Pannexin1 Drives Multicellular Aggregate Compaction via a Signaling Cascade That Remodels the Actin Cytoskeleton*

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Pannexin 1 (Panx1) is a novel gap junction protein shown to have tumor-suppressive properties. To model its in vivo role in the intratumor biomechanical environment, we investigated whether Panx1 channels modulate the dynamic assembly of multicellular C6 glioma aggregates. Treatment with carboxylate and probenecid, which directly and specifically block Panx1 channels, respectively, showed that Panx1 is involved in accelerating aggregate assembly. Experiments further showed that exogenous ATP can reverse the inhibitive effects of carbenoxolone and that aggregate compaction is sensitive to the purinergic antagonist suramin. With a close examination of the F-actin microfilament network, these findings show that Panx1 channels act as conduits for ATP release that stimulate the P2X7 purinergic receptor pathway, in turn up-regulating actomyosin function. Using a unique three-dimensional scaffold-free method to quantify multicellular interactions, this study shows that Panx1 is intimately involved in regulating intercellular biomechanical interactions pivotal in the progression of cancer.

Background: Panx1 is a novel gap junction protein with tumor-suppressive properties. Results: Panx1 channels release ATP to initiate a cascade that drives actomyosin-mediated assembly of multicellular aggregates. Conclusion: Expression of Panx1 can directly alter the biomechanical properties of three-dimensional tumor aggregates. Significance: Panx1 channels through P2X7 signaling help regulate three-dimensional biomechanics of tumor suppression.

Pannexins (Panx)2 are a novel group of membrane channel proteins homologous to the invertebrate gap junction proteins, innexins (1). To date, there are three subtypes: Panx1, Panx2, and Panx3. Although sharing no sequence homology with innexins, the predominant class of gap junction proteins, they do possess topological similarities. Both have four transmembrane domains, two extracellular loops, and cysteoplastic amino and carboxylic termini (2, 3). Although connexins have been defined by their ability to form gap junctions via the docking of apposed hemichannels (4), this may not be the case for pannexins. Evidence of their clearly distinct cell surface dynamics and cytoskeletal interactions (5) suggests that pannexins primarily function in the undocked configuration.

With research on pannexins still sparse but growing, most (if not all) of the in vitro studies thus far have employed a single cell/few cell model, such as the Xenopus oocyte, to carefully characterize the gating and trafficking properties of the channel proteins themselves. Rather than using standard two-dimensional tissue culture techniques, the goal of this study is to examine Panx1 function in a 3D multicellular system (6).

In particular, we examined the role of Panx1 in the spontaneous assembly of large multicellular structures from individual cancer cells, a dynamic phenomenon that models the morphogenesis of cancerous and normal tissues alike (7, 8). Once thought to be driven exclusively by cell adhesion molecules (CAMs) (9, 10), it is increasingly clear that although CAMs are critical in the initial cell contact/adhesive phase, other components are also involved. Specifically, the actomyosin cytoskeletal system appears to be responsible for generating the intercellular biomechanical forces that drive the compaction and stabilization of these 3D structures (11). Gap junction proteins appear to play a complex and multifaceted role. Short-term rotary shaker assays examining the initial adhesive phase have found that connexin hemichannel docking may be an adhesive event in its own right (12, 13). Within the extended time frame of aggregate assembly, we found that connexin docking and subsequent gap junction channel activity may play opposing roles, with the former accelerating and latter regulating assembly (14). Given their mechanistic distinctions from connexins, it is difficult to speculate the exact function, if any, of pannexins. Answering this question may prove worthwhile, given the well documented inverse relationship between gap junction proteins and cancer (15) and growing evidence that such biomechanical interactions within the tumor itself are intimately involved in disease progression (16).

We have shown previously shown that rat C6 glioma cells, which have reduced connexin expression (17), do not endogenously express pannexins and that instituting Panx1 expression reduces several tumorigenic parameters (18, 19). Using C6...
cells, this study shows that Panx1 dramatically accelerates the assembly of large multicellular tumor aggregates. Pharmacological disruption of Panx1 channel activity by the direct channel inhibitor carbenoxolone and the purinergic receptor antagonist suramin show that Panx1 channels, as conduits for ATP, initiate an intracellular signaling cascade. Treatment with cytochalasin B and a close examination of the F-actin microfilament network show further that it may be this downstream cytoskeletal effector that is directly accelerating assembly.

