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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,