Piezo1 forms mechanosensitive ion channels in the human MCF-7 breast cancer cell line

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Mechanical interaction between cells – specifically distortion of tensional homeostasis-emerged as an important aspect of breast cancer genesis and progression. We investigated the biophysical characteristics of mechanosensitive ion channels (MSCs) in the malignant MCF-7 breast cancer cell line. MSCs turned out to be the most abundant ion channel species and could be activated by negative pressure at the outer side of the cell membrane in a saturable manner. Assessing single channel conductance (\(G_L\)) for different monovalent cations revealed an increase in the succession: \(\text{Li}^+ < \text{Na}^+ < \text{K}^+ < \text{Rb}^+ < \text{Cs}^+\). Divalent cations permeated also with the order: \(\text{Ca}^{2+} < \text{Ba}^{2+}\). Comparison of biophysical properties enabled us to identify MSCs in MCF-7 as ion channels formed by the Piezo1 protein. Using patch clamp technique no functional MSCs were observed in the benign MCF-10A mammary epithelial cell line. Blocking of MSCs by GsMTx-4 resulted in decreased motility of MCF-7, but not of MCF-10A cells, underscoring a possible role of Piezo1 in invasion and metastatic propagation. The role of Piezo1 in biology and progression of breast cancer is further substantiated by markedly reduced overall survival in patients with increased Piezo1 mRNA levels in the primary tumor.

The single cell, as elementary building block of an organ, steadily encounters physical forces such as hydrostatic pressure, shear, compression and tension. Via force-dependent activation of signaling cascades, cells dynamically adapt and respond to mechanical cues by modifying their behavior and remodeling of the microenvironment\(^1\). Together with hormonal and growth cues, force shapes cellular architecture of the mammary gland at all stages of development and function\(^1\). The balance between forces and cellular reactions is pivotal to maintain adult tissue homeostasis and, as a consequence, distortion or loss of this equilibrium leads to pathology, including cancer\(^2\). Several molecular entities that perceive and integrate forces (mechanoreceptors) have evolved in mammalian cells, amongst them various transmembrane ion channel proteins\(^9\). The transient receptor potential (Trp) ion channel family is represented by dozens of genes within the human genome and their members are generally activated by environmental stimuli such as temperature, pH, osmolarity, pheromones and taste compounds\(^1\). In addition, ion channels formed by particular Trp subunits have also been identified to act as mechanosensitive ion channels (MSCs)\(^1\). Other promising candidates for mechanotransduction in mammals comprise TMC and Piezo proteins, which represent two recently discovered mechanically gated ion channel families\(^3\). The role of ion channels as molecular actors contributing to virtually all hallmarks of cancer cells is still emerging\(^5\). Given the importance of mechanosensation for development and tissue homeostasis of normal mammary gland, its role in cancerogenesis and subsequent metastasation, we have studied MSCs within the plasma membrane of the malignant human MCF-7 breast cancer cell line. Permeation properties of ion channel proteins, i.e. single channel conductance and selectivity for certain kinds of ions, represent highly specific features that can be used to identify peculiar types of ion channels and even to distinguish between orthologs of subtypes from different species (see e.g.: http://www.guidetopharmacology.org/). In order to identify the molecular architecture of MSCs in MCF-7 cells, we characterized and compared this biophysical fingerprint to the recently discovered MSC protein Piezo1. In order to investigate the contribution of Trp ion channel subunits, we have
engineered a cell line, based on MCF-7 wild type (MCF-7WT) that permanently overexpresses a dominant negative TrpC subunit (MCF-7TrpC-k.o) and studied whether the density ofMSCs is affected by knockout of functional TrpC channels. The benign human MCF-10A mammary epithelial cell (MEC) line was studied in order to investigate whether the existence ofMSCs is a peculiar feature ofmalignant MECs. As Ca2+ permeableMSCs have been shown to play an important role in cell motility and migration13, we investigated whether block by the tarantula toxinGmSTx-4 influenced motility of the two MEC lines studied. Finally we have analyzed expression levels for the mRNA encoding the MSC of MCF-7 cells in primary tumors has an influence on prognosis for patients suffering from breast cancer.

**Methods**

**Cell culture.** MCF-7, MCF-10A and HEK-293 cells were cultured as described13,14. Medium was changed every 2–3 days. Confluent cells were detached split 1:10 and transferred to fresh culture flasks.

