Altered translation initiation of Gja1 limits gap junction formation during epithelial–mesenchymal transition

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\textbf{ABSTRACT} Epithelial–mesenchymal transition (EMT) is activated during development, wound healing, and pathologies including fibrosis and cancer metastasis. Hallmarks of EMT are remodeling of intercellular junctions and adhesion proteins, including gap junctions. The GJA1 mRNA transcript encoding the gap junction protein connexin43 (Cx43) has been demonstrated to undergo internal translation initiation, yielding truncated isoforms that modulate gap junctions. The PI3K/Akt/mTOR pathway is central to translation regulation and is activated during EMT, leading us to hypothesize that altered translation initiation would contribute to gap junction loss. Using TGF-β–induced EMT as a model, we find reductions in Cx43 gap junctions despite increased transcription and stabilization of Cx43 protein. Biochemical experiments reveal suppression of the internally translated Cx43 isoform, GJA1-20k in a Smad3 and ERK-dependent manner. Ectopic expression of GJA1-20k does not halt EMT, but is sufficient to rescue gap junction formation. GJA1-20k localizes to the Golgi apparatus, and using superresolution localization microscopy we find retention of GJA1-43k at the Golgi in mesenchymal cells lacking GJA1-20k. NativePAGE demonstrates that levels of GJA1-20k regulate GJA1-43k hexamer oligomerization, a limiting step in Cx43 trafficking. These findings reveal alterations in translation initiation as an unexplored mechanism by which the cell regulates Cx43 gap junction formation during EMT.

\textbf{INTRODUCTION} A variety of growth factors, including transforming growth factor β (TGF-β), can induce epithelial cells to undergo the transdifferentiation process of epithelial–mesenchymal transition (EMT). Although EMT occurs normally in development and wound healing, it can also contribute to pathological processes such as fibrosis and cancer progression (Lamouille et al., 2014). During EMT, cellular junctions are disassembled and remodeled in concert with cytoskeletal alterations and acquisition of a mesenchymal phenotype (Kalluri and Weinberg, 2009). Gap junctions, composed of connexin proteins, provide direct intercellular communication between polarized epithelial cells and are subject to down-regulation with loss of polarity during EMT (Goodenough and Paul, 2009; Bax et al., 2011). Of the 21 human connexins, connexin43 (Cx43; gene name GJA1) is the most ubiquitously expressed gap junction protein (Beyer et al., 1987). A rate-limiting step in Cx43 intracellular transport occurs at the trans-Golgi network (TGN) where six Cx43 monomers must oligomerize to form Cx43 hemichannels, or connexons, to permit cytoskeleton-based trafficking to the plasma membrane (Vanslyke et al., 2009). Connexons on the surface of apposing cells dock to form continuous channels which coalesce into dense arrays, termed gap junction plaques, to effect direct intercellular flow of ions and small molecules (Kumar and Gilula, 1996). Suppression of gap junctional intercellular coupling (GJIC) was thought to be a necessary
RESULTS

Internal translation of Gja1 is suppressed during TGF-β–induced EMT

To investigate the contribution of internal translation initiation in EMT-associated alterations in Cx43 gap junctions, we utilized normal mouse mammary gland epithelial cells (NMuMG). NMuMG cells are an established cell model which readily undergoes EMT upon stimulation with TGF-β (Piek et al., 1999). Expression of mesenchymal markers including fibronectin and N-cadherin with concomitant suppression of the epithelial marker E-cadherin were detected by Western blot, reverse transcription quantitative PCR (RT-qPCR), and immunofluorescence, confirming induction of the EMT process within 24 h of TGF-β addition (Supplemental Figure 1). Phase contrast microscopy reveals morphological changes from an epithelial “cobblestone” phenotype to larger spindle-shaped mesenchymal cells by 48 h after TGF-β stimulation (Figure 1A). Given the role of PI3K/AKT/mTOR in TGF-β–induced EMT, and in regulation of GJA1-20k translation (Smyth and Shaw, 2013), we investigated the status of GJA1 translation by Western blot. Cx43 was detected with an antibody directed against the C-terminus, capable of detecting GJA1-20k and its full-length counterpart GJA1-43k. We find significantly increased levels of GJA1-43k in mesenchymal cells in comparison to controls treated only with vehicle (Figure 1B). GJA1-20k levels, however, are significantly reduced relative to GJA1-43k (Figure 1, B, quantified in C). To ask whether increased GJA1-20k protein degradation is responsible, we used replication incompetent lentiviruses to generate stable cell lines encoding LacZ, the entire GJA1 coding sequence, or GJA1-20k alone. Again, levels of GJA1-43k were increased and relative levels of GJA1-20k reduced following stimulation with TGF-β in LacZ and GJA1 cell lines. The GJA1-20k stable cells, however, maintained GJA1-20k expression following stimulation with TGF-β, where the protein is constitutively synthesized from an independent mRNA and therefore not subject to suppression of internal translation (Figure 1, D, quantified in E). These data demonstrate that altered GJA1-43k and GJA1-20k levels in mesenchymal cells are likely not due to increased degradation of GJA1-20k, but rather inhibition of translation initiation within GJA1 mRNA with concomitant enhanced GJA1-43k translation from the initial start codon. Specificity to TGF-β signaling is confirmed with the TGF-β receptor inhibitor SB431542, which inhibits EMT induction and rescues expression of GJA1-20k. Together, these data suggest a shift in translation initiation may occur during EMT to regulate Cx43 localization and function at the posttranscriptional level.

