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COMMENTARY

Shear elegance: A novel screen uncovers a mechanosensitive GPCR

Ruby M. Lam[1,2] and Alexander T. Chesler[1]

Mechanical stimuli range in timescale and intensity, from the constant force of gravity to the slow oscillations of pumping blood or the sensation of high-pitched sound waves. The array of molecular strategies that have evolved to detect these different stimuli reflect the diversity of forces experienced by cells and organisms. Our understanding of the transduction machinery involved has been propelled in recent years by the growing toolkit of approaches to study mechanobiology. Now, a study by Patapoutian et al. reports the development of a high-throughput approach that sheds light on how cells detect and respond to shearing stress. Their work provides technical and conceptual breakthroughs that significantly advance our understanding of mechanotransduction.

Although the cytoskeleton itself can respond to force (Burridge and Chrzanowska-Wodnicka, 1996), in many cases, mechanosensation relies on receptors integrated in the plasma membrane. For example, slowly oscillating strains on cellular attachments with the surrounding extracellular environment or neighboring cells are sensed by adhesion receptors (Rutishauser et al., 1988). Integrins and cadherins interact with the extracellular matrix and intracellular cytoskeletal elements as critical components of force-dependent remodeling during development (Hynes, 2002; Levayer and Lecuit, 2013; Scholz et al., 2016). On a much faster time scale, our ability to rapidly detect the slightest touch relies on stretch-gated ion channels (Ranade et al., 2015; Chesler et al., 2016; Azevedo and Wilson, 2017). Several families of ion channels have been implicated in mechanotransduction with different permeabilities and sensitivities to membrane stretch. These include, but are not limited to, the bacterial nonselective large conductance MscL channel (Sukharev et al., 1994), the epithelial amiloride-sensitive Na^+ channel (DEG/ENac) family (Goodman et al., 2002), the two-pore K channels (Bagriantsev et al., 2013), the cation-selective Transient Receptor Potential (TRP) channel family (Walker et al., 2000; Yan et al., 2013), and the Piezo channels (Coste et al., 2010).

A unifying feature of ionotropic mechanotransduction is that channel gating results in extremely fast ion flux, within milliseconds. However, slower metabotropic mechanisms involving G protein–coupled receptors (GPCRs) have also been implicated in mechanotransduction. Membrane strain can initiate signal transduction cascades involving GPCRs in several cell types, most notably in the vascular and renal systems (Zou et al., 2004; Mederos y Schnitzler et al., 2008). Angiotensin receptors (Zou et al., 2004), expressed in heart tissue, have been proposed to respond to pressure overload. Similarly, Bradykinin receptors expressed by endothelial cells have been reported to be activated by fluid shear stress (Chachisvilis et al., 2006). In these cases, mechanical strain has been proposed to activate G-protein signaling independent of ligand binding. That said, it remains unclear whether force acts directly or indirectly on these receptors.

To identify new molecules or mechanisms of mechanotransduction, Patapoutian et al. developed an unbiased screen (Xu et al., 2018) so as to limit assumptions about the kinds of receptors involved. In doing so, they addressed a major stumbling block that has stymied the study of mechanotransduction: how to deliver physiologically relevant stimuli in a manner compatible with high-throughput cellular assays. Previous studies relied on several approaches to solve this problem. For example, Coste et al. (2010) used a recording electrode to give a brief negative pressure pulse on the cell membrane while recording electrically. Although ultimately fruitful, recording one cell at a time was a painstaking approach; it took years to test ∼70 candidate genes before yielding the seminal Piezo ion channel discovery. Several other groups have tried developing alternative assays. Some have experimented with cells grown on flexible substrates that can be longitudinally or radially stretched to deliver mechanical stimuli (Banes et al., 1985; Bhattacharya et al., 2008; Ino et al., 2008). Another approach involves adhering coated paramagnetic beads to specific targets and then applying localized pulling forces on molecules associated with the plasma membrane using

[1]National Center for Complementary and Integrative Health, National Institutes of Health, Bethesda, MD; [2]Brown-National Institutes of Health Graduate Partnerships Program, Brown University, Providence, RI.

Correspondence to Alexander T. Chesler: alexander.chesler@nih.gov.

