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Using the zebrafish to understand tendon development and repair

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Abstract

Tendons are important components of our musculoskeletal system. Injuries to these tissues are very common, resulting from occupational-related injuries, sports-related trauma, and age-related degeneration. Unfortunately, there are few treatment options, and current therapies rarely restore injured tendons to their original function. An improved understanding of the pathways regulating their development and repair would have significant impact in stimulating the formulation of regenerative-based approaches for tendon injury. The zebrafish provides an ideal system in which to perform genetic and chemical screens to identify new pathways involved in tendon biology. Until recently, there had been few descriptions of tendons and ligaments in the zebrafish and their similarity to mammalian tendon tissues. In this chapter, we describe the development of the zebrafish tendon and ligament tissues in the context of their gene expression, structure, and interactions with neighboring musculoskeletal tissues. We highlight the similarities with tendon development in higher vertebrates, showing that the craniofacial tendons and ligaments in zebrafish morphologically, molecularly, and structurally resemble mammalian tendons and ligaments from embryonic to adult stages. We detail methods for fluorescent *in situ* hybridization and immunohistochemistry as an assay to examine morphological changes in the zebrafish musculoskeleton. Staining assays such as these could provide the foundation for screen-based approaches to identify new regulators of tendon development, morphogenesis, and repair. These discoveries would provide new targets and pathways to study in the context of regenerative medicine-based approaches to improve tendon healing.

Keywords

Tendon; Ligament; Scleraxis; Zebrafish; Fluorescent in situ hybridization; immunohistochemistry

Introduction

Tendons transmit force generated by the contracting muscle to the bone, enabling movement; ligaments connect bone to bone, maintaining stability. Each year, millions of

individuals in the United States suffer from tendon and ligament injuries (Gomoll, Katz, Warner, & Millett, 2004). Injuries to these tissues have a slow and limited healing potential, and the current treatment options, which include anti-inflammatory treatments, surgery, and/or physical therapy, are often plagued by complications such as re-rupture of the repair site, pain, and limited mobility (Sharma & Maffulli, 2005). In recent years, many groups have examined tissue engineering and stem cell-based approaches for treating these diseases. Although some approaches appear promising, there are currently no widespread accepted treatments for many tendon and ligament injuries (Guerquin et al., 2013; Kiapour, Fleming, & Murray, 2015; Nourissat et al., 2010; Pelled et al., 2012). Limitations likely stem from an incomplete understanding of the fundamental pathways that regulate tendon formation, growth, maturation, and healing.

The molecular signals involved in tendon development have been largely unexplored due to the absence of molecular markers. Accordingly, the discovery of *Scleraxis* as the earliest known marker of tendon and ligament progenitors in the mouse and chick paved the way for investigating the early formation of tendons and ligaments (R. Schweitzer et al., 2001). Subsequent studies in the mouse and chick have identified several factors involved in tendon development, and henceforth, provided fruitful insights into the molecular mechanisms governing tendon formation in the developing embryo (R. Schweitzer, Zelzer, & Volk, 2010). However, many questions remain regarding the molecular networks and cellular behaviors that regulate multiple stages of tendon formation and maturation. With the advent of high-throughput chemical genetic screens in zebrafish, this model offers a vertebrate genetic system amenable to discovery of novel genetic pathways relevant to tendon biology (Kaufman, White, & Zon, 2009). Such discoveries will greatly advance our understanding of the cellular and molecular mechanisms involved in tendon development and have the potential to impact regenerative medicine-based approaches to tendon injury and repair.

Tendon structure

A tendon's capacity to tolerate tensile stress is unrivaled to that of other tissues: human tendons are capable of withstanding up to 9 kN of force, the equivalence of 12.5 times the body weight (Komi, 1990). Their ability to function under such forces is due to their molecular structure. Tendons and ligaments are hierarchically organized tissues with the fundamental structural unit being the collagen fibril (Prockop & Kivirikko, 1995). Macroscopically, the collagen fibrils organize to form collagen fibers (primary bundle), fascicles (secondary bundles), tertiary bundles, and finally the tendon itself (Silver, Freeman, & Seehra, 2003). The biomechanical properties of tendons and ligaments are dependent on the intra- and intermolecular bonds of the collagen network (Butler, Grood, Noyes, & Zernicke, 1978). In adult mice with mutations in collagen or the tendon-enriched proteoglycans, the tendons have abnormal collagen fibrillogenesis (Connizzo, Yannascoli, & Soslowsky, 2013; Gaut & Duprez, 2016), further underscoring the importance of the matrix in tendon development and maturation of the adult tissue.