GJA1-43k protein localizes to nonjunctional soluble pools during TGF-β–induced EMT

Dissolution of intercellular junctions is an established hallmark of EMT, yet conflicting reports exist regarding the fate of Cx43 as cells acquire the mesenchymal phenotype (van der Heyden et al., 2000; de Boer et al., 2007; Tacheau et al., 2008; Bax et al., 2011; Hills et al., 2012). Our finding that GJA1-20k, which is known to regulate gap junction formation, occurs at lower levels in mesenchymal cells (Figure 1), would suggest alterations in GJA1-43k intracellular trafficking. Immunofluorescence confocal microscopy reveals diffuse redistribution of Cx43 to the cytosol during EMT (Figure 2A). Cx43 gap junctions still occur at cell–cell borders in mesenchymal cells, but are fewer and more punctate than those of epithelial cells. To complement immunofluorescence studies, we employed the Triton X-100 solubility assay to fractionate and quantify junc- tional GJA1-43k in mesenchymal cells and their unstimulated epithe- lial counterparts at 48 h after induction. Consistent with our
immunofluorescence data, significantly lower levels of junctional Cx43 were detected following TGF-β stimulation relative to total Cx43 (Figure 2, B, quantified in C). Together, these data demonstrate limited gap junction formation in mesenchymal cells despite increased Cx43 protein levels.

**Levels of GJA1-43k protein and Gja1 transcript increase during TGF-β-induced EMT and GJA1-43k protein is stabilized**

In Figures 1 and 2 we report that TGF-β stimulation increases GJA1-43k levels in concert with reduced GJA1-20k expression and redistributes GJA1-43k to nonjunctional compartments. Interestingly, total levels of GJA1-43k protein increase significantly over the 72 h time course of EMT induction (Figure 3, A, quantified in B). We also find by RT-qPCR that Gja1 mRNA increased continuously throughout the 72 h time course (Figure 3C). These findings led us to investigate changes in Cx43 protein regulation, where altered GJA1-43k stability could explain the lack of correlation between protein and mRNA levels, and gap junction formation. Click formation and subcellular distribution, quantification was performed by fluorescence intensity line scans across cell borders (Figure 5, B and D; Smyth et al., 2012). Cell borders are identified with a pan-cadherin antibody (red). Upon TGF-β stimulation, GJA1-20k cells are protected against cytosolic redistribution of Cx43 and gap junctions are maintained with practically no change in intensity profiles detected (Figure 5, C and D), despite comparable increases in GJA1-43k to LacZ cells (Figure 1, D and E). To complement immunofluorescence studies, we again employed the Triton X-100 solubility assay to fractionate and quantify junctional GJA1-43k protein (Figure 5, E, quantified in F). These data demonstrate that suppression of GJA1-20k translation has no apparent effect on EMT induction, but is necessary for limiting gap junction formation in mesenchymal cells.

**Ectopic stable expression of GJA1-20k rescues gap junction formation and does not alter EMT marker induction in NMuMG cells**

Having demonstrated that internal translation of GJA1-20k is suppressed during EMT, we next removed cellular control of GJA1-20k expression in order to test the necessity of GJA1-20k suppression for gap junction down-regulation. Utilizing lentivirally transduced clonal NMuMG cell lines expressing LacZ as control or the GJA1-20k coding sequence, we find that both LacZ and GJA1-20k cell lines underwent EMT following TGF-β stimulation. Both cell lines displayed comparable morphological changes by phase contrast microscopy (Figure 4A) and immunofluorescence confocal imaging reveals comparable fibronectin (green) induction with actin (red) stress-fiber formation in mesenchymal cells (Figure 4B). Induction of EMT markers fibronectin and N-cadherin was confirmed biochemically by Western blot (Figure 4, C, quantified in D). To determine effects on GJA1-43k subcellular localization and gap junction formation, confocal immunofluorescence microscopy was employed (Figure 5, A–D). We detected GJA1-43k using an antibody targeting the cytoplasmic loop of Cx43, in order to exclude a confounding signal from truncated Cx43 isoforms. To measure changes in GJA1-43k gap junction chemistry was utilized to perform nonradioactive pulse-chase experiments to determine GJA1-43k stability in the presence of TGF-β stimulation (Breinbauer and Köhn, 2003). We find that despite reduced gap junction formation, GJA1-43k half-life increased from 1.3 h in unstimulated cells to 2 h following 48 h TGF-β stimulation (Figure 3, D, quantified in E).

