SIGNIFICANCE: This research aims to develop a clinically translatable therapy to improve bone regeneration by targeting the Notch signaling pathway.

INTRODUCTION: While the majority of bone fractures heal with standard care, a significant proportion of injuries fail to produce viable callus tissue, resulting in atrophic non-union. Autologous bone grafts and bone morphogenetic proteins are commonly used to treat atrophic injuries. However, these therapies have significant limitations and a clinical need persists for the development of new methods to promote bone tissue formation.

A series of studies have shown that the Notch signaling pathway regulates murine endochondral and intramembranous bone development by stimulating progenitor cell proliferation while inhibiting differentiation\(^1\). Previous studies from our lab demonstrated that the Notch signaling pathway is upregulated during murine endochondral and intramembranous bone fracture healing, with Jagged1 as the most highly expressed and upregulated ligand\(^2\). Furthermore, the Notch signaling pathway has been shown to regulate regeneration of non-mesenchymal tissues and has been successfully targeted as a therapy to improve myocardial function in infarcted hearts\(^3\). However, the ability of Notch signaling to enhance bone tissue regeneration has not been investigated. Therefore, our objective is to develop a clinically translatable therapy using a biomaterial construct with Jagged1 ligand to activate the Notch signaling pathway to improve bone tissue formation.

We have developed a library of photocrosslinkable poly(\[\text{a}mino \text{ester}\]) and found diethylene glycol diacrylate (‘A’) mixed with isobutylamine (‘6’), or A6 for short, to have significant osteoconductive potential\(^4\). Previous studies have shown that Jagged1 and other Notch ligands must be immobilized to a surface in order to activate the Notch signaling pathway. For these experiments, we immobilized Jagged1 to A6 through direct and indirect (via antibody binding) adsorption at concentrations of 0, 2.5 and 10 \(\mu\)g/ml. We hypothesized that increased Jagged1 concentration would 1) increase activation of Notch signaling, 2) increase cell number, 3) increase early osteogenesis, but 4) ultimately inhibit in vivo overall osteogenic activity due to increased cell number. Primary human MSCs were used in these studies.

METHODS: Diethylene glycol diacrylate (‘A’) and isobutylamine (‘6’) were mixed together at a 1:2 molar ratio for 40 hours at 90°C, followed by addition of 0.5 wt% photo-initiator DMPA. The resulting A6 macromer was coated onto 24 well plates, and photopolymerized with ultraviolet light (365 nm).

For direct Jagged1 immobilization, recombinant rat Jagged1/Fc chimera protein (R&D Systems) was adsorbed to the A6 surface at 0, 2.5 and 10 \(\mu\)g/ml for 2 hours at room temp: Direct\([0]\), Direct\([2.5]\), Direct\([10]\)

For indirect Jagged1 immobilization, rabbit anti-human Fc antibody (Jackson Immunoresearch) was first adsorbed to the A6 surface at 15 \(\mu\)g/ml for 2 hours at room temp, followed by 1% BSA blocking for 2 hours. Jagged1/Fc was then adsorbed to the A6 surface at 0, 2.5 and 10 \(\mu\)g/ml for 2 hours at room temp: Indirect\([0]\), Indirect\([2.5]\), Indirect\([10]\)

Wells were plated at 5000 cells/cm\(^2\) and cultured in αMEM, 20% FBS, 1X L-Glutamine, and 1X Pen/Strep for up to 7 days. RNA was harvested at days 1, 3, 5 and 7. Alamar Blue, a cell viability assay, was performed at days 1, 3, 5 and 7. Cell viability was assessed using an enzyme produced by osteoblasts during bone formation, was assayed at day 7. Tissue-culture wells (No A6 Control\([0]\)) were included for RNA. (n=3)

RESULTS: Immobilization of Jagged1 to A6 increased expression of Notch target gene Hey1 (Fig 1 top). Direct\([10]\] was significantly upregulated relative to Direct\([0]\) at days 1, 3, and 7, and Direct\([2.5]\) was significantly upregulated at day 1. Indirect\([2.5]\) and \([10]\] were significantly upregulated only at day 7. Interestingly, No A6 Control\([0]\) showed significantly less Hey1 expression at day 1 then Direct\([0]\).

Immobilization of Jagged1 to A6 increased expression of the osteogenic differentiation marker bone sialoprotein (BSP) (Fig 1 middle). Similar to Hey1, Direct\([10]\] was significantly upregulated relative to Direct\([0]\) at days 1, 3, and 7, and Direct\([2.5]\) was significantly upregulated at day 1. Indirect\([10]\] was significantly upregulated at day 1. No A6 Control\([0]\) was significantly lower at day 1 than Direct\([0]\). Similar effects were seen with alkaline phosphatase gene expression (data not shown).

Immobilization of Jagged1 to A6 did not significantly change cell number at day 1 or 7 (Fig 1 bottom left). However, alkaline phosphatase staining was stronger with increasing Jagged1 concentration (Fig 1 bottom right). It also appears that at 10 \(\mu\)g/ml, staining was increased when Jagged1 was directly adsorbed relative to indirect adsorption.

DISCUSSION: Consistent with hypothesis 1, Jagged1 immobilization to A6 increased activation of Notch signaling. Also consistent with a previous study \(6\), direct Jagged1 adsorption at concentrations above 4.25 \(\mu\)g/ml (30 nM) activated Notch signaling at higher levels and for a longer period of time than indirect.

Interestingly, the data did not support hypotheses 2-4. Although Jagged1 increased activation of Notch signaling, it had no effect on cell number. Also, Jagged1 increased expression of osteoblast differentiation markers BSP and Alkaline Phosphatase throughout the duration of the experiment. Collectively, these results suggest that the primary effect of Jagged1-initiated Notch signaling in hMSC is enhanced osteogenic differentiation, not enhanced proliferation.

Published \textit{in vitro} studies have cultured murine MSCs on tissue-culture plastic and inhibited all Notch signaling, demonstrating that Notch inhibits differentiation\(^2\). In this experiment, we cultured human MSCs on A6 polymer and upregulated Jagged1-initiated Notch signaling, demonstrating that Jagged1-initiated Notch promotes osteogenic differentiation. Future studies will investigate the apparently divergent mechanisms of Notch signaling in these different culture conditions, including potential osteogenic synergy between Jagged1 and A6. In addition to regulation of cell proliferation, Notch plays a role in determining cell fate. For example, transient Notch activation in neural crest stem cells promotes glial differentiation while inhibiting neuronal\(^2\). We hypothesize that in hMSCs, Jagged1-initiated Notch signaling activates a pro-osteogenic pathway. Future studies will evaluate the ability of 3D porous Jagged1-A6 scaffolds to promote bone regeneration \textit{in vivo}.

ACKNOWLEDGEMENTS: The authors thank Darren Brey and Jamie Ifkovits for their work on A6. This study was supported by the NIH/NIDCR F31DE020231 and the DOD CDMRP.