**Molecular Biology.** RNA isolation and cDNA synthesis was performed as described15. Six ng cDNA were subjected to PCR for gene quantification (QuantiFast SYBER Green PCR kit, Qiagen GmbH) using Light Cycler 480 system (40 Cycles; 95°C for 10 s, 60°C for 1 min). Successful expression was monitored by an antibiotic that blocks translation; LifeTech Company; Order No.: 11811-031; 3 mg/mL 72 h after TransfastTM transfection. Successful expression was monitored by a concentration of 90 μg/mL GsMTx-4 was obtained from Alomone Labs (Cat.#: STG-100, Jerusalem, Israel) and reconstituted at 10 μg/mL 72 h after TransfastTM transfection. Successful expression was monitored by fluorescence of the chimeric k.o subunit that was fused to a yellow variant of the green protein (eGFP) using the TransfastTM reagent (Promega) and MCF-10A cells with a concentration of 90 μg/mL Aliquots were shock frozen in liquid N2 and stored at −80 °C until use. All reagents used were of reagent grade unless stated otherwise.

**Results**

Upon application of mechanical stress, functionalMSCs were detected in 54% of cell attached membrane patches derived fromMCF-7 cells (Figure 1A, Table 1). The next frequently observed ion channel within the plasma membrane of MCF-7 cells was a constitutively active, highly K+ selective, inward rectifying ion channel with an inward single channel conductance of approx. 100 pS in PFS (supplementary Figure 2). Since this ion channel, in comparison toMSCs, was observed in only 9 out of 96 patches in MCF-7WT cells (p<0.05) and other species of ion channels were observed only once, we conclude thatMSCs represent a major ion channel population of the malignantMCF-7 breast cancer cell line. MSC activity depended on the strength of negative pressure (n.p.) applied in a saturable manner. Half maximal n.p. for activation (EP50; Figure 1b, Table 2) in MCF-7 cells was similar to EP50 values observed for Piezo1, a recently discovered MSC conductance (G50) was measured for cation flux across the plasma membrane from the extracellular compartment inside the cytosol and in the opposite direction. At the same absolute value of negative pressure (Ep), Itp was used to calculate the maximum (Ip) fractional activation, as a function of negative pressure (n.p. (mbar)) at the extracellular side, to equation (2).

\[
I_{tp} = \frac{1}{1 + e^{-\frac{V_p + V_p}{k_T}}} \quad (1)
\]

Where EP50 represents the n.p. required for half maximal activation ofMSCs and b the slope of the sigmoidal function at EP50. In our initial observations ofMSCs in the MCF-7WT cell line, PFS without addition of Gd3+ ions was used in the patch clamp pipette. Addition of Gd3+ at 20 μm/L, a concentration known to block both divalent cations under standardized conditions\(^{21}\), was added to the appropriate wells immediately before frame acquisition was started. Frames were acquired every 20 minutes for a total time interval of 72 h. Individual cells were tracked using the ImageJ software (v1.47; Wayne Rasband, NIH, http://image.nih.gov/) using the manual tracking plugin. Cell coordinates were recorded and analyzed using Microsoft Excel 2010 and routines written in Visual Basic for Applications (version 7.0). The 2D motility coefficient (MC (μm^2/min)) was calculated from the slope (D/Δt) of the linear regression of the squared distance (D^2) as a function of time interval Δt (MCF-7: 72 h) according to equation (2) as described13:

\[
MC = \frac{D^2}{4t} \quad (2)
\]

**Statistical analysis and bioinformatics.** Statistical analysis was performed using SigmaPlot/SigmaStat (version 12.5, Systat Software, USA). Experimental parameters were first tested for normal distribution. Subsequently, tests for statistical significance were performed (One Way ANOVA or Kruskal-Wallis test), followed by the appropriate pairwise multiple comparison procedures. The overall survival curves based on Piezo1 (Fam38A; affymetrix ID: 202771_at) mRNA expression was calculated by the "KMPlotter" tool using the "autoselect best cutoff" function (http://kmplot.com/assays/) using the breast cancer database (version 2014; N=1115).