**FIGURE 1:** TGF-β treatment induces EMT and suppresses internal translation of Gja1 mRNA. NMuMG cells were treated with 2 ng/ml TGF-β for 48 h to induce EMT. (A) Phase contrast microscopy of NMuMG cells <xsup>+</xsup> (B) Western blot of cell lysates probed with Cx43 C-terminal antibody to detect full-length GJA1-43k and internally translated GJA1-20k. α-Tubulin serves as loading control. (C) Quantification of GJA1-20k band intensity relative to GJA1-43k from D. Graphs represent mean ± SEM. Statistical analysis performed using the Student’s t test (C, n = 3) and one-way analysis of variance (ANOVA) with Tukey’s multiple comparison posttest (E, n = 3). *p < 0.05.
transport, and Cx43 gap junction formation during TGF-β stimulation. We turned to nondenaturing blue NativePAGE gel electrophoresis and quantified the ratio of hexameric (∼258 kDa) versus monomeric (43 kDa) Cx43 in cell lines stably expressing LacZ or GJA1-20k, with and without TGF-β stimulation (Figure 6, F, quantified in G). Quantification of band intensity by densitometry reveals a modest increase in hexamer formation in LacZ cells following EMT induction. GJA1-20k cells, however, display significantly larger increases in hexamer formation than that observed in LacZ control cells (Figure 6G).

**DISCUSSION**

Research on Cx43 and gap junction regulation has historically focused upon transcription, trafficking, and posttranslational modification of the full-length GJA1-43k protein. The existence of an IRES within the 5′ UTR of GJA1 mRNA, together with the recent finding

**GJA1-20k localizes to the Golgi apparatus and regulates Cx43 hemichannel oligomerization**

Given that GJA1-20k is known to play a role in GJA1-43k trafficking, we investigated localization of GJA1-20k/GJA1-43k within subcellular compartments of the vesicular transport pathway. Confocal immunofluorescence microscopy revealed colocalization of V5-epitope tagged GJA1-20k with the Golgi apparatus marker GM130 (Figure 6A). Direct stochastic optical reconstruction microscopy (STORM) was employed to localize the Golgi apparatus (GM130) at subdiffraction limit resolution with GJA1-43k in cells stably expressing LacZ or GJA1-20k (Figure 6, B–E). Cross-pair correlation generates a probability distribution of finding a given molecule as a function of a given distance from another distinct molecule, with a pair correlation amplitude of 0 indicating no colocalization between two proteins and higher values indicating a correlation. Utilizing an antibody directed against the Cx43 cytoplasmic loop, pair correlation analysis reveals an increase in GJA1-43k colocalization with GM130 following TGF-β stimulation in cells expressing LacZ, suggesting retention of GJA1-43k in the Golgi apparatus (Figure 6C). In cells expressing GJA1-20k, however, GJA1-43k colocalization with GM130 remains comparable to that in vehicle-treated cells (Figure 6E). GJA1-43k oligomerization into hexamers at the TGN is a rate-limiting step in Cx43 trafficking (Musil and Goodenough, 1993). Given our GJA1-43k and GJA1-20k localization data, we hypothesized a role for GJA1-20k in promoting hemichannel formation, thereby enhancing Golgi exit,
by several groups that GJA1 mRNA can undergo internal translation, has highlighted translation initiation as a novel and key regulatory step in Cx43 gap junction regulation (Schiavi et al., 1999; Smyth and Shaw, 2013). Internal translation of GJA1 mRNA yields N-terminally truncated isoforms, including GJA1-20k, and their expression influences GJA1-43k localization and gap junction formation. However, implementation of this dynamic translational regulation by the cell in gap junction formation and maintenance has yet to be demonstrated. We utilized EMT as a physiologically relevant model system with which to investigate the role of internal translation and GJA1-20k on GJA1-43k gap junction formation and maintenance. The process of EMT involves reorganization of intercellular junctional structures as cells acquire mesenchymal traits (Lamouille et al., 2014). As the primary means of direct intercellular communication, gap junctions undergo significant remodeling during this process (McLachlan et al., 2006; Bax et al., 2011). In this study, we report that suppression of Gja1 mRNA internal translation in mesenchymal cells is sufficient to limit gap junction formation.