Tendon formation and differentiation

Tendon and ligament development is intimately connected with the development of the adjacent muscle, cartilage, and bone of the musculoskeletal system (Wortham, 1948). Cells are specified towards a tendon fate and then organize, initially as loose cellular aggregates and then as structurally distinct tendons, at the anatomical interface of the differentiating cartilage and skeletal tissues. The earliest marker of tendon and ligament progenitors in the embryo is *Scleraxis*, a basic helix-loop-helix transcription factor (Figure 1). *Scleraxis* is robustly expressed in the tendon and ligament lineages from the progenitor to the differentiated state (Brent, Schweitzer, & Tabin, 2003; Grenier, Teillet, Grifone, Kelly, & Duprez, 2009; R. Schweitzer et al., 2001). *Scleraxis* can activate the Collagen type I alpha 1 proximal promoter *in vitro* (Lejard et al., 2007), and upregulate the expression levels of proteoglycans enriched in the tendon matrix (Alberton et al., 2012). Moreover, *Scleraxis* positively regulates the expression of Tenomodulin, a marker of differentiated tenocytes, *in vitro* and *in vivo* (Shukunami, Takimoto, Oro, & Hiraki, 2006). *Scleraxis* null (*Scx*^{-/-}) mice are viable as adults but have impaired mobility. In *Scx*^{-/-} embryos, the force-transmitting and inter-muscular tendons are severely disrupted, whereas the ligaments and muscle-anchoring tendons are present. (Murchison et al., 2007) The presence of some populations of tendons and ligaments in *Scx*^{-/-} embryos indicates that while *Scleraxis* is a faithful marker of the lineage, it is not required for the development of all tendons and ligaments.

After specification of the tendon lineage, the progenitor cells organize and aggregate into primordia that differentiate in coordination with the neighboring muscle and skeletal tissues. Tenomodulin is a type II transmembrane protein that is expressed in the axial and limb tendons and ligaments, at a more differentiated stage of the lineage compared to *Scleraxis* (Shukunami et al., 2006). Loss of Tenomodulin results in abnormal collagen fibril structure, (Docheva, Hunziker, Fassler, & Brandau, 2005) indicating a later role for Tenomodulin in collagen matrix maturation. As the tendons mature, the cells secrete collagen type I fibers that initially grow in number and subsequently, in length and diameter, eventually creating the predominantly extracellular matrix tissue that is characteristic of adults (Birk, Zycband, Woodruff, Winkelmann, & Trelstad, 1997; Kalson et al., 2015; Zhang et al., 2005).

The craniofacial tendons and ligaments of the zebrafish (Figure 2A, B) share similar morphological, molecular, and structural characteristics from embryonic to adult stages with that of amniotes (Chen & Galloway, 2014). The tendon and ligament progenitors initiate at the junctional interface between the developing muscle and cartilage or between cartilage segments, respectively, and express *scleraxisa* (*scxa*) (Figure 2C, E). Due to genome duplication events, there exists a second *scleraxis* gene in the zebrafish, *scleraxisb* (*scxb*); however, its expression is only detected by RT-PCR after 54 hours post-fertilization (hpf). At slightly later stages, following *scxa* expression, the expression of extracellular matrix proteins *tenomodulin* (Figure 2D, F), Thrombospondin-4b, and *collagen type I alpha 2* can be observed (Chen & Galloway, 2014; Subramanian & Schilling, 2014). Moreover, *xirp2a*, an actin-binding protein, is expressed in the myosepta and all muscle attachment sites in the head and fin (Chen & Galloway, 2014; Otten et al., 2012). In the adult zebrafish, the tendon and ligaments are characterized by a *D*-periodicity, which results from a highly organized

network of collagen fibrils and resembles the mammalian tendon structure (Chen & Galloway, 2014; Docheva et al., 2005).

Tissue interactions within the developing musculoskeletal system

Tendons and ligaments develop in multiple anatomic locations: the craniofacial, limb/fin, and axial regions. The induction and maintenance of tendons and ligaments are similar across vertebrate species, with some differences in the developmental program between the craniofacial, limb/fin, and axial tendons, which arise from distinct embryonic origins. Nevertheless, tendons and ligaments in all locations originate from common progenitor populations that also will give rise to cartilage and bone (Soeda et al., 2010) but are distinct from muscle forming regions.

