mesenchyme separated from the multilayered outer epidermis by a single layer of epithelial cells termed the basal layer of epithelium (BLE) (Fig. 1). During regeneration, just beneath the BLE, collagenous fibers called the actinotrichia are synthesized that serve to support the fin folds (Thorogood, 1991; Sordino et al., 1995). Osteoblasts and joint forming cells, collectively termed the skeletal precursor cells (SPC's), reside between the actinotrichia and BLE (Grandel and Schulte-Merker, 1998; Ton and Iovine, 2013). The formation of the blastema and the regenerative outgrowth is characterized by cell division and cell differentiation to replace all lost tissues of the fin.

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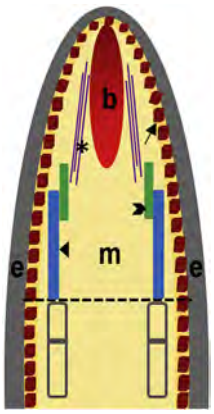

Fig. 1. Cartoon of a longitudinal section illustrating the different compartments in a regenerating fin ray. The dotted line (black) represents the amputation plane. Upon amputation, a wound epidermis (e) (gray) is formed. The arrow points to the basal layer of epithelium (brown cuboidal cells) just beneath the epidermis (e) (gray) separating it from the central mesenchyme (m) (cream). Following that, blastema (b) (red) is established that contains two zones, the distal blastema, containing relatively slowly proliferating cells that direct regenerative outgrowth, and proximal blastema, containing rapidly proliferating cells that differentiate into other cell types. The closed arrowhead points to the newly formed bony rays – lepidotrichia (blue), open arrowhead points to the skeletal precursor cells (green) and the asterix (*) denotes the actinotrichia (purple lines) extending towards the tip of the fin.

In order to evaluate changes in ECM components during skeletal regeneration, we examined ECM proteins in longitudinal fin sections at 0 dpa (uncut), 3 dpa, 5 dpa and 7 dpa regenerating fins. The uncut 0 dpa fin represents the ontogenic, non-regenerative state mostly consisting of well differentiated tissue. The early time point 3 dpa represents the time during which maximum rate of regeneration occurs (Lee et al., 2005; Hoptak-Solga et al., 2008). Later, regenerative outgrowth and fin patterning continues up to 14 days (Tal et al., 2009). The time points of 5 and 7 dpa were chosen to evaluate the expression patterns of the ECM proteins during the outgrowth and patterning phase of fin regeneration. We further paired immunohistochemical analyses with semi-quantitative analyses in order to validate the qualitative interpretation of overall changes in expression levels.

3.1. *Hapln1a dependent ECM undergoes transient dynamic modification during regeneration*

In a recent study, we showed that the ECM protein Hapln1a plays critical roles during skeletal growth and patterning by influencing cell proliferation, joint formation and the distribution of HA in the regenerating fin (Govindan and Iovine, 2014). Here we evaluate expression and localization of the link protein Hapln1a, the PGs Acan and Vcan, and HA during the time course for regeneration. Hapln1a and HA exhibit similar expression patterns during all time points of regeneration (Fig. 2A and B). In ontogeny fins, both Hapln1a and HA were low in the epidermis. In the mesenchyme, Hapln1a was almost absent but HA was observed. HA appeared to be tightly associated with epidermal cells and may be associated in a pericellular manner (Knudson et al., 2002; Toole, 2004). By 3 dpa, Hapln1a and HA were predominantly upregulated in mesenchyme and epidermis (Fig. 2A and B). The proximal end of the mesenchymal compartment showed more expression compared to the proliferative blastema. Additionally, HA was also strongly upregulated in the mesenchyme of the stump tissue (Figure S1). At 5 dpa, Hapln1a and HA were seen throughout the mesenchymal compartment and in the epidermis. During 7 dpa, the Hapln1a staining was more like the ontogeny fin where the epidermal staining was reduced significantly, but there was moderate staining in the mesenchyme. HA appeared to go down at 7 dpa compared to 3 and 5 dpa but was still moderately expressed throughout the mesenchyme (Fig. 2A and B).