Although the loss of adherens and tight junctions during EMT has been well documented, data regarding the effects of TGF-β on GJA1 transcription and Cx43 protein levels are conflicting (Larson et al., 1997; van der Heyden et al., 2000; de Boer et al., 2007; Tacheau et al., 2008; Hills et al., 2012; Chen et al., 2015b). Electrical conduction between epicardial cells is decreased significantly during and after EMT, coincident with decreased expression of Cx43 as well as other gap junction proteins (Bax et al., 2011). EMT induction in human kidney cells also results in decreased GJIC, and again results in decreased Cx43 expression as cells become mesenchymal relative to gap junctional GJA1-43k. Interestingly, we did not observe complete gap junction dissolution in mesenchymal cells, although gap junctions were less dense and more punctate than in epithelial cells. Nonchannel/junctional functions for Cx43 have been attributed its C-terminus of correlation between Cx43 protein, RNA levels, and gap junction formation, this highlights the complexity of connexin biology during EMT progression and the contribution of translational regulation.

Based on our data, the question remains as to why mesenchymal cells must harbor such high levels of intracellular GJA1-43k (Hills et al., 2012). Overexpression of Cx43 in glioma and breast cancer cells results in an epithelial morphology, supporting a role of Cx43 and gap junctions in maintaining an epithelial phenotype (Huang et al., 1998; McLachlan et al., 2006). Tumor-suppressive properties of Cx43 have been documented in a number of contexts beyond EMT, and Cx43 expression is down-regulated in many human cancers (Tsai et al., 1996; Laird et al., 1999; Simes et al., 2012). Ectopic expression of Cx43 in colon cancer epithelial cells does not appear to form gap junctions but limits cancer cell growth by antagonizing apoptotic pathways (Simes et al., 2012). In contrast, other studies have reported induction of Cx43 expression during EMT and associated high levels of Cx43 with more invasive and migratory cells (Larson et al., 1997; Bates et al., 2007; Tacheau et al., 2008; Zhang et al., 2015). Finally, overexpression of connexins has been associated with inhibition of EMT in hepatocellular and lung carcinoma cell populations resistant to chemotherapeutic agents (Yu et al., 2014, 2017). Together with our work revealing lack of correlation between Cx43 protein, RNA levels, and gap junction formation, this highlights the complexity of connexin biology during EMT progression and the contribution of translational regulation.

**Figure 3:** Levels of GJA1-43k protein and Gja1 transcript increase during TGF-β-induced EMT and GJA1-43k is stabilized. NMuMG cells were treated with vehicle, 2 ng/ml TGF-β, 5 μM SB431542 (SB), or TGF-β + SB431542 and sampled at 24, 48, and 72 h after stimulation for Western blot and RT-qPCR. (A) Western blot of GJA1-43k in NMuMG cells after treatment (left panels) with α-tubulin serving as loading control (right panels). (B) Quantification of GJA1-43k band intensity relative to α-tubulin from A. (C) qRT-PCR of Gja1 mRNA levels after treatment relative to vehicle at 24, 48, and 72 h after treatment (n = 6). (D) Western blot of total GJA1-43k and TAMRA pulsed GJA1-43k in NMuMG cells following 48 h treatment with vehicle or TGF-β. Time points indicate hours after pulse with TAMRA alkyne. (E) GJA1-43k exponential decay curves of blots described in E; vehicle t½ = 1.3 h, TGF-β t½ = 2.0 h (n = 3). Graphs represent mean ± SEM. Statistical analysis performed using one-way ANOVA with Tukey’s multiple comparison posttest (B, n = 3, C, n = 5). *p ≤ 0.05; **p ≤ 0.01.
TGF-β also activates TAK1, independent of its receptor kinase activity, leading to activation of the MAP kinases p38 and JNK (Yamaguchi et al., 1995; Watkins et al., 2006; Sorrentino et al., 2008). Following chemical inhibition of ERK1/2 signaling, but not that of p38 or JNK, we report a significant increase in the ratio of GJA1-20k to GJA1-43k. ERK1/2 signaling is necessary for TGF-β-induced EMT, further suggesting a role for internal translation of GJA1 in EMT (Xie et al., 2004). Of relevance, ERK pathway activation promotes canonical translation through ribosomal protein S6 phosphorylation (Roux et al., 2007). Our findings are also consistent with previous reports that Mnk1/2 suppresses GJA1-20k translation as ERK activation is upstream of Mnk (Salat-Canela et al., 2014). We find inhibition of Smad3 activity with the inhibitor SIS3 also rescues GJA1-20k expression, implying transcriptional effects may also regulate GJA1 translation, such as alternate UTR usage. However, our data in Figure 1D, in which we overexpress the coding sequence of GJA1 alone, support a dominant shift in cellular processes regulating translation initiation independent of changes to the mRNA. Moreover, internal translation of an independent mRNA transcript, paxillin (Pxn), has also been reported in EMT, suggesting a global shift in translation initiation during TGF-β–induced EMT (Tumbarello et al., 2005). Given the role of paxillin in EMT through focal adhesion formation, it is tempting to speculate that an entire “nonjunctional” mesenchymal and motile phenotype may be affected through suppression of internal translation relevant mRNA families (Turner, 2000).