**Cell observer and motility.** Cells were split and seeded to 24 well plates at densities of 1×10^5 cells/well (MCF-7) and 2.5×10^5 cells/well (MCF-10A), approx. 24 h before observation by the cell observer (Axiover200M, Zeiss, Germany). GmSTx-4 was added to the appropriate wells immediately before frame acquisition was started. Frames were acquired every 20 minutes for a total time interval of 72 h. Individual cells were tracked using the ImageJ software (v1.47; Wayne Rasband, NIH, http://image.nih.gov/) using the manual tracking plugin. Cell coordinates were recorded and analyzed using Microsoft Excel 2010 and routines written in Visual Basic for Applications (version 7.0). The 2D motility coefficient (MC (μm^2/min)) was calculated from the slope (D/Δt) of the linear regression of the squared distance (D^2) as a function of time interval Δt (MCF-7: 72 h) according to equation (2) as described13:

\[
MC = \frac{D^2}{4t} \quad (2)
\]
significantly reduced when Li\(^+\) or Na\(^+\) were used as permeant monovalent cations with the succession Li\(^+\) < Na\(^+\) < K\(^+\) < Rb\(^+\) < Cs\(^+\). Also divalent cations permeated considerably, but at significantly reduced \( G_L \)’s, when compared to K\(^+\) under the experimental conditions used. \( G_L \) was also significantly smaller for Ca\(^{2+}\) compared to Ba\(^{2+}\) (see Figure 3c for \( G_L \)’s for different cations). In summary, both distinct and significant differences in \( G_L \), the rate of ion permeation across an open MSC, were observed. Ion selectivity of MSCs (measured by the quotient of permeability coefficients (\( P_X / P_K \)) of the respective ion (\( P_X \)), normalized to the permeability coefficient for K\(^+\) (\( P_K \)), was calculated from the observed reversal potential of single channel currents (\( I_X \)’s; supplementary Table 1). The ratio \( P_{Ca^{2+}} / P_{K^+} \) (0.40

**Table 1 | Frequency of occurrence of MSCs in the cell lines used**

| Cell Line          | Total number of patches | Number of patches with MSCs | % of patches with MSCs |
|--------------------|--------------------------|----------------------------|------------------------|
| MCF-7\(^{WT}\)     | 291                      | 157                        | 54%                    |
| MCF-7\(^{WT}\)/TrpC\(_{k.o}\) | 39                      | 22                         | 56%                    |
| HEK-293\(^{WT}\)   | 16                       | 0                          | 0%                     |
| HEK-293\(^{k.o}\)  | 60                       | 44                         | 73%                    |
| MCF-10A\(^{WT}\)   | 30                       | 0                          | 0%                     |
| MCF-10A\(^{k.o}\)  | 45                       | 28                         | 62%                    |

\(^*\) stably expressing cell line.

**Figure 1 | Mechanosensitive ion channels in the MCF-7 cell line.** (1a): Original registration derived in the cell attached configuration (shown schematically at top). Mechanical stress was induced by applying negative pressure (n.p.) inside the pipette (indicated by arrows). (1b): Open probability (in % of maximum activation) as a function of negative pressure applied (N=18).

**Figure 2 | Single channel conductance properties of MSC in the MCF-7 cell line.** (2a): Original registrations recorded at different potentials during mechanical stress (configuration shown schematically at top). K\(^+\) ions (153 mmole/L) were carrying the inward single channel currents at negative potentials. (2b): I/V relation for the single channel currents shown in (2a).