**EXPERIMENTAL PROCEDURES**

**3D Multicellular Scaffold-free Assays**—A modified version of our 3D non-adhesive hydrogel system (20) was used to examine C6 proliferation and multicellular aggregate assembly. Briefly, agarose hydrogels were produced by pouring a sterile 3% molten agarose solution (500 μl) into 3D PetriDish™ micromolds (MicroTissues, Inc.) designed for 24-well tissue culture plates. After allowing the agarose to set for 10 min, the gels were separated from the micromolds according to the manufacturer’s protocol and transferred to the tissue culture plates with the seeding chambers facing upwards (1 gel/well). The gels were briefly degassed and then equilibrated overnight in the appropriate culture medium. For the proliferation assay, we used serum-free DMEM with 1% penicillin/streptomycin and, if applicable, the drug of interest: aggregate assembly assays, we used serum-free DMEM with 1% penicillin/streptomycin, and for appropriate culture medium. For the proliferation assay, we used serum-free DMEM with 1% penicillin/streptomycin, and if applicable, the drug of interest: carbenoxolone (CBX) (50–100 μM), probenecid (PBN) (200 μM), ATP (500 μM), suramin (100 μM), 2′,3′-(benzoyl-4-benzoyl)-ATP (BzATP) (200–400 μM), oxidized ATP (oATP) (200 μM), brilliant blue G (1–5 μM), 2-methylthio-ADP (MeSADP) (250 μM), pyridoxal-phosphate-6-axophenyl-2′,4′-disulphonic acid (PPADS) (100 μM), and UTP (100 μM). Stock solutions of drugs were prepared according to the manufacturers’ protocols, and appropriate vehicle controls were used. Spheroid micromolds with a rectangular array of 24 (3 × 8) trough-shaped round-bottom recesses (2200-μm length, 400-μm width, 800-μm depth) were used for assembly assays requiring quantification of compaction kinetics. Seeding of cells into gels, considered 0 h, was as follows. C6 cells grown to near confluence in DMEM with 10% FBS and 1% penicillin/streptomycin in 37 °C and 5% CO₂ were trypsinized for 5 min, counted, and resuspended to the desired seeding density: 100 cells/60 μl for the proliferation assay, 0.12 × 10⁶ cells/60 μl for spheroid-based assembly assays, and 0.6 × 10⁶ cells/60 μl for rod-based assembly assays. Cells were seeded by pipetting 60 μl of cell suspension into the seeding chamber and allowed to settle for 10 min before they began to spontaneously assemble into aggregates. 1 ml of additional culture medium was added before time-lapse microscopy began.

**Microscopy and Image Analysis**—Proliferation and aggregate compaction kinetics were quantified by analyzing time-lapse phase contrast images taken with an Axio Observer Z1 microscope equipped with an AxioCam mRM camera and a humidified chamber with temperature (37 °C) and CO₂ (5%) control (Carl Zeiss MicroImaging). To quantify proliferation kinetics, images were taken every 24 h for 13 days. The fit ellipse tool in ImageJ (National Institutes of Health) was then used to measure the area of the growing tumor spheroid. To quantify rod aggregate compaction, images were taken every hour for 9 to 24 h. The line tool in ImageJ was used to measure rod length, defined as the long axis (x axis) length normalized to the 0 h length. Percent inhibition by drug treatment was defined as the percent difference between the drug-treated and control rod lengths. Visualization of specific proteins (Panx1-eGFP, calcein acetoxyethyl, F-actin) within aggregates was achieved by harvesting the aggregates via gel inversion and brief centrifugation followed by imaging with a LSM 510 meta confocal laser scanning microscope (Zeiss).