The similar expression pattern of Hapln1a and HA during regeneration is expected as these two components are dependent on each other to associate with the PG aggregates. In particular, HA by itself is known to play a crucial role during matrix remodeling. It is well documented that HA synthesis and HA dependent signaling play a critical role during tissue regeneration, implying that it could be an evolutionarily conserved process that takes place in response to tissue loss to promote regeneration. The HA expression pattern in the regenerating zebrafish fin strongly corroborates with newt skeletal muscle and *Xenopus* tail regeneration studies where HA is expressed strongly in the distal stump and proximal blastema, as compared to the distal end of the regenerate. This, in turn is thought to regulate the proliferative state of mesenchymal cells in the regenerating tissue by modulating the distance between the cells (Thorogood and Hinchliffe, 1975; Kosher et al., 1981; Singley and Solursh, 1981; Contreras et al., 2009; Calve et al., 2010). Also, the immediate up regulation of HA proximal to the amputation plane and in the distal stump could play an important role to promote smooth migration of the progenitor cells into the regenerating blastema. For example in vitro studies using newt myoblasts have shown that myoblasts in an HA rich environment show reduced fusion and differentiation and a significant increase in migration (Calve et al., 2010). In contrast, the comparatively reduced expression of HA in the blastema could facilitate cell proliferation (Knudson and Knudson, 1993; Lee and Spicer, 2000; Spicer and Tien, 2004; Calve et al., 2010).

The aggregating PGs Acan/Vcan are present surrounding the bones and are important for matrix organization, cell motility and growth and are effective inhibitors of mineral deposition because of their ability to sequester calcium and impose steric hindrance (Gokhale and Boskey J, 2001). Furthermore, changes in GAG composition are now considered a signature event during various physiological and pathological remodeling processes, such as bone formation, regeneration, scarring, osteoarthritis, and cancer metastasis (Toole, 2009; Salbach et al., 2012). We have analyzed the expression pattern of both Acan and Vcan in regenerating fins.

Acan expression shows a distinct pattern in each of the different stages during fin regeneration. In ontogeny fins, Acan strongly localized to the mature joints and was poorly expressed in the mesenchyme and epidermis (Fig. 2C). On 3 dpa, we observed a moderate up regulation of Acan expression surrounding the mesenchymal cells, and around the stump bone matrix. Epidermal staining was reduced compared to the mesenchyme. Around 5 dpa, Acan expression was very strongly associated with lepidotrichia consisting of SPC's and, to a lesser extent with actinotrichia and mesenchyme. Acan expression goes down by 7 dpa, but was still observed in the lepidotrichia and to a lesser extent surrounding the mesenchymal cells (Fig. 2C). Even though the skeletal elements in fin rays are considered to be of dermal origin and formed by intramembranous ossification (Haas, 1962; Geraudie and Landis, 1982), studies have shown the expression of chondrogenic markers that are specific to cartilage, implying that the fin skeleton could be an intermediate between intramembranous and endochondral type of bone (Johnson and Weston, 1995; Padhi et al., 2004; Smith et al., 2006). There is evidence showing that Acan contributes to functional properties such as mechanical stiffness, and to the normal development and growth of membranous bones (Wong et al., 1992; Spicer and Tien, 2004). These data are consistent with our findings. In 5 dpa sections, Acan is expressed in the area of lepidotrichia where the bone forming osteoblasts reside. This suggests that the fin skeleton requires relatively higher Acan