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electromagnets. (Wu et al., 2016). Although both the flexible substrate and attached magnetic bead techniques hold promise, further engineering is required to adapt them for screening.

Xu et al. (2018) provide a new and clever way to deliver physiologically relevant mechanical stimuli to multiple cells at a time. They engineered a platform that applied shear stress to cells grown in microwell plates via an array of 384 vibrating pins dipped into the wells. Each vibrating pin was coupled to a solenoid, and a subwoofer driver controlled their stimulation frequency, amplitude, and duty cycle. The vibrations generated by each pin mechanically perturbed the media covering the cells in the assay plate, creating a disturbed flow. Cellular responses to this shear stress was recorded optically through the bottom of the plate by using a calcium-sensitive dye to monitor intracellular calcium concentration in real time before, during, and after controlled delivery of mechanical stimuli.

Because cancer cells grow well in culture and are dysregulated such that many genes are being ectopically expressed, Xu et al. (2018) examined a panel of 60 cancer cell lines from the National Cancer Institute (NCI-60) to identify lines intrinsically mechanosensitive to shear stress. They excluded lines expressing the known mechanoreceptors Piezo1 or Piezo2 to generate a pool of 25 cell lines. Of these, two produced calcium responses to shear stress, with transients from the breast cancer cell line MDA-MB-231 being particularly robust. Next, they generated a list of targets to knock down by small interfering RNA (siRNA). Their assumption was that the unknown mechanoreceptor would be localized to the plasma membrane and contain at least two transmembrane domains. Bioinformatic analyses yielded a list of 2,907 potential target genes that met their criteria. A quick back-of-the-envelope calculation to estimate controls and combinations of multiple siRNAs per gene allows one to appreciate the transformative nature of the ability to screen in microwell plates. All aspects of the experiment, including liquid handling, siRNA screening, and plate reading could be automated.

Of all the genes tested, knockdown of the G protein–coupled orphan receptor GPR68 significantly and repeatedly diminished the calcium influx after shear stress was applied. Critically, transfection of normally mechano-insensitive HEK293 cells with this GPCR resulted in calcium responses in the disturbed shear stress assay, indicating that expression of this GPCR was sufficient to endow mechanosensitivity. Perhaps most impressive was the amplitude of the mechanical responses seen in responses to laminar flow (a different type of mechanical stimulation) after both human and mouse GPR68 expression. In contrast, neither expression of the mechanosensitive Piezo1 nor the osmosensitive TRPV4 resulted in calcium responses to laminar flow above baseline. Even more surprising, overexpression of other GPCRs in HEK293 cells, including those previously proposed to be mechanosensors, did not result in calcium transients to shear force. Collectively, these findings provide the most compelling evidence to date for a bona fide mechanosensitive GPCR and, by doing so, open the door to several interesting questions.

How does force activate GPR68? Interestingly, GPR68 was originally identified as a proton-sensitive GPCR (Ludwig et al., 2003). Preliminary findings from Xu et al. (2018) suggest that pH and force may converge on a conserved protein domain in the extracellular region of the molecule. Several histidine residues that have been shown to be required for proton sensitivity (Ludwig et al., 2003) are also required for the mechanical response. Indeed, the responses to shear stress were shown to be pH dependent with the strongest calcium responses in more acidic environments of pH ≤ 6.5. Notably, mutations of the histidine residues required for pH sensing also blocked sensitivity to shear stress. Alternatively, it remains possible that shear stress liberates protons locally and the receptor is indirectly responding to mechanical stimulation. Because protons are difficult to track using common biochemical, electrophysiological, and structural methods, it will take future studies to work out the details. That said, it is tempting to speculate that the electrostatic interactions perturbed by protons could be similarly affected by mechanical stress. Given advances in our general understanding of GPCR structure/function and the diversity of tools that have been recently developed, it will be exciting to probe the mechanism for mechanosensation and compare it to what has been proposed for stretch-gated ion channels.