The jaw develops into a functioning musculoskeletal system early in the zebrafish, with cartilage and muscle progenitors present in the pharyngeal arches by 48 hpf and feeding evident by 96 hpf. The pharyngeal cartilage and muscle progenitor populations develop in synchrony and in close proximity to each other, as is observed in musculoskeletal development of amniotes (Schilling, 1997; Schilling & Kimmel, 1997). Fate mapping experiments have determined that the cartilage and connective tissue of the jaw in the zebrafish and higher vertebrates originate from the cranial neural crest, a vertebrate-specific multipotent cell population (Chen & Galloway, 2014; Couly, Coltey, & Le Douarin, 1993; Kontges & Lumsden, 1996; Le Lievre, 1978; Noden, 1978; Schilling & Kimmel, 1994). Similarly, the limb tendons/ligaments and skeletal structures of amniotes originate from the lateral plate mesoderm-derived limb mesenchyme (Pearse, Scherz, Campbell, & Tabin, 2007; Wachtler, Christ, & Jacob, 1981). Although both skeletal and tendon lineages arise from common progenitor populations, our understanding of the dynamics of their interactions has been limited until recently. In zebrafish, the specification of tendons and ligaments occurs independently of the cartilage program, though a properly formed cartilage template is critical for proper tendon/ligament organization (Chen & Galloway, 2014). This finding gains support from studies in the mouse, wherein the tendons of the middle limb segment, the zeugopod, form in *Sox9*-deficient limbs lacking cartilage although the digit tendons in the distal autopod segment are dependent upon cartilage (Huang et al., 2015). In the context of bone eminences, the initiation of the *Sox9*-positive cartilage and *Scleraxis*-positive tendon populations is independent of each other as well as the enthesis domain (Blitz, Sharir, Akiyama, & Zelzer, 2013; Sugimoto et al., 2013).

The molecular crosstalk between the muscle and the associated tendon has been well-characterized in vertebrates. Studies in mice revealed that, in the head, *Scleraxis*-positive tendon progenitors initiate independently of the branchiomic muscles, although muscle is required for continued maintenance of tendon fate (Grenier et al., 2009). In the limb, tendon progenitor specification is also muscle-independent, whereas the maintenance of limb tendon fate is muscle-dependent (Brent, Braun, & Tabin, 2005; Edom-Vovard, Schuler, Bonnin, Teillet, & Duprez, 2002; Kardon, 1998; Shellswell & Wolpert, 1977). Divergent from this developmental archetype are the distal tendons of the autopod, which initiate and form in muscle-less limbs (Huang et al., 2015; Hurler et al., 1990; Kieny & Chevallier, 1979). Conversely, the myogenic program initiates independently of tendon progenitors in all

anatomic locations, but the proper patterning and differentiation of the myofibers is dependent on the tendon and connective tissues (Kardon, 1998; Rinon et al., 2007).

We have shown that a similar program exists in the zebrafish, wherein initiation of *scxa* expression in cranial and pectoral fin regions is muscle-independent, but its continued expression is muscle-dependent (Chen & Galloway, 2014). Together, the dissection of these interactions suggests a complex interdependence of the musculoskeletal tissues at distinct stages of development. Although FGF signaling has been implicated in some of these interactions (Brent et al., 2003; Brent & Tabin, 2004; Edom-Vovard et al., 2002; Rinon et al., 2007; Smith, Sweetman, Patterson, Keyse, & Munsterberg, 2005), we still have a limited understanding of the molecular crosstalk regulating the coordination of musculoskeletal development, assembly, and growth.

The axial musculoskeletal tissues arise from the somites, which differentiate into the dermamyotome (gives rise to the dermis and muscle) and the sclerotome (gives rise to the vertebrae and ribs) (Brand-Saberi & Christ, 2000; Christ & Ordahl, 1995). In amniotes, the somite is comprised predominantly of the medially-positioned sclerotome, and vertebral elements develop synchronously with the associated musculature (Christ, Huang, & Wilting, 2000; Grotmol, Kryvi, Nordvik, & Totland, 2003). Axial tendons originate from the syndetome, a somitic compartment that is established by myotomal FGF signals to the adjacent sclerotome to induce formation of tendon progenitors (Brent et al., 2003). Specification of the sclerotome to a tendon-fated syndetome is mutually exclusive of a cartilage fate (Brent et al., 2005). In teleosts, by contrast, the somite is composed predominantly of myotome with a relatively small ventral sclerotome (Stickney, Barresi, & Devoto, 2000). The formation of the axial cartilage and myotome is not synchronized – differentiated myofibers are present in the embryo, whereas the cartilage template forms in the larvae (Bird & Mabee, 2003; Devoto, Melancon, Eisen, & Westerfield, 1996). The myomeres are separated along the dorsal-ventral axis by the horizontal myoseptum and along the anterior-posterior axis by the vertical myosepta. The horizontal and vertical myosepta both function in force transmission during undulatory movement (Nursall, 1956; Westneat, Hoese, Pell, & Wainwright, 1993). Together, they are thought to be homologous to the axial tendons of higher vertebrates based on similarities in architecture and a gene expression profile enriched in mammalian tendon matrix proteins (Bricard, Ralliere, Lebret, Lefevre, & Rescan, 2014). However, there appear to be some differences in their origins that are distinct from higher vertebrates. Early expression of extracellular matrix (ECM) molecules in zebrafish horizontal myosepta by 24 hpf is thought to derive from the muscle pioneer cells, which originate from the myotome (Devoto et al., 1996; Felsenfeld, Curry, & Kimmel, 1991; Hatta, Bremiller, Westerfield, & Kimmel, 1991; J. Schweitzer et al., 2005). Although lineage tracing of the myosepta in teleosts is lacking, the sclerotome is postulated to give rise to the *scxa*-expressing cells in the vertical myosepta. Consistent with this idea, the induction of axial *scxa*-expressing tendon progenitors in zebrafish is muscle-dependent (Chen & Galloway, 2014), which is consistent with the mode of amniote axial development.