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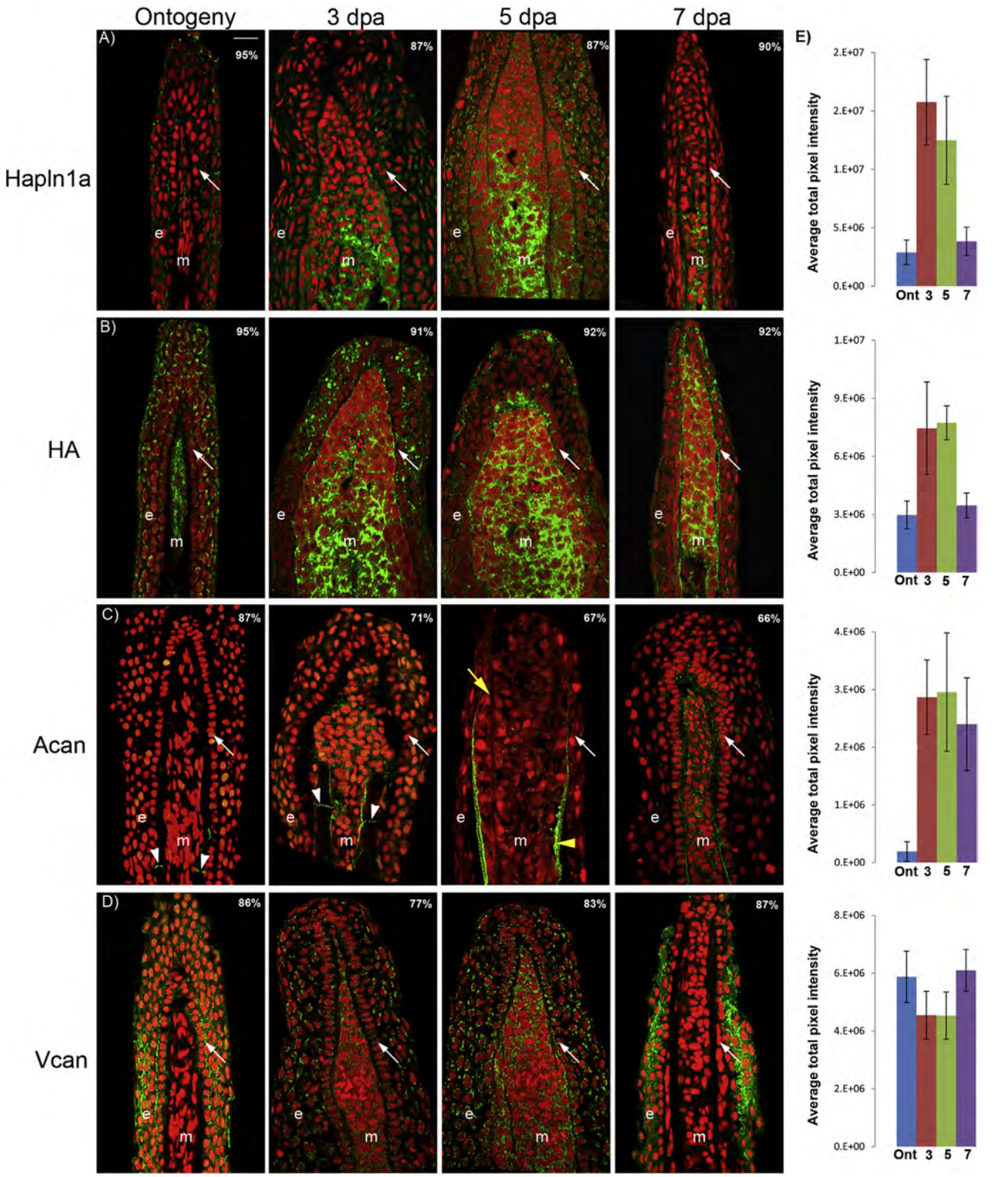

Fig. 2. Immunostaining and histochemistry for Hapln1a-ECM components during the time course of regeneration. Longitudinal fin sections were treated with the respective primary antibodies and detected using the corresponding secondary antibody conjugated with Alexa Fluor— 488 (green). Propidium iodide (nuclei) is used as the counter stain (red). For each time point the percentage of sections showing similar expression pattern is denoted in each panel (n = 40–65 sections). (A) Immunostaining for Hapln1a; (B) histochemical detection of HA using biotin-HA binding protein; (C) immunostaining for Acan, the arrowhead identifies the joints and (D) immunostaining for Vcan. (E) The graph illustrates the overall change in the expression level during the time course of regeneration for each component. Efforts to compare expression levels between components were not completed. Arrows identify the basal layer of epithelium (BLE); yellow arrowhead identifies lepidotrichia and yellow arrow identifies actinotrichia; m, mesenchyme; e, epidermis; dpa, days post amputation. Scale bar is 20 μm.

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expression levels at specific tissue compartments as part of a coordinated program of ECM synthesis and remodeling during skeletal regeneration.

