Intravital imaging of osteocyte membrane dynamics using fluorescent nanoparticles shows perturbation with dynamin and cholesterol inhibitors.

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INTRODUCTION: Osteocytes are the resident mechanosensory cells in bone, maintaining skeletal health and homeostasis. Integrins are dynamic heterodimer proteins on the plasma membrane that facilitate cell adhesion, and in osteocytes, mediate strain amplification and mechanotransduction during physiological fluid flow stimulation[1,2]. Integrins are maintained via endocytosis and recycling in numerous cell types *in vitro*[3]. Binding of integrins activates their internalization and subsequent trafficking to the lysosome or recycling back to the plasma membrane[4]. This process provides spatiotemporal regulation of integrin signaling and modulates cell adhesion/motility; together, these characteristics implicate endocytosis in cell mechanotransduction[5]. However, whether integrin cycling and endocytosis pathways impact the function and mechanotransduction of osteocytes has not been studied. In previous work we validated the use of non-targeted and integrin-targeted ultrabright, ultrasmall, fluorescent nanoparticles (C'Dots) as novel *in vivo* osteocyte and integrin imaging tools[6]. Here, we use pharmacological perturbation of select endocytosis components to interrogate trafficking of fluorescent nanoparticles in osteocytes by visualizing uptake, subcellular localization, and clearance kinetics. Specifically, we target dynamin GTPase activity (implicated in clathrin endocytosis) and cholesterol-based lipid rafts (implicated in micropinocytosis) [4].

METHODS: Silica core poly(ethylene glycol) shell (core-shell) nanoparticles covalently encapsulating Cy5 dye (PEG-C'Dots) were created using a modified Stöber condensation in water[7]. RGD-C'Dots were created by surface functionalizing C'Dot surface with amino acid motifs that bind to the extracellular RGD domain of integrin proteins. Skeletally mature C57BL/6J mice age 16-18 weeks were used for these studies (n=4-5/group/sex). C'Dot Injections: Under inhaled isoflurane anesthesia, PEG- or RGD-C'Dots were injected subcutaneously above the third metatarsal (MT3) in the mouse hind paw at a concentration of 10μM (Figure 1). C'Dots were incubated for 45 minutes with mice allowed normal cage activity. Pre-incubation with Dyngo-4a (Abcam) or Methyl-β-cyclodextrin (Sigma Aldrich) for 30 minutes prior to C'Dot injection was used to inhibit dynamin activity or cholesterol lipid raft formation in treated groups. Imaging: After incubation, the MT3 was surgically isolated and stabilized using a metal 3-pin system in re-anesthetized mice[6]. The entire paw was submerged in a PBS bath and placed under a two-photon microscope (Bergamo II, ThorLabs). Osteocytes in the MT3 cortical bone were imaged with a 20x water immersion objective (XLUMPLFLN, Olympus), excitation wavelength of 1090nm, and >647nm long pass filter acquisition. Image Quantification: Mean intensity and cell count of the C'Dot tagged osteocytes were quantified in ImageJ (NIH) using 3D segmentation and were normalized to background intensity. Subcellular localization of C'Dots was quantified by eye from a single frame of the first z-stack of each clearance experiment. Cells were given a binary score for saturated or discretely localized. Clearance study z-stacks (35μm depth) were taken every 15 minutes for 2.5 hours. Statistical analyses used GraphPad Prism software (2-Way ANOVA with multiple comparisons, $p<0.05$). All procedures were IACUC approved.