**Table 2 | Activation of MSCs by negative pressure in the different breast cancer cell lines studied**

| Cell Line          | EP\(_{50} \) ± SEM (mm Hg) | b ± SEM (mm Hg\(^{-1}\)) | N  |
|--------------------|-----------------------------|---------------------------|----|
| MCF-7\(^{WT}\)     | 40.8 ± 1.1                  | 9.1 ± 1.0                 | 18 |
| MCF-10A\(^{k.o}\)  | 38.7 ± 1.1                  | 5.9 ± 1.1                 | 4  |
was significantly lower for Ca\textsuperscript{2+} when compared to K\textsuperscript{+}, but Ca\textsuperscript{2+} permeation was still substantially as indicated by \(G_L\). Ion permeability ratios were close to 1 for the other cations tested (Supplementary Figure 2). The specific differences in \(G_L\)'s observed for different cations led us to overexpress hPiezo1 in HEK-293 cells and to characterize the resulting MSCs. q-PCR analysis revealed that mRNA encoding Piezo1 was overexpressed 100 fold in transiently transfected cells, when compared to HEK-293 WT (Figure 4e). Accordingly, MSCs that were not observed in HEK-293\textsuperscript{WT} cells were frequently recorded from transfected HEK-293hP1 ones (Figure 4a&b; Table 1). Next, \(G_L\)'s of the resulting MSCs at chosen ion compositions were characterized (Figure 4c&d). Single channel conductance was significantly smaller (p < 0.001) when Li\textsuperscript{+} was used as charge carrier. Ca\textsuperscript{2+} alike MSCs recorded from MCF-7\textsuperscript{WT}, permeated also. \(G_L\)'s assessed for Li\textsuperscript{+}, K\textsuperscript{+}, Ca\textsuperscript{2+} and Na\textsuperscript{+}:K\textsuperscript{+} (at a molar ratio of 1:1) were indistinguishable from those of MSCs recorded from MCF-7 cells (Figure 4d). The peculiar \(G_L\)'s for different cations observed by us strongly suggest that MSCs in MCF-7 are composed of Piezo1 protein. In order to generate additional data that would allow MSC identification, we have engineered a MCF-7 based cell line overexpressing a dominant negative TrpC subunit (MCF-7\textsuperscript{TrpC_k.o}). Overexpression of the ion permeation deficient subunit is expected to eliminate currents through channels formed by TrpC proteins that are known homo- or heteromerization partners of TrpC1, TrpC3, TrpC4, TrpC6 and TrpC7\textsuperscript{20}. Provided involvement of TrpC subunits in MSC formation, a significant reduction in the number of functional ion channels is expected in the MCF-7\textsuperscript{TrpC_k.o} cell line. After all, the frequency of occurrence of functional MSCs in both cell lines was similar, suggesting that TrpC subunits are not involved (Table 1).

MCF-7 cells have been cultivated from invasive ductal carcinoma and exert the luminal gene cluster subtype signature\textsuperscript{26}. Subsequently we investigated whether non-cancerous breast cells do also possess functional MSCs at the surface. Accordingly, the MCF-10A line, derived from human fibrocystic mammary tissue and representing an immortal non neoplastic MEC line\textsuperscript{27} was used as a model for normal mammary gland cells. Under the experimental conditions used we could not detect functional MSCs in MCF-10A cells (Figure 5a; Table 1). In addition, q-PCR revealed that mRNA encoding Piezo1 is substantially reduced in MCF-10A compared to MCF-7 cells, but not entirely absent (Figure 5d, right). When cDNA encoding human Piezo1 was overexpressed transiently in MCF-10A cells, endogenous Piezo1 mRNA levels increased >10 fold (Figure 5D, left). Consequently MSCs with single channel conductance indistinguishable from those obtained from HEK-293 cells were observed (Figure 5c; supplementary Figure 3). The results indicate that MCF-10A cells are able to express MSCs formed by Piezo1 protein, but endogenous expression is not sufficient to form functional channels.

Migration, motility and invasion represent essential hallmarks of cancer cells, important for malignancy and metastasation\textsuperscript{28}.

| Table 3 | Single channel properties of MSCs in the MCF-7\textsuperscript{WT} cell line (PFS) |
|----------|-------------------------------------|
| \(E_{rev}\) (mV) | Mean ± SEM | N |
| K\textsuperscript{+} inward | 7.3 ± 1.2 | 8 |
| \(G_L\) (pS); inward | 25.6 ± 0.4 | 8 |
| \(G_L\) (pS); outward | 20.6 ± 2.1 | 8 |

Figure 3 | Ion selectivity profile of MSC in MCF-7 cell line. (3a): Original registrations of single channel inward currents recorded in the cell attached configuration at three different potentials during mechanical stress. Different ions were used as charge carriers for the inward currents (153 mmole/L were used in case of monovalent cations and 100 mmole/L in case of divalent cations). (3b): Average I/V relations for single \(I\textsubscript{in}\) inward currents carried by different cations (N = 6–10). Symbols denote mean values ± SEM, line denotes linear regression through the data. (3c): Ion selectivity profile of inward single channel conductance carried by different mono- and divalent cations. Number of individual experiments is shown at the top of each bar in parenthesis. Mean values ± SEM are shown.
Moreover, Ca\(^{2+}\) permeable MSCs have been shown to be pivotal for cell motility and migration\(^{16}\). Subsequently we investigated whether MSCs formed by Piezo1 may regulate these cellular properties. Motility and velocity of MCF-7WT cells were studied in the absence and in the presence of GsMTx-4 (Figure 6a&b), a peptide toxin from Chilean rose tarantula venom, known to block functional Piezo1 channels\(^{29}\). Both cellular velocity and motility of MCF-7WT cells were reduced by the presence of GsMTx-4 (Figure 6c&d). In contrast to MCF-7, GsMTx-4 did not affect velocity or motility of MCF-10AWT cells (supplementary Figure 4). This finding further supports a role of Piezo1 in motility of the cancerous MCF-7 cell line. The observations derived from models of benign and malign MECs prompted us to investigate whether overexpression of mRNA encoding Piezo1 in the primary tumor may be related to clinical outcome in breast cancer patients. A dataset generated from GEO and comprising overall survival data for 1115 patients was used (dataset version 2014)\(^{23}\). Overall survival times of breast cancer patients with low mRNA expression for Piezo1 in the primary tumor turned out to be significantly longer when compared to patients with high expression levels (Figure 7). This finding is of strong support that high levels of Piezo1 in the tumor have causal and profound impact on disease progression.