The architecture of the vertebrate body axis presents the myosepta as a model for the formation of the myotendinous junction (MTJ), the primary site of force transmission in skeletal muscle. As MTJ-associated defects lead to a subset of muscular dystrophies (Berger,

Berger, Hall, Lieschke, & Currie, 2010), a more comprehensive understanding of the ECM-dependent interactions between the muscle sarcolemma and tendon cells should prove fruitful in developing therapeutics for such conditions. Indeed, drug screening approaches for chemical modulators of a muscular phenotype in a zebrafish dystrophy model have identified molecules and pathways that could be candidate therapeutic targets for these diseases (Kawahara et al., 2011; Kawahara & Kunkel, 2013).

The highly organized collagenous vertical myosepta are a region important in development of the myomeres, functioning specifically as attachment sites for the sarcolemmal basement membrane (Charvet, Malbouyres, Pagnon-Minot, Ruggiero, & Le Guellec, 2011; Henry, McNulty, Durst, Munchel, & Amacher, 2005). Studies in the zebrafish have identified several factors with a role in the stabilization, maintenance and repair of the MTJ, including *collagen type XXII alpha 1*, *laminin alpha 2*, *thrombospondin-4b*, *focal adhesion kinase* and *beta-dystroglycan* (Charvet et al., 2013; Hall et al., 2007; Snow & Henry, 2009; Subramanian & Schilling, 2014). These findings contribute to our understanding of the cellular and molecular mechanisms involved in development of the MTJ and have the potential to advance therapeutic-based approaches to congenital muscle disorders.

Methods to study the embryonic tendon program in zebrafish

Here we describe technologies to assess the development, differentiation, and patterning of tendon and ligament tissues. Our whole-mount protocol combines fluorescent *in situ* hybridization (FISH) and immunohistochemistry (IHC) to evaluate expression of multiple genes and/or proteins (Figure 3) in zebrafish embryos. We employ tyramide signal amplification (TSA) technology to robustly label nucleic acid sequences and proteins of interest *in situ*, thereby facilitating spatial resolution at a cellular level using confocal microscopy (Wang, Achim, Hamilton, Wiley, & Soontornniyomkij, 1999). TSA uses enzymatic activity to amplify the signal intensity of a fluorescent-labeled target protein or nucleic acid. We have employed antibodies to stain for muscle proteins, but staining for cartilage can be performed using the Col2a antibody (Clement et al., 2008).

Our strategy can be modified to evaluate tendon and ligament development in the context of the musculoskeletal system when assessing gene function using pharmacological agonists and inhibitors, morpholino oligonucleotides to prevent mRNA translation, or CRISPR/Cas 9 (clustered regularly interspaced short palindromic repeats (CRISPR)/ CRISPR-associated protein 9) knockout/knockin methods (Kok et al., 2015; Lawson & Wolfe, 2011; Varshney, Sood, & Burgess, 2015). Because zebrafish provide a robust regenerative model system, our methods could also be applied to cell ablation and regeneration experiments pertinent to musculoskeletal tissues.

Materials

Preparation of embryos

1. 50X E3 Buffer: 250 mM NaCl, 8.5 mM KCl, 16.5 mM CaCl₂, 16.5 mM MgSO₄ in distilled water. Prepare as 50X stock and dilute to 1X working solution in distilled water. Store at room temperature.

2. 0.003% *N*-Phenylthiourea (PTU; Sigma-Aldrich #P7629) in E3 Buffer. To dissolve, mix on stir plate overnight at room temperature. Store at room temperature. Use caution – phenylthiourea is a toxic chemical and is an irritant to the skin.
3. Pronase (Sigma-Aldrich #10165921001): prepare as 50 mg/mL stock in distilled water. Store at –20°C.
4. 4% paraformaldehyde (PFA): 4% (wt/vol) solution of paraformaldehyde (Electron Microscopy Sciences #RT19202) in 1X phosphate buffered saline. To dissolve, heat to 95°C (do not boil) with constant mixing on a stir plate. Store at –20°C. Thaw fresh in 37°C waterbath before use. Use caution – paraformaldehyde is a corrosive, toxic chemical and is an irritant to the skin, eyes, and respiratory tract.
5. 10X phosphate buffered saline (PBS): 1.37 M NaCl, 27 mM KCl, 100 mM Na₂HPO₄, 18 mM KH₂PO₄ in distilled water. Adjust the pH to 7.4. Autoclave and store at room temperature. Prepare as 10X stock and dilute to 1X working solution in distilled water.
6. 1X phosphate buffered saline with 0.1% Tween-20 (PBT). Store at room temperature.
7. Tween-20 (Sigma-Aldrich #P1379).
8. Bleach solution: 0.8% KOH, 0.9% H₂O₂ (Fisher Scientific #H325) in distilled water. Prepare fresh on the day of use. Use–caution hydrogen peroxide (H₂O₂) is a corrosive, toxic chemical and is an irritant to the skin and eyes.
9. Methanol. Use caution–methanol is a flammable, toxic chemical and is an irritant to the skin and eyes; it is harmful to organs if ingested.

