Q17: Are there any animal-free *in vitro/in silico* approaches in development that can replace animal models for studying mechanisms of OA pain?

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Response: YES. Nociceptors are sensory neurons that express specialized receptors for noxious chemical, thermal, and mechanical stimuli. The nociceptive response to molecules released due to osteoarthritis (OA) tissue damage or the altered response by nociceptors that have become sensitized to mechanical or thermal stimuli can be modeled *in vitro* using human-derived sensory neurons. Interactions between human neuronal and non-neuronal cell types can also be modeled in sophisticated co-culture systems/joint-on-a-chip. These types of models and defined culture conditions may facilitate the discovery of novel pain mediators and therapeutic targets and may be useful for screening drugs designed to inhibit neuronal responses. *In silico* models also offer the opportunity to screen compounds for protein-protein binding and for toxicology testing. However, pain is defined as 'an unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage.' Therefore, in contrast to neuronal activity, the emotional/affective experience of pain cannot be fully recapitulated *in vitro*.

Level of Evidence: Moderate

Rationale: A PubMed search for (("microphysiological system") OR (microfluidic*) OR ("chip") OR (in vitro model) OR (animal-free) OR (in silico model) OR (iPSC) OR (nociceptor) OR (spinal neuron)) AND (osteoarthritis) AND (pain) yielded 538 papers, which were manually curated for relevant publications and summarized here. In addition, a web search was performed to find non-OA papers.

Animal-Derived Sensory Neuron Approaches to Modeling OA Nociceptive Responses

Cultured dorsal root ganglia (DRG) sensory neurons isolated from animal models of OA have been shown to sensitize to ligands present in OA joints and/or exhibit intrinsic hyperexcitability, *i.e.*, decreased threshold for action potential firing, or spontaneous firing [1-6]. Animal-derived DRG neurons have also been shown to have differential responses to inflammatory vs. non-inflammatory animal-derived fibroblast-like synoviocytes (FLS) co-cultured in the same dish [7, 8] or to conditioned medium derived from these cells [9].

Sensory neurons isolated from naïve animals have also been used to test whether conditioned medium generated from human OA cells or human OA synovial fluid modulates neuronal activity. For example, conditioned medium generated from OA FLS cells taken from painful sites induced rat DRG neurite outgrowth more than non-painful sites [10]. Similarly, synovial cell conditioned medium from OA patients with worse pain increased cell stress in rat DRG neurons [11]. Conditioned medium from RA synovial fibroblasts also stimulated axonal outgrowth from mouse DRG neurons [12]. Moreover, analysis of human OA synovial fluid has identified a range of dysregulated mediators [13-17], and a subset of these molecules has been shown to activate/sensitize rodent DRG neurons [1, 4-6, 18-20]. Additionally, OA synovial fluid can directly sensitize mouse DRG neurons [21]. One challenge with using human tissue for *in vitro* studies is that donor heterogeneity should be considered when determining sample sizes.

Microfluidic systems can enable the development of more complex co-culture systems that separate the neuronal cell bodies from their axons and from other types of non-neuronal cells [22], which more closely mimics the *in vivo* scenario [23]. Initial work has used human derived joint tissues to study the effects on axonal outgrowth and sensitization of animal-derived DRG neurons [24, 25].

Nociceptor Differences Across Species

Preclinical models of OA have been useful for studying the basic underlying mechanisms of pain [26], but while many aspects of pain transmission are conserved across species, molecular and physiological differences exist between humans and other species. Emerging work has shown that primary human DRG neurons express many of the same ion channels and receptors as in other species, but the exact expression patterns across sensory neuron subsets are different [27, 28]. Similarly, while the types of current generated by activation of these neurons are largely conserved across species, there are differences that could be relevant for drug development [29].