RESULTS SECTION: Discrete subcellular localization of RGD C'Dot signal is seen in ~75% of osteocyte cell bodies in untreated controls for both sexes (Figure 2). Pre-incubation with dynamin inhibitor Dyngo4a reduced the number of cells with discrete localization to 25% in males (ANOVA, $p\leq0.0001$), and had no statistically significant impact in females. As we have previously reported, C' Dot clearance in untreated mice was sex-dependent (Figure 3), with males losing signal much more rapidly than females[6]. Pre-incubation with Dyngo4a significantly increased RGD C'Dot signal intensity and retention in the male group ($p\leq0.05$, 2-Way ANOVA) with no effect in females. Pre-incubation with cholesterol-inhibitor MβCD resulted in a significant increase in RGD C'Dot signal in the female group ($p\leq0.05$, 2-Way ANOVA) and a no effect in males. In both drug treatments, patterns in RGD C'Dot kinetics were replicated with PEG C'Dot clearance, but to a lesser extent and with more inter-animal variation.

DISCUSSION: Nanoparticle uptake and clearance can be modulated by small molecule endocytosis disruptors, as indicated by these novel intravital imaging studies. Dynamin is critical for rapid clearance and subcellular localization of RGD C'Dots in male mice, but appears to be less impactful for females. Cholesterol and lipid raft formation is necessary for normal RGD C'Dot clearance in female mice but is not significant in males. These data suggest that osteocyte endocytic pathways, specifically receptor mediated endocytosis of integrins, are differentially regulated between the sexes. Variability and reduced impact in PEG data supports that integrin targeting is distinct compared to non-targeted C'Dots. Together, these results imply that endocytosis of mechanosensitive transmembrane proteins like integrins is unique between the sexes. If localization and availability of mechanosensitive proteins can be controlled, pharmacological disruption of specific pathways may be a new way to modulate osteocyte reaction to mechanical stimulation.

SIGNIFICANCE/CLINICAL RELEVANCE: These results represent the first quantification of endocytosis and membrane dynamics in osteocytes *in vivo*. These studies directly motivate future work to assess the impact of endocytic perturbation on osteocyte mechanosensitivity. Sex differences in endocytic pathway utilization for mechanosensitive proteins represents a potential novel therapeutic target for modulating osteocyte response to loading.

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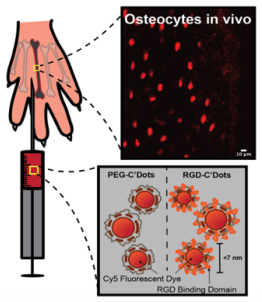

Figure 1: RGD C'Dots allow intravital imaging of osteocytes in mice after a local injection above the 3MT bone.

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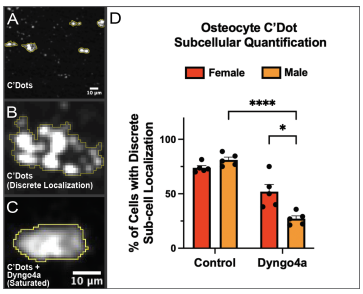

Figure 2: Discrete subcellular localization of RGD C'Dots can be visualized in the cell bodies of osteocytes in vivo. Dynamin inhibition significantly reduces subcell localization in males. (****$p\leq0.0001$).

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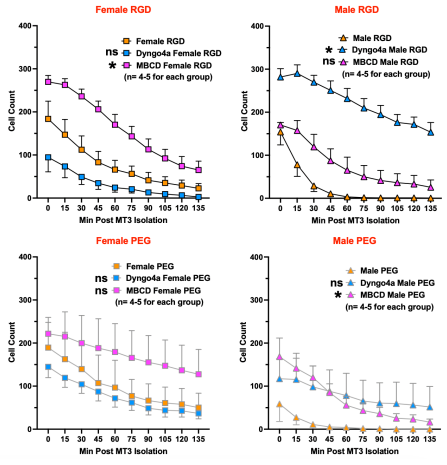

Figure 3: Kinetics of C'Dot clearance can be modulated with pre-incubation of dynamin inhibitor (blue) or cholesterol inhibitor (pink) compared to untreated control (orange). Dynamin inhibition effects C'Dot clearance in opposing ways in male and female mice. Stats compare to untreated control (ANOVA, $p\leq0.05$, Error = SEM.)