**Discussion**

Here we report for the first time the existence of functional mechanosensitive ion channels in a malignant human MEC line. Single channel analysis revealed that Ca\(^{2+}\) permeation of MSCs in the MCF-7 line is substantial. Furthermore, characteristic differences in G\(_A\) for Li\(^+\) and Na\(^+\) (the smallest alkali metal ions tested), were found when compared to G\(_A\) for other alkali metal ions (K\(^+\), Rb\(^+\), Cs\(^+\)). Among monovalent and divalent cations studied, G\(_A\) was inversely related to the radius of the hydrated ion suggesting that these ions may pass the open pore in the hydrated configuration. Several facts prompted us to identify Piezo1 as a component of MSCs.
in MCF-7 cells: (i) $G_l$’s were indistinguishable between MSCs from MCF-7 and MSCs formed by overexpressed Piezo1 in HEK-293 cells. (ii) The particular ion permeation properties with respect to $G_l$’s for different ions as described above were identical between MSCs from MCF-7 and Piezo1 overexpressed in HEK-293 cells. (iii) MSCs in cell attached patches of MCF-7 cells exerted similar mechanical sensitivity when compared to MSCs formed by overexpression of Piezo1 in HEK-293 as well as in MCF-10A cells and (iv) MSCs in MCF-7 disclosed themselves to be completely unreactive to global knockout of MSCs formed by canonical Trp subunits.

When the benign MEC line MCF-10A was screened via the patch clamp method, no functional MSCs were observed. Expression levels for mRNA encoding Piezo1 were substantially lower when compared to MCF-7, but not negligible. In this context it is worth mentioning that the cDNA encoding human Piezo1 has been initially cloned from wild-type HEK-293 cells. This cell line is, however, frequently used as a negative control, both in biochemical and electrophysiological experiments, demonstrating that its endogenous mRNA levels are not sufficient to produce high amount of Piezo1 protein. Thus a low density (or even absence) of functional MSCs in the plasma membrane despite of moderate mRNA levels is not an unusual situation. Several scenarios may account for the absence of functional MSCs in the plasma membrane of malignant MECs: (i) Endogenous mRNA encoding Piezo1 does not produce MSCs at sufficient high numbers to allow reliable detection by the patch clamp method. (ii) The resulting small amount of protein is not inserted into the plasma membrane. (iii) Endogenous Piezo1 protein is directed towards protein complexes, where it cannot be activated by simple mechanical stimulation and only heterologous Piezo1, inserted somewhere else, is accessible to this stimulation or (iv) endogenous Piezo1 is blocked by endogenous factors that become rate limiting upon overexpression (see e.g.: ref. 30).