**Protein Visualization and Immunostaining**—Panx1-eGFP was visualized within homocellular aggregates directly without fixation. For live-cell visualization of Panx1-eGFP and Panx2-eGFP in heterocellular aggregates, Panx2 cells were first stained with 5 μM CellTracker Blue 7-amoino-4-chloromethylcoumarin (Invitrogen) for 45 min before being mixed with Panx1 cells and seeded into gels. For immunostaining of F-actin, aggregates seeded 12 h earlier were washed in PBS, fixed in 4% paraformaldehyde for 1 h, and washed again. Samples were then permeabilized with 0.5% Triton X-100 for 30 min, followed by additional washes with PBS. Aggregates were then stained with Alexa Fluor 568 phalloidin (Invitrogen) for 45 min. After a final wash, aggregates were imaged. Aggregate viability with drug treatment was determined using the live/dead viability/cytotoxicity kit (Invitrogen) as described previously (21).

**RESULTS**

**Panx1 Channels Accelerate Compaction of Multicellular C6 Glioma Aggregates**—To determine whether our 3D system can recapitulate tumor behavior in vivo, we developed an assay investigating the role of Panx1 on C6 glioma tumorigenicity to compare with our previous in vivo findings (18). Briefly, wild-type C6 cells and those stably transfected with Panx1-eGFP were trypsinized and seeded into micromolded agarose gels with circular recesses so that there was only 1 cell/recess initially (100 cells into 96 recesses/gel). The area of the growing tumor spheroids was measured over 13 days (Fig. 1A). Mirroring our in vivo results, there was minimal growth within 48 h, but from day 7 onwards, Panx1 spheroids were significantly smaller than their wild-type counterparts (day 13, 23,983 ± 9851 μm² versus 60,706 ± 17,810 μm² (n = 20).

To quantify the role of Panx1 in 3D biomechanics and the assembly of large multicellular structures, an alternate assay was employed. Mono-dispersed wild-type and Panx1 cells, as well as C6 cells expressing Panx2-eGFP, were seeded into gels with rod-shaped recesses so that there were ~25,000 cells/recess (0.6 × 10⁶ cells into 24 recesses/gel). Panx2 cells were chosen as an additional control, given their intracellular localization (19) and subsequent lack of channel activity (22). Aggregation and compaction kinetics were quantified by measuring the length of the contracting rod aggregates and normalizing this value to its initial length over 24 h, prior to any significant proliferation in our 3D system (Fig. 1B). Panx1 multicellular rods displayed significantly accelerated compac-
FIGURE 1. **Panx1 accelerates multicellular C6 aggregate compaction.** A, an *in vitro* 3D proliferation assay shows that 13-day growth of Panx1-expressing C6 tumor spheroids is significantly slower than that of wild-type C6 spheroids (n = 20). Scale bar = 200 µm. B, a multicellular rod compaction assay shows that Panx1-expressing C6 aggregates contract significantly faster than wild-type or Panx2 aggregates (n = 10). Scale bar = 200 µm. C, drug treatment of multicellular rods with CBX (50–100 µM) and PBN (200 µM) shows that only Panx1 rods were affected (n = 10). D, CBX treatment causes noticeable internalization of Panx1-eGFP, normally expressed uniformly on the cell membrane, whereas Panx2-eGFP is unaffected and remains intracellular. Scale bar = 20 µm.
tion compared with wild-type and Panx2 rods, whose kinetics were comparable. Panx1 aggregate compaction was most dynamic from 3–15 h and slowed toward equilibrium.

To determine whether the acceleration in 3D assembly was directly correlated with Panx1 channel activity, cells were seeded into gels pre-equilibrated with CBX (50–100 μM) or PBN (200 μM). CBX is a potent gap junction inhibitor that directly blocks Panx1 channels (23) in a dose-dependent manner, whereas PBN is specific for Panx1 (24). The percent inhibition of rod contraction with drug treatment was determined at 12 h, within the dynamic range of assembly, and shows that spontaneous Panx1 rod contraction was inhibited by both PBN (10.7 ± 2.1%) and CBX, with the latter displaying a dose-dependent relationship (16.8 ± 7.0% for 50 μM and 34.5 ± 2.1% for 100 μM) (n = 10) (Fig. 1C). As expected, wild-type and Panx2 rods were unaffected by drug treatment. Drug effects were observed in Panx1 aggregate compaction as early as 3 h and maintained through 24 h, with live/dead analysis confirming that viability was not compromised (data not shown). Interestingly, we observed that CBX caused an internalization of Panx1-eGFP, normally localized in a uniform manner on the cell membrane, in cells within aggregates (Fig. 1D).