We next evaluated the expression of Vcan, another major PG that forms aggregates with HA and is expressed during bone development (Nakamura et al., 2005). We found that, in ontogeny fin sections, Vcan staining was minimal in the mesenchyme, but was prominent in the epidermis (Fig. 2D). During 3 dpa and 5 dpa, Vcan was slightly upregulated in the expanded mesenchyme and also was found associated with the cells of BLE and epidermis. At 7 dpa, the Vcan levels in the mesenchyme were reduced to ontogeny levels while appearing slightly upregulated in the epidermis. Overall, the Vcan expression pattern did not show robust changes compared to the other components of the Hapln1a ECM during regeneration. Vcan was found mostly associated with the epidermis during ontogeny and the later time point 7 dpa, but was slightly upregulated in the mesenchyme during the earlier time points of regeneration (Fig. 2D). Even though previous studies have shown Vcan expression in mesenchymal condensations of chick limb cartilage (Yamagata et al., 1986; Shinomura et al., 1990), epiphyseal ends of long bones (Shibata et al., 2003) and in mouse presumptive joints (Snow et al., 2005), the exact role of Vcan is not clearly known. It has been suggested that the ternary stable complex of Vcan-HA-Link protein has anti-adhesive effects, creating a highly hydrated environment conducive for cell migration (Lee et al., 1993; Yamagata et al., 1993; Yamagata and Kimata, 1994). Further studies are required to elucidate the functional role of Vcan during fin regeneration.

A semi-quantitative analysis of Hapln1a-ECM components during the time course of regeneration shows that all components exhibit a change in the expression pattern during early regeneration compared to the ontogeny state (Fig. 2E). In particular, Hapln1a and HA show more robust changes than Vcan. Acan showed distinct temporal and spatial expression patterns and was maximal at 5 dpa. Moreover, by 7 dpa the overall Acan expression levels appear reduced compared to earlier time points. Thus, it is possible that components of the Hapln1a-ECM contribute more to the earlier stages of skeletal regeneration versus later stages.

3.2. TNC, FN and LAM show distinct expression patterns during fin regeneration

Earlier studies in zebrafish fin regeneration have used TNC as a counter stain, to evaluate the integrity of the mesenchyme (Jazwinska et al., 2007; Chablais and Jazwinska, 2010; Stewart and Stankunas, 2012), and to define and demarcate the distal mesenchymal region of the regenerating vertebrate appendages (Onda et al., 1991; Jazwinska et al., 2007; Chablais and Jazwinska, 2010). Here, we show that TNC exhibits a dynamic expression pattern during fin regeneration and, hence, could play a combined role with other components in defining the regeneration specific matrix. In ontogeny fins, TNC was expressed poorly (Fig. 3A). During 3 dpa, extensive staining was seen just beneath the BLE, in the blastema, and in the mesenchyme throughout the regenerating fin tissue. At 5 dpa, the expression still seemed very strong and was similar to 3 dpa. At the later time point 7 dpa, the pattern still resembled 3 and 5 dpa, but the strength of staining was lower than 5 dpa showing that it is down regulated during the later time point of regeneration (Fig. 3A). During all regenerative time points that were under study, TNC expression was found to be present and clearly demarcate the mesenchyme from the epidermis. TNC is known to be upregulated in injured tissue sites where the outcome is regeneration rather than scarring (Murphy-Ullrich, 2001; Harty et al., 2003). Previous studies on newt muscle and heart regeneration have shown that TNC in the regeneration specific transitional matrix promotes cell migration and suppresses differentiation by coordinating function with HA and creating a hydrated environment to facilitate cell motility. Further, in vitro studies using newt myoblasts and in vivo studies in regenerating newt heart and muscles have shown that TNC promotes cell cycle re-entry thereby promoting cell proliferation (Calve et al., 2010; Mercer et al., 2013). Moreover, TNC alone has been shown to be sufficient to induce cardiomyocyte proliferation in vitro suggesting that ECM derived signals can induce cell proliferation during regeneration (Mercer et al., 2013). During zebrafish fin regeneration we observed an initial up regulation of HA and TNC. Based on the previous studies, we hypothesize that the initial up regulation of these components can be an important event for progenitor cell migration and the down regulation at later time points could be a required step to promote differentiation.