Fluorescent in situ hybridization and immunohistochemistry

- 1 Phosphate buffered saline (PBS). See above.
- 2 1X phosphate buffered saline with 0.25% Tween-20 (PBST). Store at room temperature. NB: A higher concentration of Tween-20 is used for the FISH portion of the protocol.
- 3 Methanol. See above.
- 10 Proteinase K (Sigma-Aldrich #3115879001): prepare as 10 mg/mL stock in distilled water. Store at –20°C.
- 4 0.2% glutaraldehyde (Sigma-Aldrich #G6257)/4% paraformaldehyde/1X PBS. Prepare fresh day of use. Use caution–glutaraldehyde is a toxic chemical and is an irritant to the skin, eyes, and respiratory tract.
- 5 Hybridization solution: 50% Formamide (Fisher Scientific #F841), 5X SSC pH 4.5, 2% SDS, 2% Blocking Reagent (Sigma-Aldrich #11096176001), 250 µg/mL yeast RNA (Life Technologies #15401029), 100 µg/mL Heparin (Fisher

Scientific #BP2524100. Store solution at -20°C . Use caution – formamide is a toxic chemical and is an irritant to the skin, eyes, and respiratory tract.

- 6 20X SSC Buffer: 3 M NaCl, 300 mM sodium citrate in distilled water. Adjust the pH to 4.5 with citric acid. Prepare as 20X stock and dilute to 5X and 2X working solutions in distilled water. Store at room temperature.
- 7 Sodium dodecyl sulfate (SDS): prepare as 10% stock in distilled water. Store at room temperature. Use caution – sodium dodecyl sulfate is a flammable, toxic chemical and is an irritant to the skin, eyes, and respiratory tract; it is harmful to organs if ingested.
- 8 Dextran sulfate (Sigma-Aldrich #S4030).
- 9 Solution I: 50% Formamide, 2X SSC pH 4.5, 1% SDS in distilled water. Store at 4°C .
- 10 2% H_2O_2 : dilute 30% stock 1:15 in respective solution. Prepare fresh day of use.
- 11 TNT: 0.1 M Tris-HCl pH 7.5, 0.15 M NaCl, 0.5% Tween-20 in distilled water. Store at room temperature.
- 12 TBSTB: TNT with 0.5% Blocking Reagent. Prepare fresh the day of use and store at 4°C for short-term storage.
- 13 Anti-Digoxigenin-Peroxidase (Sigma-Aldrich #11207733910).
- 14 Anti-Fluorescein-Peroxidase (Sigma-Aldrich #11426346910).
- 15 TSA Plus Cyanine 3 and Fluorescein system (Perkin Elmer #NEL753001KT).
- 16 TSA Plus Cyanine 5 system (Perkin Elmer #NEL745001KT).
- 17 Newborn calf serum (NBCS; ThermoFisher Scientific #16010159). Store at -20°C and thaw in 37°C waterbath prior to use. Prepare as 10% solution in PBST fresh the day of use and store at 4°C for short-term storage.
- 18 *Optional*: Hoechst 33342 (Invitrogen #H1399).
- 19 *Optional*: DAPI (Invitrogen #D1306).

Preparation of embryos for Imaging

1. Glycerol (Sigma-Aldrich #G7757).
2. Glass microscope slides (Fisher Scientific #12-550-A3).
3. Microscope cover glass, 18 X 18 mm (Fisher Scientific # 12-542A).
4. Modeling clay non-hardening (EZ Shape).
5. Probe (Fine Science Tools #10140-01).
6. Dumont #55 forceps (Fine Science Tools #11255-20).
7. *Optional*: glass bottom culture dishes (MatTek #P50G-1.5-14F)

Methods

Breeding of embryos—Zebrafish husbandry is performed as described in The Zebrafish Book (Westerfield, 1995). Eggs are collected from mating pairs approximately 10–30 minutes after laying. The embryos develop in E3 Buffer at 28.5°C. In embryos >24 hpf, pigmentation is inhibited by the prior addition of 0.003% PTU to the E3 Buffer at 15–22 hpf. The chorions are removed by incubation with 1 mg/mL pronase for 20 minutes at 28.5°C, and embryos are rinsed twice with E3 Buffer. Alternatively, chorions may be removed using forceps. The embryos continue to develop in E3 Buffer at 28.5°C until the desired developmental stage. For observing developing cranial tendons, 48–72 hpf are ideal stages.