Panx1 within the Heterocellular Microenvironment—Having examined Panx1 in only homocellular aggregates, it was important to extend our investigation to include a heterocellular environment. To this end, we observed the behavior of heterocellular Panx1/Panx2 aggregates by titrating the proportion of Panx1 cells in 10% increments while keeping the total cell number constant. The normalized rod lengths at four different times over 24 h (6 h, 12 h, 18 h, 24 h) were measured as a function of the proportion of Panx1 cells (Fig. 2A). For all times measured, we found that increasing the Panx1 cell percentage resulted in a linear shortening in rod length (6 h r²0.949, 12 h r²0.967, 18 h r²0.980, 24 h r²0.991). The slopes of these four curves were nearly constant (−0.0013, −0.0015, −0.0015, and −0.0014, respectively), suggesting that heterocellular Panx1/Panx2 aggregate compaction is dependent on the proportion of the two constituent cell types.

To determine whether localization of either Panx1 or Panx2 proteins had been altered, unlabeled Panx1 cells were mixed with Panx2 cells labeled with CMAC (blue) at 50%:50% (Fig. 2B). Confocal images at 24 h confirm that trafficking of Panx1-eGFP and Panx2-eGFP were unchanged in the heterocellular aggregates. Interestingly, we also observed a tendency of Panx1 cells to compartmentalize to the center of these heterocellular aggregates.

Given reports of possible heteromeric Panx1/Panx2 interactions when coexpressed within the same cell (25), we compared the assembly kinetics of C6 cells expressing both Panx1 and Panx2 to C6 rods expressing Panx1 alone (Fig. 2C). The Panx1 + 2 rods compacted as quickly as the Panx1 rods, both significantly faster than the wild-type control.

Panx1-mediated ATP Release Drives Compaction via a Signal Cascade That Up-regulates Cytoskeletal Function—Rather than forming gap junctions, recent evidence suggests that Panx1 channels release ATP into the extracellular space, which then binds to cell surface purinergic receptors (26). A modified dye transfer assay confirmed the absence of gap junction-mediated coupling within Panx1 aggregates (supplemental Fig. 1). Having established the relationship between Panx1 channel activity and aggregate assembly, we then determined if this effect was facilitated by the specific release of ATP. We found that addition of exogenous ATP significantly, although not completely, reverses the inhibitive effects of CBX on Panx1 aggregate compaction (Fig. 3A). Panx1 cells in gels pre-equilibrated with ATP (500 μM) and CBX (50 μM) formed more compact aggregates, resembling the control rather than the loose aggregates seen with CBX treatment alone. Live (green)/dead (red) images at 24 h confirm that viability was not compromised.
by treatment with CBX and/or ATP. Furthermore, the addition of exogenous ATP (500 μM) alone could accelerate compaction of both wild-type and Panx1 aggregates (Fig. 3B). Although the effect on wild-type aggregates was immediate and consistent through 24 h, its onset was delayed but more pronounced for Panx1 aggregates.

Given that extracellular ATP can bind to P2X7 cationic channel (27) and P2Y1/P2Y2 G-protein coupled (28) purinergic receptors, both shown to be expressed in C6 glioma cells (29, 30), we then determined which receptor pathway is dominant in aggregate compaction. Wild-type and Panx1 rods were first treated with suramin (100 μM), a nonspecific purinergic antagonist. Although wild-type rods were unaffected, suramin immediately inhibited Panx1 rod compaction (Fig. 4A). To differentiate between the P2X7 and P2Y1/P2Y2 pathways, samples were then treated with a complimentary series of agonists/antagonists specific for P2X7 or P2Y1/P2Y2. When treated with the P2X7 agonist BzATP (200–400 μM), Panx1 rods displayed a significant acceleration in assembly that was dose-dependent (Fig. 4B) and more pronounced than that with ATP (500 μM) at 12 h. When concomitantly treated with BzATP (200 μM) and the P2X7 antagonist oATP (200 μM), the BzATP-induced effect was mitigated as these Panx1 rods compacted similarly to the untreated control. Wild-type aggregates were unaffected by treatment with BzATP and/or oATP. Interestingly, treatment with the P2X7 antagonist BBG (1–5 μM) did not affect Panx1 rod compaction (supplemental Fig. 2). Wild-type and Panx1 rods were then treated with reagents specific for P2Y1 (agonist MeSADP, antagonist PPADS) and P2Y2 (agonist UTP) (Fig. 4C). Neither wild-type nor Panx1 aggregates were affected. Taken together, these findings implicate the involvement of Panx1-mediated ATP release and subsequent P2X7 purinergic receptor activation in aggregate assembly.