Several studies have addressed the effects of mechanical stress on cancerogenesis and tumor progression in benign and malign MECs and thereupon may shed light on potential roles of increased densities of MSCs in the plasma membrane of malignant MECs: When the effect of compressive stress on proliferation, apoptosis, migration and cytoskeletal architecture of several MECs, including MCF-10A and MCF-7, was studied, malign breast carcinoma cell lines, but not MCF-10A responded to compressive stress with the development of a more aggressive phenotype. The authors of the study concluded that non-tumorigenic MCF-10A cells are less mechanosensitive as their malign counterparts. Using a microlithography based approach it was found that proliferation and invasion of several lines of malignant MECs, embedded in non-malignant tissue, occurred preferentially in regions characterized by high endogenous mechanical stress; this observation substantiates the role of mechanical stress in promoting the malignant phenotype. In another study the impact of long-range mechanical interaction exerted via collagen lines on the disorganization of ras-transformed mammary acini formed by MCF-10AT cells was investigated. Mechanical interaction between MCF-10AT acini was shown to facilitate the transition to the invasive phenotype whereas mechanical isolation of acini impaired it. Taken together, the general role of mechanical stress in etiology and progression of breast cancer as well as the impact of mechanosignaling on growth, invasion and differentiation of pecu-
The findings described above fit reasonably to the hypothesis that a high density of MSCs in malignant MECs intensifies their reaction to mechanical cues, thereby promoting malignancy. It must be stated, however, that mechanosensation by MECs is generally considered to be based on a molecular machinery that does not necessarily entail ion channels: The molecular chain of events comprises ligands of the extracellular matrix or from neighboring cells that bind to integrins thereby inducing integ-

Figure 6 | Effect of GsMTx-4 on migration and velocity of MCF-7 cells. (6a): left: Migration trajectories of five single MCF-7WT cells over the entire observation interval of 72h. Right: Squared distance as a function of time for the five cells shown to the left. (6b): similar to 6a but in the presence of 300 nmole/L GsMTx-4. (6c): statistical analysis of motility coefficients. Black line in box marks median, upper and lower borders of box mark 25th and 75th percentiles, whiskers mark 10th and 90th percentiles, respectively; black crosses mark individual single cell velocities below and above the 10th and 90th percentiles. Grey line marks mean value. Number of individual cells studied is shown in parenthesis above each box. The dataset was checked for statistical significant differences using ANOVA based on ranks. (6d): similar to 6c but cellular velocities are shown.
xerocytosis and distal arthropathy24,26. In addition to our findings, other data suggest that Piezo1 protein is also involved in cancer: Most intense expression of mRNAs encoding Piezo1, amongst the tissues studied, has been found in the lung and also in lung epithelial cells, where its physiological role still remains to be shown25,27. Loss of Piezo1 in normal lung epithelial cells promoted an amoeboid, reduced integrin-dependent, mode of cell migration, that is a typical phenotype of small lung cancer cells where Piezo1 expression was found to be greatly reduced28. In the prevailing study we observed the opposite, i.e. that mRNA expression was low in benign MECS, but higher expression levels led to functional MSCs within the plasma membrane of the malignant MCF-7 cell line, affecting cell migration. Similar to our results, knockdown of Piezo1 in gastric epithelium cancer cells has been shown to reduce cell migration and the authors suggested that Piezo1 overexpression promotes invasion and metastasis of gastric cancer29. Piezo1 was also amongst the most profoundly upregulated genes in thyroid cancers following Iodine-131 exposure after the Chernobyl accident30, indicating that deregulated Piezo1 expression may contribute to cancer of several tissue types. The role of Piezo1 in progression of breast cancer is substantiated by the significantly increased hazard ratio and corresponding shorter overall survival times of breast cancer patients upon high mRNA expression levels in the primary tumor reported here. This novel role of Piezo1 in cancer biology seems to be a peculiar manifestation in breast cancer that is different to the role of Piezo1 in lung cancer. Further research will reveal whether Piezo1 is causally involved in cancerogenesis and progression of breast cancer, represents a potential therapeutic target or can be used as a prognostic factor.

Figure 7 | Overall survival of breast cancer patients with low and high expression of Piezo1 mRNA in the primary tumor. Kaplan–Meier plot showing overall survival of breast cancer patients with low and high expression levels of Piezo1 mRNA. Grey line: patients with high expression of Piezo1; black line: patients with low expression (cutoff value was 1760 tpm (transcripts per million)). Hazard ratio (HR) was 1.63 (1.26–2.09; 95% confidence interval), P < 0.000013, N = 1115.

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Author contributions
C.L. and W.S. performed patch clamp experiments. S.R., S.K., A.G. and S.J. performed molecular biology experiments. C.L., S.R., A.S., T.dV. and W.S. performed experiments with and analyzed data from the cell observer. S.R., A.S., H.H., C.W. and W.S. performed data mining and biostatistics. C.L., A.S. and W.S. prepared the figures. C.L. and W.S. wrote the main manuscript text. C.L., S.R., S.K., A.S., T.dV., A.G., S.J., H.H., K.G., C.W., E.M., T.B. and W.S. participated in discussions on and planning of experiments. All authors reviewed the manuscript.

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