Fixation, bleaching, and dehydration of embryos

1. Transfer the embryos to 1.5 mL Eppendorf tubes.
2. Embryos are euthanized using a buffered tricaine solution.
3. Fix the euthanized embryos in 4% PFA/1X PBS overnight with gentle rocking (BioExpress GeneMate Rocker #R-3200-1) at 4°C. The fixation step preserves the morphology of the tissue, but over-fixation will result in a reduced hybridization signal.
4. Rinse and wash embryos twice in PBT for 5 minutes each at room temperature. Tween-20 is a mild surfactant that helps to prevent the embryos from sticking to the sides of the tube.
5. Wash embryos in bleach solution with rocking at room temperature until the melanin pigment of the embryos is mostly lost. The bleaching interval varies between 5–20 minutes depending on the developmental stage of the embryo.
6. Rinse embryos in PBT for at least 5 minutes.
7. Fix the embryos in 4% PFA/1X PBS for at least 1 hour with rocking at room temperature.
8. Dehydrate the embryos into 100% methanol through a graded methanol/PBT series (25%, 50%, 75% methanol in PBT), allowing 5 minutes with each solution at room temperature. Wash embryos twice in 100% methanol for 5 minutes each at room temperature.
9. Store in 100% methanol at –20°C for at least 2 hours (and up to several months) prior to proceeding with *in situ* hybridization.

Riboprobe generation—To detect two different RNA transcripts, synthesize the probes with different epitopes: digoxigenin-UTP and fluorescein-UTP. Instructions for synthesis of digoxigenin-labeled (Sigma-Aldrich #11175025910) and fluorescein-labeled (Sigma-Aldrich #11685619910) riboprobes may be obtained from the manufacturer. The fluorescein fluorophore is stronger than the Cy3 fluorophore, so we recommend synthesizing the riboprobe of the transcript with the weakest signal with digoxigenin-labeled NTPs, followed by detection of the riboprobe with anti-digoxigenin-peroxidase and amplification of the

signal with fluorescein-tyramide. Conversely, the riboprobe of the transcript with a stronger signal should be synthesized with fluorescein-labeled NTPs, followed by detection of the riboprobe with anti-fluorescein-peroxidase and amplification of the signal with Cy3-tyramide. We perform the protocol with the antibodies in the following order – Tyr-Cy3, Tyr-Fluorescein, and Tyr-Cy5. We have never detected fluorescence from the fluorescein-labeled riboprobe alone, indicating the fluorescein-labeled riboprobe does not interfere with fluorescein-tyramide signal detection.

Day 1. Hybridization of riboprobe(s)

1. Rehydrate the embryos into PBST through a graded methanol/PBST series (75%, 50%, 25% methanol in PBST) with 5 minutes each at room temperature. Wash 4 X 5 minutes each in PBST at room temperature.
2. Incubate the embryos with Proteinase K in PBST to permeabilize the tissue to allow access to the riboprobe(s). Optimal concentration and duration is dependent on the tissue type, activity of the enzyme, and developmental stage of the embryo (suggestions below are for embryos to be processed for both FISH and IHC). If your procedure does not include antibody staining, we recommend using concentrations and durations known to be effective. Insufficient treatment will reduce hybridization signal; whereas overly aggressive treatment will result in reduced tissue integrity, which may lead to shearing of specimen in subsequent washing steps and/or problems with the antibody detection.

Stage	Time	Concentration
24 hpf	10 min	1 µg/mL
30 hpf	20 min	1 µg/mL
36 hpf	30 min	1 µg/mL
48 hpf	45 min	1 µg/mL
55 hpf	1 hour	1 µg/mL
60 hpf	25 min	10 µg/mL
72 hpf	30 min	10 µg/mL
> 72 hpf	~ 1 hour	10 µg/mL

3. Fix the embryos in 0.2% glutaraldehyde/4% PFA/1X PBS for 20 minutes with rocking at room temperature to stop Proteinase K digestion.
4. Wash embryos 4 X 5 minutes each in PBST at room temperature.
5. Incubate embryos with pre-warmed hybridization solution containing 5% dextran sulfate for at least 1 hour with rocking at 68–70 C. Dextran sulfate increases effective riboprobe concentration, thereby promoting hybridization (Matthiesen & Hansen, 2012; Wahl, Stern, & Stark, 1979).
6. Prepare a mix of 1 µL riboprobe (no more than 1 µg of each probe) per 100 µL pre-warmed hybridization solution containing 5% dextran sulfate for each sample. Incubate the riboprobe/hybridization mix for 5–10 minutes at 68–70 C.