Pharmacological tests by Xu et al. (2018) provided evidence that GPR68 exerts its effects by canonical G-protein signaling (Gq/11) involving phospholipase C (PLC) activity and calcium release from intracellular stores. Several ion channels are downstream of PLC signaling, which hydrolyses and cleaves PIP2 into DAG and IP3, two potent second messenger molecules. Intriguingly, the DAG pathway activates TRPC ion channels (Hofmann et al., 1999), which have been implicated in mechanotransduction in several tissues (Sexton et al., 2016). Notably, removing extracellular calcium reduced the responses from GPR68-expressing cells, so it would be very interesting to determine whether TRPC channels are coexpressed with GPR68 and mediate these effects. In addition, Xu et al. (2018) also observed residual transients in calcium, even when the ion was excluded from their extracellular solution. Thus, it seems likely that the remaining calcium signal is a result of IP3-induced release of intracellular calcium stores, as indicated by the blocking effect of thapsigargin. Here again, there are several potential downstream effectors, such as Stim and Orai (Putney and Tomita, 2012; Vashisht et al., 2015), that could mediate the effect of GPR68 signaling.

One of the most exciting aspects of identifying GPR68 as a novel mechanoreceptor is that it offers opportunities to explain unknown physiological mechanisms. As a first step, Xu et al. (2018) examined the mRNA distribution in a reporter mouse strain engineered to express GFP under the GPR68 promoter (GPR68-GFP) to uncover the cells and tissues in which the receptor is expressed. They identified GPR68 in several tissues throughout the body, with the highest expression found in immune cell populations from the spleen. It will be fascinating to uncover how GPR68 contributes to immune function and whether the knockout mice are immunocompromised. Recently, it was suggested that mechanotransduction is important for antibody T-cell recognition and that the functional contribution of the mechanosensitive ion channel Piezo1 may be important for normal immune responses (Liu et al., 2018). siRNA knockdown of Piezo1 attenuates the ability of T cells to be primed and proliferate properly. Furthermore, mechanical shear stress may increase the chemical sensitivity of T-cell receptors (Lee et al.,

2015). Because GPCR68 was also observed abundantly in the spleen, it could function in parallel with Piezo channels in modulating the immune response.

In addition to the spleen, Xu et al. (2018) found GPR68 expressed in a subset of endothelial cells in small vessels, which raises the possibility that GPR68 signaling is important for regulating vascular tone in response to blood pressure. Indeed, primary cultures of GPR68-positive endothelial cells show calcium transients in response to shear stress that are attenuated in the absence of the receptor. Furthermore, they found that flow-mediated dilation of third-order branches of mesenteric arteries were dependent on Gpr68. Intriguingly, application of Ogerin, a molecule previously identified as a selective positive allosteric modulator of GPR68 (Huang et al., 2015), potentiated vessel dilation in vivo, suggesting a new pharmacological approach to modulating vascular tone. GPCRs are attractive "druggable" targets, so it is easy to imagine a modification of the high-throughput shear stress platform to screen for novel GPR68 agonists and antagonists.

The dorsal root ganglion (DRG) also showed pronounced expression of GPR68. This ganglion contains the cell bodies of the sensory neurons that are the primary sensors for environmental and internal stimuli. Notably, the majority of DRG neurons detect mechanical stimuli, and previous work established that Piezo2 is required for touch discrimination, vibration sensitivity, and proprioception in mice and humans (Ranade et al., 2014; Woo et al., 2015; Chesler et al., 2016). However, other types of mechanosensitivity, such as those that respond to mechanical pain, are largely Piezo2 independent. Is GPR68 expressed in nociceptors, and is it important for pain responses? Tissue acidosis modulates the function of several ion channels known to be important for pain, including acid-sensing ion channels (Bohlen et al., 2011; Wemmie et al., 2013) and TRP channels (Caterina and Julius, 2001; Holzer, 2009), so it is a notable coincidence that protons also activate GPR68.

In summary, Xu et al. have demonstrated the power of an unbiased large scale and automated screening assay to discover shear stress–sensitive mechano-transducers. Looking forward, we anticipate that the development of new screening assays that use other types of mechanical stimulation, such as compression tensile forces or membrane stretch, will yield the discovery of even more receptors. Furthermore, many of the well understood mechanosensory mechanisms involve protein complexes (Scholz et al., 2016), suggesting that further screening might uncover essential subunits. Lastly, the assay used by Xu et al. (2018) was based on measuring calcium fluctuations. We already know of mechanosensitive ion channels selective for potassium that would not have been found with this approach. The continuous improvement of fluorescent indicators for other messenger pathways offers a wealth of new possibilities for future screens.

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