Direct posttranslational modification through phosphorylation of the GJA1-43k C-terminus is known to promote gap junction internalization and degradation of GJA1-43k (Hesketh et al., 2010; Smyth et al., 2014; Solan and Lampe, 2014). A phosphorylation cascade encompassing Akt, PKC, and MAPK converges at the GJA1-43k C-terminus to effect internalization from the plasma membrane (Smyth et al., 2014). Indeed, alterations in GJA1-43k phosphoforms are observed in our biochemical solubility studies where we find reduced junctional GJA1-43k in mesenchymal cells. Together with our localization data and the fact that we report GJA1-43k is stabilized during TGF-β–induced EMT, however, it seems probable that GJA1-43k predominantly remains within intracellular compartments in these cells, the majority most likely not having reached the cell surface where such phosphorylation events are understood to occur. Moreover, the internalization of gap junctions occurs rapidly after activation of growth factor signaling cascades and so may play a role in dissolution of gap junctions earlier in the EMT process than the 48–72 h time points examined in this study (Dunn and Lampe, 2014; Fong et al., 2014; Smyth et al., 2014).

Oligomerization of GJA1-43k into hemichannels occurs at the TGN, later in the vesicular transport pathway than most ion channels (Musil and Goodenough, 1993). Only two proteins are known to

FIGURE 4: NMuMG cells transduced with pLenti6.3-LacZ or pLenti6.3-GJA1-20k undergo EMT. Stable NMuMG cells transduced with pLenti6.3-LacZ or pLenti6.3-GJA1-20k were treated with vehicle, 2 ng/ml TGF-β, 5 μM SB431542 (SB), or TGF-β + SB431542 for 48 h. (A) Phase contrast microscopy at 48 h after stimulation, ×20. Scale bar: 100 μm. (B) Fixed cell confocal immunofluorescence (>100) of cells labeled with antibody directed against EMT markers actin (red) and fibronectin (green). Nuclei counterstained with DAPI (blue). Scale bar: 20 μm. (C) Western blot probed for EMT markers fibronectin and N-cadherin with α-tubulin serving as loading control. (D) Quantification of Western blot band intensity in C. Graphs represent mean ± SEM. Statistical analysis performed using one-way ANOVA with Tukey’s multiple comparison posttest (n = 3). ns: p value not significant.
may complement ERP29/Rab20 activity by chaperoning GJA1-43k through the Golgi apparatus transport pathway, spatially and temporally regulating Cx43 hemichannel oligomerization and vesicular loading at the TGN. The stoichiometry of GJA1-43k/GJA1-20k is difficult to measure in the biochemical assays herein, but it does seem that relatively low levels of GJA1-20k are sufficient to promote GJA1-43k transport. Given that GJA1-20k has not been detected on the cell surface and resides primarily within the Golgi apparatus, it is likely that one GJA1-20k molecule may interact transiently with several GJA1-43k monomers/hemichannel intermediates as they pass through the Golgi apparatus.

As a membrane channel, Cx43 hemichannels can exchange molecules such as ATP and glutamate with the extracellular milieu in addition to its more established function as a gap junction (Stout et al., 2002; Orellana et al., 2011). Although our biochemical Triton X-100 solubility findings demonstrate a relative reduction in Cx43 gap junction formation during EMT, they do not exclude the possibility of increased surface hemichannel expression. Accrual of surface Cx43 hemichannels into gap junction structures is limited by binding of the C-terminus to ZO-1, a scaffolding protein down-regulated during TGF-β-induced EMT (Rhiett et al., 2011). Therefore, in mesenchymal cells we would expect the absence of negative regulation of gap junction accretion by ZO-1, but did not observe increases in gap junction size with TGF-β stimulation. Furthermore, data presented in Figures 3 and 6 show that TGF-β stimulation alters Cx43 protein stability and oligomerization, which are key limiting steps in the trafficking of hemichannels to the cell surface. These findings, together with previous studies indicating GJA1-20k expression is restricted to intercellular compartments, lead us to conclude that the observed increases in soluble GJA1-43k is primarily intracellular. The role of Cx43 internally translated isoforms in regulation of surface hemichannel activity and/or accrual into gap junctions, however, is an interesting topic of future research.