Human Primary DRG Neurons vs. iPSC-derived Sensory Neurons vs. DRG Cell Lines

Part of the rationale for using animal-derived DRG neurons for research has been the difficulty in accessing human DRGs since they are not available from routine surgical procedures. The collection of live cells rapidly from organ donors is resource-intensive and not widely adopted; however, protocols have recently been described for storage and shipping, which decreases the necessity for geographical proximity [30]. Primary DRG neurons also cannot be propagated as these cells no longer divide. Moreover, compared to rodent DRG, human DRG will show more variability due to the life history of the individual from which they are taken, especially any clinical conditions. Finally, while one

human DRG cell line has been reported [31, 32], it is not commercially available.

For these reasons, the development of human induced pluripotent stem cell (iPSC)-derived sensory neurons has been an exciting avenue of research, and commercial investment in this area has also helped to increase the number of publications reporting new information about these cells [33]. These iPSC-derived sensory neurons have been reported to express many of the same ion channels and receptors as primary human DRG neurons. However, there are some important differences such as variability in expression of $Na_V1.8$ and differences in the action potential profiles generated [34, 35]. Because iPSCs are immature neurons, the expression profiles change rapidly over time and so the time point used is important. Ongoing work is examining how to more closely mimic primary DRG neurons [36]. There are also reports demonstrating the translational relevance of using these cells by relating the findings back to improved analgesia in patients [37, 38]. Finally, the ability to scale up these cells for use in high-throughput screening is being pursued for drug development [39]. However, it should be noted that the genetic background of iPSCs may be a limitation as cells are often generated from a few individuals. In the future, iPSCs would ideally be generated from each patient just as non-neuronal joint tissue is currently collected on a patient-by-patient basis.

Fully-Humanized Microfluidic Modeling of OA Nociceptive Responses

As the FDA and NIH continue to encourage the adoption of new approach / animal-free methodologies, interest in developing on-chip, organoid, and microfluidic platforms to model the crosstalk between nociceptive neurons and OA joint tissues using a fully humanized approach is expected to grow. One example that has successfully used this approach demonstrated that human nucleus pulposus cells stimulated with degenerative conditions induced more axonal outgrowth in human iPSC derived sensory neurons compared to non-stimulated cells [40]. Expanding modeling of the synovium by using a fully humanized approach will be of interest. Systems that recapitulate subchondral bone are also likely to receive attention, given the substantial evidence for altered nerve innervation in subchondral bone marrow and across the osteochondral junction [41-45]. Current attempts to engineer such humanized neuron-joint interfaces are still at an early stage [46]. Because any of these platforms will require rigorous validation of their histological, biochemical, and functional mimicry [47], the path toward a robust, functional *in vitro* system for pain research will likely span many years.

Modeling the Central Nervous System In Vitro

While options for modeling the human sensory nervous system are being more widely adopted, few *in vitro* models of the human central nervous system currently exist. The first synapse made by sensory neurons occurs in the spinal cord, and a recent report suggests an approach to model this circuitry using human cells [48]. In addition, two other recent publications focus on modeling the different brain regions using human cells [49, 50], including the assembly of a four-part system generated from human pluripotent stem cells that integrates somatosensory, spinal, thalamic and cortical organoids to model the entire spinothalamic pathway [49]. An ultimate end goal in the future would be to develop human brain organoids that are coupled to DRG neuron – spinal cord synapsing circuitry, as well as the “innervated” joint tissues, thus enabling the humanized ‘OA pain pathway’ to be fully reconstructed *in vitro*.

In Silico Opportunities

Investment in generating large databases of human expression data is helping to create a more complete picture of all human signaling pathways, including in OA [51, 52]. One example of how these data can be used is the NCATS initiative, which is seeking to construct an informatics platform enabling browsing of these pathways to perform *in silico* toxicology screening in the future [53]. New tools are also becoming available that can predict protein-protein interactions to screen new drugs or drug libraries *in silico* for efficacy [54-56] and modeling of pharmacokinetics [57]. Other work is demonstrating that pharmacological modeling can be used to predict more effective therapeutic interventions by combining existing drugs for improved pain relief [58].

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