7. Incubate each sample with sufficient riboprobe/hybridization mix to completely submerge the embryos.
8. Incubate samples overnight with rocking at 68–70 C.

Day 2. Post-hybridization washes and antibody incubation for the first probe

1. Remove riboprobe/hybridization mix and store at –20C for future reuse.
2. Rinse and wash samples 4 X 30 minutes each in Solution I with rocking at 68–70 C.
3. Wash samples 2 X 10 minutes each in PBST at room temperature.
4. Incubate samples in 2% H₂O₂/PBST for 1 hour with rocking at room temperature.
5. Wash samples 4 X 5 minutes each in TNT at room temperature.
6. Incubate samples in TBSTB blocking buffer for 1–4 hours with rocking at room temperature.
7. Replace blocking buffer with anti-Fluorescein-POD diluted 1:4000 (if double FISH) or anti-DIG-peroxidase diluted 1:1000 (if single FISH) in TBSTB blocking buffer.
8. Incubate samples overnight with rocking at 4 C.

Day 3. Antibody detection of first probe and antibody incubation for second probe

1. Wash 8X in TNT over the course of 1–2 hours at room temperature with rocking.
2. Wash 5 minutes in 50 µL Amplification Diluent at room temperature.
3. Prepare Tyr-Cy3 (if double FISH) or Tyr – Fluorescein (if single FISH) diluted 1:50 in Amplification Diluent, using 50 µL per sample. Centrifuge each solution for 3 minutes at high speed in a microcentrifuge to pellet any precipitate. Avoid pipetting from bottom of tube in the next step.
4. Incubate each sample in freshly prepared 50 µL Tyr-Cy3 or Tyr-Fluorescein mixture for 1 hour (do not exceed time) in the dark with tubes upright on a rocking platform at room temperature. All subsequent steps in the protocol are performed in the dark to avoid photo-bleaching of fluorophores. Incubation time is dependent on the riboprobe and may be shortened to increase signal-to-background ratio.
5. Wash each sample 2 X 5 minutes each in TNT at room temperature.
6. Incubate in 2% H₂O₂/TNT for 1 hour with rocking at room temperature. (If doing single FISH and IHC, proceed to the IHC section)
7. Wash samples 4 X 5 minutes each in TNT at room temperature.

8. Incubate in TBSTB blocking buffer for 1–4 hours with rocking at room temperature.
9. Replace the blocking buffer with anti-digoxigenin-peroxidase diluted 1:1000 in TBSTB blocking buffer.
10. Incubate samples overnight with rocking at 4 C.

Day 4. Antibody detection of second probe

1. Wash samples 8X with TNT over the course of 1–2 hours at room temperature with rocking.
2. Wash samples for 5 minutes in 50 μ L Amplification Diluent at room temperature.
3. Dilute Tyr-Fluorescein 1:50 with Amplification Diluent, allowing 50 μ L per sample. Centrifuge the solution for 3 minutes at high speed in a microcentrifuge to pellet any precipitate. Avoid pipetting from bottom of tube in the next step.
4. Incubate each sample in freshly prepared 50 μ L Tyr-Fluorescein mixture for 1 hour (do not exceed time) in the dark with tubes upright on rocking platform at room temperature. Wash samples 2 X 5 minutes each in TNT at room temperature.
5. Incubate samples in 2% H_2O_2 /TNT for 1 hour rocking at room temperature. (If doing double FISH and IHC, go to IHC section)
6. Wash samples 2 X 5 minutes each in TNT at room temperature.
7. Wash samples 2 X 5 minutes each in PBST at room temperature.
8. Store the embryos in PBST at 4 C.

Day 5. Immunohistochemistry (IHC) – Primary Antibody—Detection of protein begins after completion of the peroxide inactivation step of the FISH protocol. The IHC protocol may need to be optimized for each antibody – troubleshooting may involve adjusting variables such as the blocking buffer, proteinase K digestion, and antibody dilution.

1. Wash samples 2 X 5 minutes each in TNT at room temperature.
2. Wash samples 2 X 5 minutes each in PBST at room temperature.
3. Incubate samples in 10% NBCS/PBST blocking buffer for 1 hour with rocking at room temperature.
4. Replace solution with primary antibody diluted in 10% NBCS/PBST blocking buffer. If using peroxidase-conjugated secondary antibodies, which we recommend for proteins that are expressed at low levels, dilute the primary antibodies 1:500. If using Alexa Fluor-conjugated secondary antibodies, dilute the primary antibodies 1:100. These dilutions are optimized for Myosin Heavy Chain antibodies A4.1025 and MF20 (Developmental Studies Hybridoma Bank (DSHB), Iowa City, Iowa); other antibodies may require additional optimization.