Several internally translated isoforms of Cx43 have been detected in addition to GJA1-20k (Smyth and Shaw, 2013). In this study, we focus on GJA1-20k as the most predominantly expressed isoform but given that all three preceding methionine codons (M100, 125, and 147) must also be mutated to limit GJA1-43k trafficking, one GJA1-20k molecule may interact transiently with several GJA1-43k monomers/hemichannel intermediates as they pass through the Golgi apparatus.
remodeling in the heart during ischemia can be prevented through rapidly at the posttranscriptional level. Indeed, pathological Cx43 tune Cx43 hemichannel oligomerization and thus gap junctions indicate that levels of GJA1-20k provide a means for the cell to fine-
important in regulation of GJA1-43k transport. Together, our data which is intact in this mutant and contained in GJA1-20k as most
SEM. Statistical analysis performed using the Student’s (F) Western blot of blue NativePAGE probed for Cx43 hexamers (∼43 kDa) and monomers (∼258 kDa) and monomers (−43 kDa). (G) Quantification of Western blot band intensity in F (n = 5). Graphs represent mean ± SEM. Statistical analysis performed using the Student’s t test. *p ≤ 0.05; **p ≤ 0.01.
which is intact in this mutant and contained in GJA1-20k as most important in regulation of GJA1-43k transport. Together, our data indicate that levels of GJA1-20k provide a means for the cell to fine-tune Cx43 hemichannel oligomerization and thus gap junctions rapidly at the posttranscriptional level. Indeed, pathological Cx43 remodeling in the heart during ischemia can be prevented through vectors were created using TOPO cloning to insert the GJA1 coding sequence (forward primer GTGGTGCC CAGGCAAC; reverse primer ATGCTGGTGGTGTCCCTTG) and reverse primer GGGGACCACTTTTGTGAGAAGAGAGCTGGGTTCTAGATCTCCAGGTCATCAG GCC to create an entry clone (BP clonase II; Thermo Scientific). GJA1 was then inserted into pcDNA3.2-V5/DEST from pDONR221/hGJA1 entry clone using Gateway LR cloning to generate pcDNA3.2/ hGJA1. pcDNA3.2/GJA1-20k-V5 was generated using forward primer GGGGACAAGTTTGTACAAAAAAGCAGGGTCATCAGGCCG to insert the GJA1-20k coding sequence (forward primer GGGGACCACTTTTGTGAGAAGAGAGCTGGGTTCTAGATCTCCAGGTCATCAG GCC to create an entry clone (BP clonase II; Thermo Scientific). GJA1-20k was then inserted into pcDNA3.2/V5-DEST from pDONR221/hGJA1 entry clone using Gateway LR cloning to generate pcDNA3.2/ hGJA1-20k-V5.盗版发布者：Thermo Scientific). Lentiviral expression vectors were created using TOPO cloning to insert the GJA1 coding sequence (forward primer GTGGTGCC CAGGCAAC; reverse primer CTAGATCTCCAGGTCATCAGGCC) or GJA1-20k (forward primer ATGCTGGTGGTGTCCCTTG) and reverse primer CTAGATCTCCAGGTCATCAGGCC into plenti6.3/V5-TOPO (Thermo Scientific). plenti6.3/V5-GW/LacZ and pcDNA3.2/GW-CAT served

FIGURE 6: GJA1-20k promotes GJA1-43k oligomerization and release from the Golgi apparatus. NMuMG cells were transfected with C-terminally V5 tagged GJA1-20k and subsequently fixed for confocal immunofluorescence imaging. (A) Fixed cell confocal immunofluorescence (>100) of NMuMG cells 24 h posttransfection. The Golgi apparatus is labeled with an antibody directed against GM130 (green) and GJA1-20k detected with antibody directed against the V5 epitope tag (red). (B, D) Stable NMuMG cells transduced with pLenti6.3-LacZ or pLenti6.3-GJA1-20k were treated with vehicle or TGF-β for 48 h and subsequently fixed for superresolution immunofluorescence imaging. 3D STORM imaging detecting GJA1-43k (green) and GM130 (pink) with localizations depicted as 50-nm point clouds in an x-y cross-section. Scale bars: 2 μm. (C, E) Cross-pair correlation functions for GJA1-43k and GM130 from stable NMuMG cells treated with vehicle (black) or TGF-β (red). Graphs represent mean ± SEM, n = 10 cells. (F) Western blot of blue NativePAGE probed for Cx43 hexamers (∼43 kDa) and monomers (∼258 kDa) and monomers (−43 kDa). (G) Quantification of Western blot band intensity in F (n = 5). Graphs represent mean ± SEM. Statistical analysis performed using the Student’s t test. *p ≤ 0.05; **p ≤ 0.01.

MATERIALS AND METHODS

Molecular biology

Human GJA1 cDNA was obtained from GE Dharmacon and cloned into pDONR221 using Gateway BP cloning (forward primer GGGGACAAAGTTTGTACAAAAAAGCAGG-GCTTAGTGGTGGTCCTTG; reverse primer GGGGACCACTTTTGTGAGAAGAGAGCTGGGTTCTAGATCTCCAGGTCATCAGGCC) to create an entry clone (BP clonase II; Thermo Scientific). GJA1 was then inserted into pcDNA3.2-V5/DEST from pDONR221/hGJA1 entry clone using Gateway LR cloning to generate pcDNA3.2/hGJA1-20k-V5 was generated using forward primer GGGGACAAGTTTGTACAAAAAAGCAGGGTCATCAGGCCG to insert the GJA1-20k coding sequence (forward primer GGGGACCACTTTTGTGAGAAGAGAGCTGGGTTCTAGATCTCCAGGTCATCAGGCC) or GJA1-20k (forward primer ATGCTGGTGGTGTCCCTTG) and reverse primer GGGGACCACTTTTGTGAGAAGAGAGCTGGGTTCTAGATCTCCAGGTCATCAGGCC into plenti6.3/V5-TOPO (Thermo Scientific). pLenti6.3/V5-GW/LacZ and pcDNA3.2/GW-CAT served

