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Dynamics of bone extracellular matrix assembly and mineralization

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The extracellular matrix (ECM) of skeletal tissues provides a structural framework for mineralization and modulates functions such as cell adhesion/migration and growth factor signaling. Using live cell computational imaging, we have recently shown that the ECM in living osteoblast cultures is highly dynamic and that cells continually stretch and contract the ECM fibrils. We have also shown that fibronectin is a key regulator of osteoblast mineralization, via its role in orchestrating assembly of multiple bone ECM proteins. To gain further insights into the mechanisms of bone ECM assembly and mineralization, we have performed dynamic time lapse imaging in living osteoblasts using fluorescent probes for fibronectin and for other bone ECM components. Alizarin red was also used as a vital stain for mineralization. Computational techniques were developed to analyze cell, ECM and mineralization dynamics.

Primary rat osteoblasts were incubated with fluorescent probes for fibronectin, collagen and latent TGF-β binding protein-1. Cells and fibrils were imaged every 15 minutes for 24-48 hours. Time-lapse movies showed surprisingly large cell movements resulting in stretching, contracting and occasional breaking of ECM fibrils. Motile cells appeared to actively participate in ECM assembly and reorganization by moving and adding "packets" of fibrillar material onto growing fibrils, shunting fibrillar material from one location to another and/or exchanging material between fibrils. Time lapse imaging showed that fibronectin assembly, either *de novo* or in the presence of a mature ECM, initiated as short fibrils/patches on the cell surface that progressively coalesced to form larger fibrils. Multiple cells contributed to the formation of individual fibrils. Quantitative analyses confirmed a progressive increase in fibril size with time and a decrease in the number of fibronectin particles, consistent with a hierarchical mechanism for fibril assembly through coalescence of smaller units. Particle Image Velocimetry analysis showed that cell and ECM fibril motions were correlated. The dynamic properties of ECM fibrils were also found to change with ECM maturation.

We next performed pulse-chase studies in which existing fibronectin fibrils were labeled with a green fibronectin probe and newly assembled fibronectin was labeled with a red probe. These studies showed that newly assembled fibronectin incorporated in small cell surface fibrils that were distinct from pre-existing fibrils but were then actively reorganized by motile cells to become co-localized with existing fibrils. Image correlation analysis confirmed that red and green fibronectin images became progressively more correlated with time. To validate observations in primary osteoblasts, fibronectin assembly was also imaged in mouse calvarial explants. Osteoblasts on the bone surface appeared to assemble fibronectin via a similar hierarchical mechanism as seen in cell cultures and the process was mediated by motile cells within the surface layer.

Mineralization was examined using primary osteoblasts isolated from 5-day-old transgenic mice expressing green fluorescent protein (GFP) under control of the dentin matrix protein-1 (Dmp1) promoter. After 10-14 days in culture small foci of GFP-positive cells were observed, which delineated the sites where mineralization would occur. Time lapse imaging of mineralization was performed using alizarin red as a vital stain. After the addition of beta glycerol phosphate, mineralization initiated from small focal areas within clusters of GFP-positive cells and radiated outwards. The mineralization process was rapid and quantitative analysis of alizarin red fluorescence showed that the process was essentially complete within a 10-hour period. We also performed dynamic imaging on whole calvarial explants from 5-12-day-old Dmp1-GFP mice. Embedded osteocytes expanded and contracted their cell body within the boundaries of their lacunae

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and extended and retracted their dendrites. Partially embedded osteocytes sent out long pseudopodia over the bone surface. A subpopulation of the surface cells, that presumably represent a late osteoblast/preosteocyte, was also GFP positive. These cells were highly motile and appeared to interact with multiple osteocytes.

Together, these studies suggest that cells within the bone environment may be more dynamic than previously thought and highlight novel cell-mediated mechanisms for the assembly and subsequent reorganization of the ECM. Our findings also suggest a critical role for cell motility and cell-generated mechanical forces in shaping ECM fibrils.