FN is another ECM component that is shown to be specifically upregulated in the transitional matrix that is formed during regeneration of newt heart and skeletal muscle (Calve et al., 2010; Mercer et al., 2013) and in zebrafish heart (Wang et al., 2013). FN promotes tissue regeneration in multiple ways. It is known to be involved in clearing the tissue debris at the site of injury and influences both cell proliferation and differentiation by serving as a scaffold for cell adhesion and migration (Stoffels et al., 2013). We observed that FN is expressed during zebrafish fin regeneration (Fig. 3B). In both epidermis and mesenchyme, FN expression was minimal in ontogeny fins. During 3 dpa, FN expression was upregulated in the mesenchymal compartment and remained low in the epidermis. At 5 dpa, the expression was much stronger and was observed strongly in the blastema and, to a lesser extent, in the epidermis up to the apical tip of the fin. At 7 dpa, the expression of FN appeared to go down and was more like the ontogeny pattern (Fig. 3B). Compared to TNC and HA which are upregulated throughout the regenerative time course, FN exhibited a more transient and modest temporal up regulation followed by a decrease in expression during later time points of fin regeneration. Previous in vivo and in vitro studies have shown that FN promotes differentiation and also might be playing a combined role with TNC to promote DNA synthesis and proliferation (Calve et al., 2010; Mercer et al., 2013). Loss of function experiments have shown that FN is essential for proper heart regeneration and could play a role in signaling during cardiomyocyte migration (Wang et al., 2013). Expression of FN in regenerating fins is intriguing and further studies will help us to understand the role of FN expression during fin regeneration.

In addition to the above mentioned ECM proteins, we also looked at the expression of the protein LAM that is characteristic of differentiated tissues. LAM is one among several glycoproteins present in the basement membranes that self assembles to form the basal laminae or BLE, adjacent to the epithelial layers of the epidermis. In ontogeny fins, LAM was expressed both in mesenchyme and epidermis throughout the fin section, typical of a differentiated tissue (Fig. 3C). During 3 dpa and 5 dpa, we observed prominent expression of LAM surrounding the cells of BLE and importantly reduced and dispersed expression in mesenchyme and epidermis. At the later time point 7 dpa, specific expression surrounding BLE disappeared and the pattern resembled ontogeny fin sections (Fig. 3C). Overall, we observed only a modest change in the expression of LAM during the time course of fin regeneration. BLE has several functions, including boundary formation to separate and connect several tissue types, providing a mechanical scaffold and molecular information to regulate cell migration and proliferation (Webb et al., 2007; Godwin et al., 2014). Additional in vitro and in vivo studies will reveal the importance and functional roles of specific up regulation of LAM expression surrounding the BLE during regeneration.