5. Incubate samples overnight with rocking at 4 C.

Day 6. Immunohistochemistry – Secondary Antibody

1. Rinse and wash samples 4 X 30 minutes each in PBST at room temperature.
2. Incubate samples in 10% NBCS/PBST blocking buffer for 1 hour with rocking at room temperature.
3. Replace solution with secondary antibody diluted in 10% NBCS/ PBST blocking buffer. If using peroxidase-conjugated secondary antibody, dilute 1:500. If using Alexa Fluor-conjugated secondary antibodies, dilute 1:400. To confirm the specificity of the secondary antibody for the primary antibody, prepare control samples containing the secondary alone.
4. Incubate overnight with rocking at 4 C.

Day 7. Detection of secondary antibody

1. Rinse and wash samples 4 X 30 minutes each in PBST at room temperature. (If using an Alexa Fluor-conjugated secondary antibody, go to step 8. Continue to step 2 if using a peroxidase-conjugated secondary antibody.)
2. Wash samples for 5 minutes in 50 μ L Amplification Diluent at room temperature.
3. Prepare Tyr-Cy5 diluted 1:50 in Amplification Diluent, using 50 μ L per sample. Spin down for 3 minutes at high speed in a microcentrifuge to pellet any precipitate. Avoid pipetting from bottom of tube in the next step.
4. Incubate each sample in freshly prepared 50 μ L Tyr-Cy5 mixture for 30 minutes in the dark with tubes upright on a rocking platform at room temperature.
5. Wash samples 6 X 10 minutes each in PBST at room temperature.
6. Fix the embryos in 4% PFA/1X PBS for 30 minutes with rocking at room temperature.
7. Rinse and wash samples 2 X 5 minutes each in PBST at room temperature.
8. *Optional:* To counterstain the embryos, incubate in Hoechst 33342 diluted 1:2000 or DAPI diluted 1:1000 in PBST for 30 minutes with rocking at room temperature. Wash samples 2 x 5 minutes each in PBST at room temperature.
9. Store the embryos in PBST at 4C.

Preparation of embryos for Imaging

1. Equilibrate the embryos into 100% glycerol for imaging.
2. Dissect the head region of an embryo away from the trunk and yolk sac with forceps.
3. Prepare a coverslip by applying clay around edges to create a slight elevation between the microscope slide and the coverslip when mounting the embryos.

4. Place a small drop of glycerol onto a microscope slide and transfer the head region of the embryo to the center. Orient the embryo to the desired orientation with forceps and apply the clay-covered coverslip.
5. As an alternative, glass bottom culture dishes may be used for mounting on inverted microscopes. This method does not necessitate clay-covered coverslips.
6. High magnification images are taken with a Zeiss LSM710 NLO or comparable laser-scanning confocal microscope. Digitized images are saved as TIFF files and subsequently processed in Photoshop.

Conclusion

This chapter describes the zebrafish as a model for the study of tendon development and details a method to examine the expression of mRNA and protein in the forming zebrafish musculoskeletal system. The recent development of high-throughput platforms has prompted the use of small molecule chemical screening to identify novel regulators of developmental processes in zebrafish these – molecules may have therapeutic benefits (Rennekamp & Peterson, 2015). The genetic tools available in the zebrafish, in combination with transgenic approaches, make the zebrafish system amenable for rapid functional analysis of candidate molecules and target pathways. In addition, the robust regenerative capacity of zebrafish will facilitate future studies aimed at understanding the molecular and cellular mechanisms of tendon healing and regeneration. We believe such studies will lead to new discoveries that can be placed within the larger framework of vertebrate musculoskeletal formation and serve to advance the development of regenerative-medicine-based solutions to tendon injuries and disease.

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Figure 1. Expression of *Scx* in wild-type mouse embryos. (A) *Scx*-GFP mouse embryo at E10.5. (B) Section *in situ* hybridization of *Scx* expression (arrowheads) between cartilage and muscle in the forelimb at E14.5.

Figure 2.

Expression of tendon genes in the zebrafish musculoskeleton. Schematic (A) ventral and (B) lateral views of craniofacial region at 72 hpf with muscle, cartilage, and tendon/ligament depicted. Expression of *scxa* (C, E) and *tnmd* (D, F) in the craniofacial region (77 and 99 hpf, respectively) and myosepta (48 and 96 hpf, respectively). Ventral (C, D) and lateral (E, F) views of flat-mounted embryos were processed for the respective transcripts by fluorescent *in situ* hybridization.

Figure 3.

Tendon genes (*tnmd* and *xirp2a*) are co-expressed at the sternohyoideus muscle attachment (arrow) in zebrafish craniofacial region at 99 hpf. (A) Maximum-intensity projection. (B–E) Single channel optical sections from confocal image. Fluorescent *in situ* hybridization was performed for *tnmd* and *xirp2a* to visualize the tendons. Muscle is indicated by A4.1025 antibody staining for Myosin Heavy Chain 1 (MYH1). Ventral view of flat-mounted embryo.