The mechanical regulation of Sema3A in may drive neuroplasticity and pain in osteoarthritis.

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INTRODUCTION: Despite copious research demonstrating alterations in biomechanical loading, such as chronic knee varus malalignment, and dysregulation of joint tissue biology as driving factors in osteoarthritis (OA) progression, mechanistic links between these driving factors of OA are lacking. Changes in subchondral bone are biomechanically driven and a key factor in long term joint degeneration[1]. Bone resorption and necrosis, immune cell influx and angiogenesis all occur in OA bone and directly contribute to joint destruction and pain [2]. Profound neuroplasticity and nociceptor sprouting is also displayed within OA subchondral bone and are one of the few disease characteristics to correlate with OA pain [3]. Bone is inherently mechanically sensitive [4]; however, the molecular cause of this pathological dysfunction is unknown. An essential coupling exists between cells of the skeletal and nervous systems. Correct innervation patterns are indispensable for bone growth, homeostasis, and repair. Axonal guidance factor, Sema3A is an anabolic agent in bone expressed by mechanically sensitive osteocytes, and a known inhibitor of sensory nerve, blood vessel and immune cell invasion [5] and essential for the correct innervation patterning of bony tissues. The Sema3a pathway is also differentially expressed in human OA patients[6].

HYPOTHESIS: Pathological mechanical overloading and inflammation of in vitro 3D osteocyte cultures mimics that observed in the medial knee compartment in varus knee malalignment patients causing dysregulation of bone derived Sema3A signaling, nociceptor plasticity, and pain directly linking joint biomechanics to pathology and pain.

METHODS: **In Vitro**: Human KOLF2-C1 iPSC derived nociceptors were generated by TALEN-mediated insertion of transcription factors NGN2+Brn3A and modified chambers differentiation protocol [7] to produce nociceptor-like cells. Nociceptor phenotype was confirmed by immunocytochemistry. Human Y201 MSC cells [8] were embedded in 3D type I collagen gels (0.05 x 106 cell/gel) in 48 well plates and differentiated to osteocytes for 7 days before stimulation with IL-6 (5ng/ml) with soluble IL-6 receptor (sIL-6r (40ng/ml) (IL6/sIL6r) and mechanical load mimetic Yoda1 (5μM) or unstimulated (n=5/group). Duplicate cultures were mechanically loaded in silicone plates (5000μstrain, 10Hz, 3000 cycles) or not loaded (n=5/group). Conditioned media transfer was performed from osteocyte to nociceptor cultures assessed by continuous 24-hour phase contrast confocal microscopy. 24-hours after stimulation RNA was quantified by RT-qPCR (IL6) or RNAseq whole transcriptome analysis/DEseq2 analysis (Load). Protein release was quantified by ELISA. Normally distributed data with homogenous variances was analyzed by two-tailed t test. **Human Patients:** Synovial fluid obtained from 30 subjects with medial knee OA (KL grade II-IV) undergoing high tibial osteotomy surgery (HTO) had synovial fluid analyzed by multiplex (mesoscale discovery) and simple plex ELISA analysis for inflammatory, neural and bone turnover markers. This study was approved by an Ethics Committee. A subset of these HTO patients had been previously analyzed in a patient-specific musculoskeletal model of gait used to estimate joint contact location, pressure, forces, and medial-lateral condyle load distribution in a published data set included in analyses (n=6; [9]). Data analysis was performed using Pearson's correlation matrices and principal component analyses (eigenvalues greater than 1).

RESULTS: **In Vitro**: IPSC-derived nociceptor-like cells display elongated (>5mm) interconnected dendritic projections and nociceptive molecular markers such as TUJ1, PrPH and Neun and TrkA. Sema3A signaling ligands were expressed in 100% of osteocyte cultures. Under mechanical loading, Sema3 pathway displayed regulation; Sema3A (0.4-fold, $p<0.001$), Sema3B (13-fold, $p<0.001$), Sema3C (0.4-fold, $p<0.001$). Under inflammatory stimulation by IL6/IL6sR, SEMA3A (7-fold, $p=0.01$) and its receptor Plexin1 (3-fold, $p=0.03$) show significant regulation. IL6/sIL6r+Yoda1 stimulation significantly downregulated Sema3A release (2-fold, $p=0.02$). Continuous 24-hour phase contrast confocal microscopy measuring the number of extending/retreating dendritic projections revealed that sensory nerve cultures exposed to media from osteocytes stimulated with IL-6/sIL-6R+Yoda1 displayed significantly more invading dendritic projections ($p=0.0175$, 12-fold±SEM 3.5) across 3 random fields of view within a single stimulated neural culture and significantly fewer retracting dendritic projections ($p=0.0075$, 2-fold±SEM 0.33) compared to controls.

Human Patients: PC1 (32.94% of variation) and PC2 (25.79% of variation) from PCA analysis and correlation matrices separated patients according to correlated clusters of established inflammatory markers of OA pain and progression (IL6/IL8, $r=0.754$, $p<0.001$) and anti-inflammatory mediators (IL4/IL10, $r=0.469$, $p=0.005$). Synovial fluid Sema3A concentrations showed separate clustering from all OA progression markers and was inversely correlated with TNF-α ($r=-0.423$, $p=0.022$) in HTO patients. Sema3A was significantly inversely correlated with total predicted force in the medial joint compartment ($r=-0.621$, $p=0.041$), mean ($r=-0.63$, $p=0.038$) and maximum ($r=-0.613$, $p=0.045$) calculated medial compartment joint pressures during the first phase and mean ($r=-0.618$, $p=0.043$) and maximum ($r=-0.641$, $p=0.034$) medial compartment joint pressures during midstance outputs of patient-specific musculoskeletal model.

DISCUSSION: Here we show osteocytic regulation of Sema3A under pathological mechanical loading and the ability of media pathologically loaded osteocyte cultures to induce the branching and invasion of cultured nociceptor-like cells as displayed in OA subchondral bone. Synovial Sema3A concentrations are inversely correlated to patient-specific musculoskeletal model estimations of pathological medial overloading.

SIGNIFICANCE/CLINICAL RELEVANCE: This study reveals Sema3A as a biological mediator with capacity to induce OA pain and disease progression, directly linking gait mechanical loading to pain.

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