INTRODUCTION. We are interested in characterizing structure-function relationships of embryonic tendon to elucidate mechanisms involved in the elaboration of mechanical properties during development. We previously quantified changes in small-scale elastic modulus of embryonic chick tendon during normal development using force-volume atomic force microscopy (FV-AFM)1. We found that average tendon modulus increases by 3-10 fold over development when measured with nano- or micro-scale tips, respectively, and that modulus nearly doubles every 24 hours during late stages of development. Others have shown similar trends in tendon modulus during late embryonic development2, but a defined correlation between mechanical and compositional changes in developing tendon has not yet been established. In adult tendon, increases in dry mass content are coincident with increases in tensile stiffness3 and cell remodeling of adult ligament has been shown to reduce tensile stiffness compared with normal ligament4. Additionally, several reports have shown that inhibition of enzymatic collagen crosslink formation using the lysyl oxidase inhibitor BAPN significantly reduces mechanical properties in tissues such as the vertebral column5, skin and bone6, and aorta7. Thus, to provide insight into the relationships between tissue structure and mechanical properties of embryonic tendon, we investigated trends in cell and matrix content and also the effects of β-aminopropionitrile (BAPN) treatment as a function of developmental stage. Building upon our previous work1, the goal of this study was to characterize cellular and matrix contributions to tissue elasticity of embryonic tendon.

METHODS. Chick culture and limb harvest: All animal procedures received approval from the university IACUC review board. Chick embryos were treated with 5 mg BAPN/g dry embryo weight via injection in ovo and incubated for 12 hours. For explant cultures, chick embryos were sacrificed, staged at HH 408, and whole limbs were harvested and cultured at 37° C in complete medium of DMEM/10% FBS for 12 hours. Explant culture conditions included: (i) culture in complete medium or (ii) complete medium with 5 mg BAPN/g dry tissue weight. Tendon dry/wet mass and DNA/dry mass ratios: Tendon dry-to-wet mass ratio was obtained by weighing the wet mass after lightly blotting the tissue, and then weighing the dry mass after lyophilization. The DNA mass from these dry tissues was assayed spectrophotometrically using an established PCI-ethanol isolation and precipitation method8. Multiphoton imaging: To assess the morphology and density of collagen in tendon, fresh tendons in saline were scanned with an 800 nm laser source on a confocal microscope. Forward second harmonic generation (SHG) was captured through a 400 nm hardware filter using a 63x water immersion lens.

FV-AFM: A 20 nm radius AFM probe with 0.06 N/m spring constant was employed. A 16x16 indentation array was captured over a 10x10 µm area on tissue that had been cryosectioned at 20 µm thickness. Linear regions of AFM force curves were analyzed and compared with a calibration curve developed with agarose gels to derive elastic modulus. AFM measurements were performed on tendon tissue in PBS.

RESULTS. We found that tendon dry-to-wet mass ratio varied considerably during development, with rapid increases during late development (Fig. 1a). These tissues also showed a progressive 3-fold decline in DNA-to-dry mass ratios with time (Fig. 1b). We characterized the normal development of tendon collagen fibers using SHG imaging (Fig. 2) and observed progressive accumulation of collagen density and organization as a function of time, beginning at HH 35. When treated with BAPN in ovo, no significant differences in tendon collagen density or morphology were observed qualitatively, as compared to saline treated controls (Fig. 2). However, BAPN treatment reduced average tendon elastic modulus by 45%, as measured via FV-AFM (Fig. 3).

DISCUSSION. Recently, collagen crosslinking density in adult human tendon has been shown to increase with age9 and vary with tendon location10, providing insight into adult tendon material properties. In contrast, very little is known about embryonic tendon compositional changes and their correlation with mechanical properties throughout development. In this study we investigated the potential contributions of tissue constituents to apparent elasticity of embryonic tendon. We show that during the rapid increase in apparent modulus in late tendon developmental stages (HH 38-43), dry-to-wet mass and DNA-to-dry mass ratios are inversely related with time: dry mass content increases and cellularity decreases (Fig. 1). These results suggest that cellular contributions to tendon elasticity may be more significant during early development, and that dry mass content may be related to late stage elasticity. Also, our preliminary results from BAPN treatment (Figs. 2-3) suggest that collagen crosslinks may contribute to tendon elasticity. We found that BAPN treatment resulted in little qualitative difference in collagen fiber density or morphology (Fig. 2), but reduced average elastic modulus by 45% when measured with a nanoscale tip (Fig. 3). Overall these results provide initial insight into potential mechanisms responsible for the development of tendon elasticity. We are currently investigating these and other elements that may contribute to tendon mechanical properties during embryonic development.