Synergistic Regulation of Chondrocytes by Progranulin Growth Factor and Low Intensity Pulsed Ultrasound

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Introduction: Osteoarthritis (OA) is a degenerative disease of the joints, initiated by trauma, autoimmune response, infection and/or aging. OA is the most common cause of disability in American population. According to recent estimates, treatment for arthritis costs approximately $128 billion per year to the US economy alone [1]. The objective of this study it to use a novel chondrogenic and anti-inflammatory growth factor, PGRN, with external Low Intensity Pulsed Ultrasound (LIPUS) as biomechanical stimuli to i) initiate migration, proliferation and anabolism of native chondrocytes, ii) inhibit TNFα and IL-1β induced inflammation and catabolism and iii) understand the underlying mechanism for combined chondroprotective effects.

Methods: Ultrasound Setup: LIPUS stimulation were applied using an acoustic device, Sonicator 740 (Mettler Electronics, Anaheim, CA) with a 10cm2 transducer (35mm diameter) with characteristic frequency of 1Mhz, pulse duration of 200μs, with repetitive frequency of 100 Hz at an intensity of 30mW/cm2 for 20 min per day.

Migration Assay: Chondrocytes cells (C2812) were cultured in chondrogenic media supplemented with 10%FBS. At 80% confluency, 4mm scrapper head was used to draw a vertical line in the middle to create an empty zone in cell layer. Cells were divided in four groups i)Control, ii) US iii)IL-1beta (10ng/ml) and iv) US + IL-1beta (10ng/ml). Cells were imaged at 24 hrs , 48hrs and 72hrs for chondrocytes migration into empty region. ImageJ was used to count the number of cells and area covered by cells at each time point.

Differentiation: BMP2 adenovirus infected C3H10T1/2 cells were cultured as micromass in 10%FBS supplemented chondrogenic media. Cells were divided in four groups as listed above. Cells were treated for 4 days and Real time quantitative PCR analysis were conducted to study the differential level of Runx2, Col II, Sox 9 and Col X.

Chondro-Protection effects of LIPUS: Human primary chondrocytes micromass cultures were treated with US and IL-1beta for 4 days. Quantitative PCR analysis were done to study the expression of catabolic markers, MMP13, ADAMTS4,ADAMTS5 and anabolic markers, Col-II, Aggrecan and Comp.

Combined Effects of Progranulin and LIPUS: To study anabolic effects of LIPUS and PGRN, human chondrocytes were treated with LIPUS, PGRN and LIPUS+PGRN for 4 days and mRNA expression of Col II, Aggrecan and Comp too was analyzed using quantitative PCR. To study catabolic effects ex-vivo explants of human cartilage were treated with LIPUS, PGRN,LIPUS+PGRN, IL-1beta, TNF-alpha, LIPUS+IL-1beta, PGRN+TNF-alpha and LIPUS+PGRN+IL-1beta+TNF-alpha for 7 days. Safranin O histological staining was used to analyze the GAG content in cartilage ex-plants

Results: LIPUS induces Chondrocytes migration: LIPUS stimulation significantly increase the rate of migration in chondrocytes as LIPUS treated cells show 80% confluency relative to 40% in control samples at 48hrs. LIPUS also neutralizes IL-1beta effects (10% confluency) by restoring chondrocyte...
migration to approximately 25% at 48 hr (Fig 1a). Similar trend was also observed with respect to number of cells in region of interest.

LIPUS increase rate of chondrogenesis and neutralize effects of IL-1beta: Application of LIPUS significantly increased the expression of Runx2, Col II, Sox 9 and Col X in BMP2 infected C3H10T1/2 cells. IL-1beta treated cells showed significantly reduced chondrogenesis, which was rescued with application of LIPUS (Fig 1C)

LIPUS enhances anabolism and protect against IL-1beta induced catabolism: Quantitative PCR analysis of MMP13, ADAMTS4, ADAMTS5, Col 11, Aggrecan and Comp showed that LIPUS regulates chondrocytes metabolism by enhancing anabolism and suppressing expression of catabolic enzymes. Furthermore, application of LIPUS neutralizes the catabolic effects of IL-1beta in human primary chondrocytes.

Progranulin and LIPUS combined therapy show synergistic and chondro - protective effects: Application of LIPUS and PGRN to gather showed synergistic effects in by increasing expression of Col II, Aggrecan and COMP by 5-6 folds relative to control human primary chondrocytes (Fig 2a). Same trend were observed in Safranin O staining of human cartilage ex-plants with most apparent GAG content in LIPUS+PGRN treated ex-plants. Treatment with IL-1beta and TNF-alpha showed apparent reduction in Safranin O Staining. Application of LIPUS and PGRN rescued the catabolic effects of cytokines with most obvious effects in LIPUS+PGRN combined treated samples.

**Discussion:** The objective of the study was to study the effects of LIPUS and PGRN combined treatment on Chondrocytes migration, differentiation and metabolism. Furthermore the study also analyzed the therapeutic effects of LIPUS and PGRN in presence of chondro-degenerative cytokines, IL-1beta and TNF-alpha. Our previous studies have shown pro-chodrogenic and chondro-protective effects of PGRN in both in vivo and in vitro models [2-4]. Current data show LIPUS alone can enhance chondrocyte migration, and differentiation in mesenchymal stem cells in in vitro setup. IL-1beta has been shown to reduce chondrocyte migration and chondrogenesis in MSCs [5, 6], the stimulation of LIPUS in presence of IL-1beta rescues chondrocytes migration and chondrogenesis. The analysis of metabolism associated markers showed LIPUS enhances anabolism and show chondro-protective effects against IL-1beta induced catabolism. The combination of PGRN and LIPUS show up to 6 fold increase in anabolic markers and apparent increase in GAG content of cartilage explants with apparent chondro-protective role against IL-1beta and TNF-alpha. Both IL-1beta and TNF-alpha has been associated with onset and progression of osteoarthritis. The current study show that combine treatment of LIPUS and Progranulin has therapeutic potential to slowing down cartilage degradation and enhancing matrix depiction in damage cartilage by inhibiting TNF-alpha and IL-1beta associated inflammatory pathways and activating TNFR2 and Integrin associated chondro-protective pathways (Fig 3). Data from current study along with our extensive published studies on progranulin

**Significance:** The combination treatment of LIPUS and Progranulin has therapeutic potential and treatment of osteoarthritic
Fig 1A: LIPUS enhances chondrogenesis and neutralizes IL-1beta. B) LIPUS restores IL-1beta induced chondrocytes migration inhibition.

Figure 2: A) Combination treatment of PGRN and LIPUS show synergistic effects in chondrocytes anabolism. B) LIPUS + PGRN treatment enhances safron O staining and protect against cytokines induce cartilage degradation.

Figure 3 – PGRN and LIPUS role in inhibition of inflammatory and activation of chondrogenic pathways.

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