Actin stabilization by ectopic GJA1-20k expression, suggesting internal translation is a common pathway for gap junction regulation across diverse cell types and tissues (Basheer et al., 2017). At least 600 human genes are now understood to undergo alternate mechanisms of translation initiation and this study supports the importance of this biological process in health and disease (Ingolia et al., 2011; Weingarten-Gabbay et al., 2016; Karginov et al., 2017). Regulation of translation initiation can be thought of globally, whereby signal transduction cascades can shunt the entire translational landscape of the cell, or gene-specifically, where alternate promoter usage/splicing can lead to changes in specific mRNAs and render them subject to such translation initiation. Our data reveal that a shift in the translational landscape of the cell occurs during EMT affecting the proteome and contributing to alterations in gap junction formation. This suggests a potent mechanism whereby the cell can rapidly regulate Cx43 transport and gap junction formation at the point of GJA1-20k translation, without inducing transcription of mRNA or synthesis of full-length GJA1-43k.
as control expression vectors (Thermo Scientific). All recombinant DNA constructs were verified by Sanger sequencing.

**Cell culture**
NMuMG cells were a kind gift from Rik Derynck (University of California, San Francisco) and maintained in DMEM+GlutaMax supplemented with 10% fetal bovine serum (FBS), sodium pyruvate, nonessential amino acids (Thermo Scientific), bovine insulin solution (5 µg/ml; Sigma) and MycoZAP (Lonza). MycoZAP is included to prevent Mycoplasma contamination. Cells were maintained in a humidified atmosphere of 5% CO₂ at 37°C. For experiments, cells were plated at 1.6 × 10⁴ cells/cm² in 100-mm plates or six-well dishes for protein harvesting and eight-well chamber slides for imaging. Cells were incubated for 24 h before addition of media containing one of the following treatments: TGF-β1 (2 ng/ml; Humanzyme), the TβR1 blocker SB 431542 (SB; 5 µM; Sigma), Smad3 inhibitor SIS3 (10 µM; Selleckchem), p38 inhibitor SB 202190 (20 µM; Cayman), JNK inhibitor SP 600125 (20 µM; Cayman), ERK1/2 inhibitor SCH 772984 (0.1 µM; Cayman), or vehicle (veh; dimethyl sulfoxide [DMSO]).

**Western blotting**
Cells were lysed in RIPA buffer (0.1% SDS, 50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 2 mM NaF, 200 µM Na₃VO₄) supplemented with HALT Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific) and Western blotting conducted as previously described (Smyth et al., 2010, 2012). Briefly, cells were scraped into RIPA buffer and sonicated before centrifugation at 10,000 × g for 20 min at 4°C. Protein concentration was quantified using the Bio-Rad DC Protein Assay and lysates normalized to the same concentration. 4X Bolt LDS sample buffer (Thermo Scientific) supplemented with dithiothreitol (DTT; 400 mM) was added and samples heated for 10 min at 70°C before SDS–PAGE and Western blotting with the following primary antibodies: mouse anti–N-cadherin and mouse anti–E-cadherin (BD Biosciences), rabbit anti-Cx43 (1:3000; Sigma), rabbit anti-fibronectin (1:3000; Sigma), and mouse anti–α-tubulin (1:3000; Sigma). Goat secondary antibodies conjugated to Alexa Fluor 555 and 647 (Thermo Scientific) were used at 1:1000 and membranes imaged on a ChemiDoc MP (Bio-Rad).

**Immunofluorescence**
Cells were fixed in 37°C 4% paraformaldehyde for 20 min and stored in PBS at 4°C until immunostaining was conducted as previously described (Smyth et al., 2012). Briefly, cells were incubated for 24 h before addition of media containing one of the following treatments: TGF-β1 (2 ng/ml; Humanzyme), the TβR1 blocker SB 431542 (SB; 5 µM; Sigma), Smad3 inhibitor SIS3 (10 µM; Selleckchem), p38 inhibitor SB 202190 (20 µM; Cayman), JNK inhibitor SP 600125 (20 µM; Cayman), ERK1/2 inhibitor SCH 772984 (0.1 µM; Cayman), or vehicle (veh; dimethyl sulfoxide [DMSO]). 293FT cells were obtained from Thermo Scientific and maintained in DMEM+GlutaMax supplemented with 10% FBS, sodium pyruvate, nonessential amino acids (Thermo Scientific), and MycoZAP (Lonza).