The actomyosin cytoskeleton system is involved in aggregate compaction upon initial cell-cell adhesion (11). We therefore investigated whether this Panx1/ATP/P2X7 cascade modulated the F-actin cytoskeletal network (Fig. 5A). In comparison to wild-type aggregates, which possess a moderate cortical F-actin network and spherical cell morphology, we found that Panx1 aggregates possessed a significantly more robust F-actin cytoskeleton. In addition, the F-actin distribution extended to noncortical regions, whereas cell morphology became flattened. Not surprisingly, these same changes became even more pronounced with the addition of exogenous ATP. Although CBX reduced F-actin expression, the addition of exogenous ATP to CBX-treated aggregates reverted the cytoskeletal phenotype to resemble that of the untreated control. Within heterocellular Panx1/Panx2 aggregates, the F-actin network of Panx1 cells is noticeably more developed than that of Panx2 cells, which remains mostly cortical (Fig. 6A). To further explore this relationship, wild-type and Panx1 aggregates were treated with cytochalasin B (5 μM), a potent inhibitor of actin polymerization (Fig. 5B). Although assembly was nearly arrested by drug treatment in both samples, Panx1 aggregates alone were able to resume compaction upon drug washout at 3 h. Confocal images at 12 h confirm that cytochalasin B disrupted the F-actin network within Panx1 aggregates and that this effect was reversed with washout (Fig. 5C).
DISCUSSION

In this study, we show that Panx1 channels, as conduits for ATP release, initiate a signaling cascade that accelerates the formation and stabilization of large multicellular C6 glioma tumor aggregates. The 3D multicellular approach employed facilitates a close look at the biomechanical interactions within the tumor itself in response to evidence that such interactions and the subsequent stress forces generated play a pivotal role in disease progression (16). Although standard two-dimensional techniques have recapitulated such forces with some success, showing that external compressive forces can modify expression of genes involved in tumor invasion and metastasis (namely, Caveolin 1, β-integrin, phosphatase and tensin homolog, and Rac (31)), 3D tumor models are advantageous in that such forces are intrinsic. 3D models such as ours can generate internal stresses greater than 10 kPa and strains larger than 40% (32), which can alter the proliferative (33) and intercellular adhesive properties (34) of the developing tumor.

We found that 3D proliferation within our in vitro assay mirrored our previously reported findings of tumor growth in vivo (18). Both studies demonstrated nearly identical growth profiles, with minimal growth in the first few days before showing significant differences between wild-type and Panx1-expressing C6 tumors beyond day 8.

The effects of Panx1 on 3D biomechanics was also evident in multicellular C6 aggregate assembly, whereby thousands of monodispersed cells adhere and spontaneously compact further into a single multicellular unit within a short time, typically

![Figure 4](image-url)