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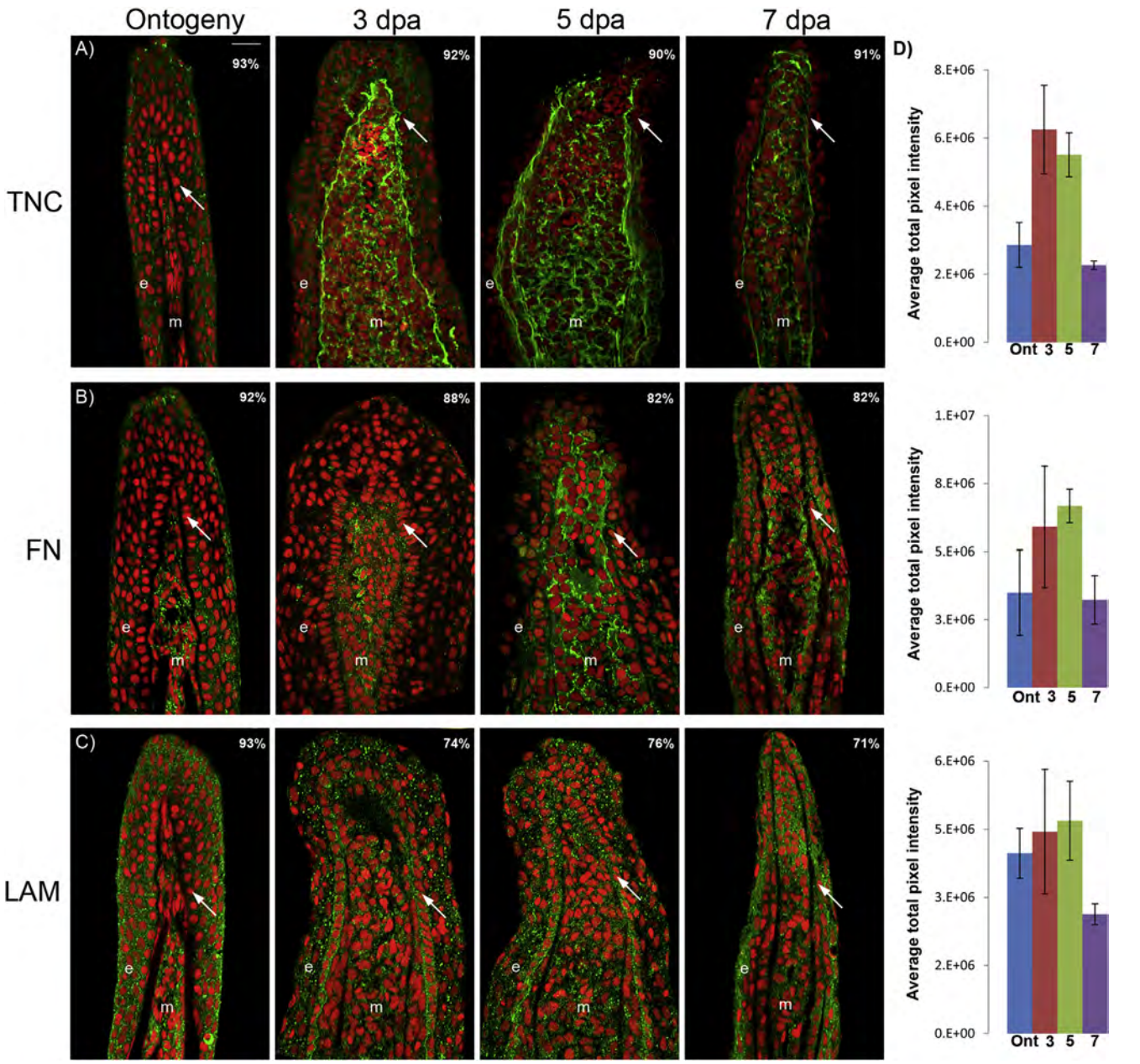

Fig. 3. Immunostaining for TNC, FN and LAM during the time course of regeneration. Longitudinal fin sections were treated with the respective primary antibodies and detected using the corresponding secondary antibody conjugated with Alexa Fluor− 488 (green). Propidium iodide (nuclei) is used as the counter stain (red). For each time point under study the percentage of sections showing similar expression pattern is denoted in each panel (n = 40–65 sections). (A) Immunostaining for TNC; (B) immunostaining for FN and (C) immunostaining for LAM. (D) The graph illustrates the overall changes in the expression levels of each of the component under study during the time course of regeneration. Efforts to compare expression levels between components were not completed. Arrows identify the basal layer of epithelium (BLE); m, mesenchyme; e, epidermis dpa, days post amputation. Scale bar is 20 μm.

A semi-quantitative analysis of the overall expression levels of TNC, FN and LAM during the time course of regeneration shows robust up regulation of TN and FN during the earlier time points compared to the ontogeny state (Fig. 3D). In contrast, LAM expression shows minimal changes between ontogeny and regeneration and during the time course of regeneration. These findings suggest that TNC and FN are functionally more important during earlier stages of fin regeneration. Since LAM is present at relatively constant levels, LAM may not provide a specific functional requirement for regenerating tissue.

4. Conclusions

Adult mammals have very limited regeneration capacity following tissue injury. In contrast, some animals like axolotls, newts and zebrafish possess an unlimited and remarkable level of tissue regenerative ability. Recent studies have highlighted the importance of dynamic extracellular modifications that occur during newt heart and skeletal muscle and zebrafish heart regeneration. In this study, we have shown that zebrafish fin regeneration also recapitulates a similar response following fin amputation.

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This indeed provides additional evidence for the evolutionarily conserved ECM alterations that take place during tissue repair and regeneration. Future work will be directed towards understanding the mechanism of how the involved players contribute individually and function together to regulate cell behavior. For example, combined in vivo and in vitro studies will provide new insights into how the biochemical properties of the changing ECM, plays a role in regulating proliferation and differentiation during the fin skeletal tissue regeneration.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.gep.2015.06.001.

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