FIGURE 4. Panx1 rod compaction is inhibited by the non-specific purinergic antagonist suramin (100 μM) (n = 5) (A). B, Panx1 rod compaction is accelerated by the P2X7 agonist BzATP (200–400 μM), an effect reversed with concomitant addition of the P2X7 antagonist oATP (200 μM) (n = 10). C, Panx1 rod compaction is unaffected by reagents specific for P2Y1 (agonist MeSADP, antagonist PPADS) and P2Y2 (agonist UTP) (n = 10). In all three experiments, wild-type rods were unaffected by drug treatment.
24 h. With our scaffold-free system, which minimizes cell-matrix interactions, we could quantify these intercellular biomechanical forces prior to proliferation. We found that expression of Panx1 significantly accelerated C6 aggregate compaction, an effect sensitive to CBX and PBN. The inhibitive effects of the two drugs, known to directly (23) and specifically (24) block Panx1 channels, respectively, confirms the connection between channel function and up-regulated aggregate compaction. Interestingly, the capacity of the drugs to inhibit Panx1 aggregate assembly mirrored their relative documented efficacies in blocking Panx1 channel currents (CBX IC50, 5 μM (22); PBN IC50, 350 μM (24)), suggesting that the acceleration in assembly may be a function of channel permeability.

Given their uniform distribution rather than plaque-based membrane localization and glycosylated status (35), it is unlikely that Panx1 channels are mediating these effects via gap junction formation. We found no evidence of coupling within homocellular Panx1 and heterocellular Panx1/Panx2 aggregates and observed that coexpression of Panx2 in Panx1-expressing C6 cells did not affect compaction, supporting growing data that Panx1 acts through an alternative mechanism. Instead, recent evidence suggests that Panx1 channels primarily facilitate the release of ATP, which then binds to purinergic receptors (26). The expression of Panx1 in Xenopus oocytes (36) has been found to increase ATP release, whereas siRNA directed against Panx1 suppresses basal ATP release in mouse pituitary At-t20 cells (37).

The current study uses several complementary experiments to show that Panx1 accelerates the 3D compaction of C6 glial aggregates via ATP release. Although wild-type C6 cells cannot release ATP (38), they do possess surface purinergic receptors. Not surprisingly then, we found that addition of exogenous ATP alone accelerated the assembly of both wild-type and Panx1 aggregates whereas, it also reversed the inhibitive effects of CBX-mediated Panx1 channel closure in the latter. Although the effects of ATP treatment (without CBX) were comparable at least in order of magnitude, it is interesting to note that this effect was delayed in Panx1 aggregates, whereas it appeared immediately in wild-type cells. This difference may be due to the intrinsic capacity of Panx1 cells to release ATP, in turn mitigating the effect of the exogenous ATP and reflecting receptor sensitization/saturation. Alternatively, this could also suggest that Panx1 channel expression alters purinergic receptor expression and function.

Because C6 cells express P2X7 (29) and P2Y1/P2Y2 (30) purinergic receptors, both of which can bind ATP, it was important to explore which of these receptor pathways were involved in C6 aggregate compaction. Treatment with the nonspecific
extracellular space. A mechanical stimulus causes Panx1 channels to open and release ATP into the cells, forming initial cell-cell adhesive contacts via CAM interactions. This biomechanical stimulus causes Panx1 channels to open and release ATP into the extracellular space. ATP binds to cell surface P2X7 purinergic receptors, initiating a signaling cascade that increases intracellular calcium. These calcium waves stimulate actin microfilament organization, up-regulating the intercellular tensile forces that drive aggregate compaction.

**FIGURE 6.** F-actin microfilament network (red) of Panx1 cells (unlabeled) is noticeably more developed than that of Panx2 cells (blue) in heterocellular aggregates (A). Scale bar = 50 μm. B, Panx1 signaling cascade and 3D multicellular aggregate compaction. Upon seeding (t0), monodispersed cells form initial cell-cell adhesive contacts via CAM interactions. This biomechanical stimulus causes Panx1 channels to open and release ATP into the extracellular space (t). ATP binds to cell surface P2X7 purinergic receptors (2), initiating a signaling cascade that increases intracellular calcium (3). These calcium waves stimulate actin microfilament organization (4), up-regulating the intercellular tensile forces that drive aggregate compaction (5).

Panx1 Signaling Controls 3D Biomechanics

purinergic antagonist suramin immediately inhibited compaction of Panx1 rods without affecting wild-type rods, suggesting that ATP released via Panx1 may be interacting with surface purinergic receptors. A complementary series of experiments involving agonists/antagonists specific for P2X7 and P2Y1/P2Y2 was then employed to differentiate between the two pathways. Treatment with BzATP, a potent P2X7-specific agonist, significantly accelerated Panx1 rod compaction in a dose-dependent manner. Of note, the accelerative effects of BzATP were more pronounced than those of ATP, supporting earlier reports that BzATP is a more potent P2X7 agonist (39). We also found that the effects of BzATP could be inhibited with concomitant addition of the P2X7 antagonist oATP. Additional experiments using agonists/antagonists specific for P2X1 and P2Y1 (MeSADP/PPADS and UTP, respectively) showed no effect on aggregate compaction. It appears, then, that the P2X7 purinergic receptor pathway plays the dominant role in aggregate assembly, which makes sense because Panx1-mediated ATP release has been primarily associated with P2X7, not P2Y1/P2Y2 (40).

However, this signaling cascade does not directly explain how Panx1 can modulate aggregate assembly because it is the actomyosin system that drives aggregate compaction. Once cell-cell contact has been established by CAMs, the contractile actomyosin network transmits intercellular tensile forces through a direct connection with CAMs, facilitated by proteins such as α/β catenins (cadherins) (41) and zonula occludens-1 (connexins) (42). Evidence of cytoskeletal involvement can be found in the temporal congruency between aggregate assembly and microfilament organization. At 1h, when the initial adhesive interactions have already occurred, assembly and cytoskeletal organization are both minimal. By 8h, when assembly is most active, the microfilament network has also begun to resemble its steady-state configuration (38). Not surprisingly, aggregate assembly is extremely sensitive to cytochalasin B and Y27632 (43), drugs that inhibit the polymerization of F-actin (44).

The proposed connection between the Panx1/ATP/P2X7 signaling cascade and the actomyosin system may be mediated by intracellular calcium. Binding of ATP to P2X7 receptors results in the elevation of intracellular calcium (45) (so-called “calcium waves”) which occur in direct parallel to actin microfilament organization in C6 cells (38). In addition, mechanical stimuli such as those generated by the actomyosin system can cause Panx1 channels to open (36). Accordingly, we found that Panx1 aggregates possess a significantly more robust F-actin network than their wild-type counterparts that extends beyond cortical regions. Cell morphology is flattened and non-spherical, similar to that in aggregates of other cells with well developed cytoskeletons (14, 46). As expected, the addition of exogenous ATP to Panx1 aggregates further amplifies these changes while also reversing the effects of CBX treatment, not known to directly interact with actin microfilaments. In conjunction with evidence that the actin cytoskeleton facilitates Panx1 cell surface trafficking (4), such a cascade may explain the observed internalization of Panx1 upon CBX treatment.

A summary of this cascade is provided in Fig. 6B. Given reports that three points within the cascade cause further Panx1 channel opening (the initial mechanical stimulus, initial binding of ATP to purinergic receptors, and changes in intracellular calcium (26)), it is important to note that this would create a positive feedback loop. Without a regulatory mechanism this could have deleterious effects, as essential cell contents could be lost and critical gradients rapidly diminished. Reports of channel attenuation with prolonged ATP release and binding (28) may indeed point to such a mechanism. This may also explain the behavior of heterocellular Panx1/Panx2 aggregates. We found that compaction was directly proportional to the Panx1/Panx2 ratio, suggesting minimal interaction between the two cell types. If Panx1 cells were able to significantly confer their augmented kinetics onto their Panx2 neighbors, as is the case with fibroblasts and epithelial cells (11, 47), then small numbers of Panx1 cells could dramatically accelerate compaction. In conjunction with the markedly different actin microfilament networks of adjacent Panx1 and Panx2 cells within heterocellular aggregates, these findings suggest...
that this carefully regulated ATP-initiated cascade is localized to Panx1 cells.

Through a series of complementary experiments employing a scaffold-free, non-adhesive microenvironment, we have demonstrated that Panx1 channels modulate the biomechanics of aggregate assembly. To our knowledge, this is the first study using a 3D multicellular approach to connect the ATP releasing capabilities of Panx1, the subsequent signaling cascade, the downstream augmentation of the actomyosin system, and the resultant effects on multicellular biomechanics.

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