**Lentivirus production, cell transduction, and stable cell line generation**
Lentivirus was created from pLenti6.3-hGJA1, pLenti6.3/GJA1-20k, and pLenti6.3-LacZ according to the manufacturer’s instructions (ViraPower Lentiviral Expression System; Thermo Scientific). Titered and normalized viruses were used to infect NMuMG cells plated in six-well dishes in the presence of hexadimethrine bromide (4 µg/ml; Sigma). After 48 h, cells were split 1:40 into 100-mm dishes and 10 µg/ml blasticidin added to medium for selection. Medium was changed every 2 d and healthy colonies picked using cloning rings (Scienceware). Clones were expanded and screened for stable expression by Western blotting and immunofluorescence.

**FIGURE 7:** TGF-β activates Smad and ERK signaling to alter translation initiation and limit gap junction formation during EMT. NMuMG cells were treated with vehicle (veh; DMSO), 10 μM SIS3, 20 μM SB 202190 (SB2), 20 μM SP 600125 (SP6), or 0.1 μM SCH 772984 (SCH), for 15 min followed by the addition of 2 ng/ml TGF-β for 24 h. (A) Western blot of cell lysates probed with Cx43 C-terminal antibody to detect full-length GJA1-43k and internally translated GJA1-20k. (B) Quantification of GJA1-20k band intensity relative to GJA1-43k from A. (C) Schematic illustrating suppression of GJA1-20k translation during TGF-β–induced EMT to limit GJA1-43k oligomerization and gap junction formation. Graph represents mean ± SEM. Statistical analysis performed using one-way ANOVA with Dunnett’s multiple comparison posttest (B, n = 5–7). ***p ≤ 0.0005; ****p ≤ 0.0001.
antibodies: rabbit anti-fibronectin (Sigma; 1:500), mouse anti–N-cadherin (BD Biosciences; 1:200), mouse anti–E-cadherin (BD Biosciences; 1:1000), rabbit anti-Cx43 ( Sigma; 1:3000), and mouse anti–α-tubulin (Sigma; 1:3000). Secondary antibodies used were goat anti-rabbit Alexa Fluor 488 and anti-mouse Alexa Fluor 647 (Thermo Scientific; 1:500) with Alexa Fluor 647 phalloidin employed to detect actin (Thermo Scientific; 1:20). For immunofluorescence using antibody directed against the Cx43 cytoplasmic loop, cells grown on 35-mm glass-bottomed dishes (Mat-Tek) or eight-well chambered slides were fixed in methanol for 5 min on ice. Antibodies used were rabbit anti-Cx43 cytoplasmic loop (Abcam; 1:500), mouse anti-pan cadherin (BD Biosciences; 1:500), and anti-rabbit Alexa Fluor 488 and anti-mouse Alexa Fluor 647 (ThermoScientific; 1:500). To localize GJA1-20k, NMuMG cells were transfected in 35-mm glass-bottomed dishes with 2 µg pcDNA3.2/hGJA1-20k-V5, or pcDNA3.2/GW-CAT (Thermo Scientific). Posttransfection (24 h) cells were fixed in methanol for 5 min on ice. Immunostaining was performed as described above using the following primary antibodies: rabbit anti-V5 (Sigma; 1:1000) and mouse anti-GM130 (BD Biosciences; 1:250).

Superresolution microscopy
Cells plated in 35-mm Mat-Tek dishes were fixed in methanol for 5 min on ice. Cells were washed three times in PBS before blocking for 1 h at room temperature in 5% normal donkey serum (Jackson ImmunoResearch), 0.5% Triton X-100, in PBS (blocking buffer). Cells were incubated with the following primary antibodies diluted in blocking buffer: mouse anti-GM130 (BD Biosciences; 1:1000) and rabbit anti-Cx43 cytoplasmic loop (Abcam; 1:500). Following PBS washes, cells were incubated with donkey secondary antibodies conjugated to Alexa Fluor 647 (Thermo Scientific) or CF568 (Biotium) diluted in blocking buffer. After PBS washes, two-color direct STORM imaging was conducted using a Vutara 350 (Bruker). Cells were imaged in 50 mM Tris-HCl, 10 mM NaCl, 10% (wt/vol) glucose buffer containing 20 mM mercaptoethanol, 1% (vol/vol) 2-mercaptoethanol, 168 active units/ml glucose oxidase, and 1404 active units/ml catalase. Videos of 5000 frames were acquired for each probe and three-dimensional (3D) images reconstructed in Vutara SRX software. Intracellular regions of interest were selected and coordinates of localized molecules used to calculate pair correlation functions in the Vutara SRX software (Sengupta et al., 2011, 2013).

Image analysis
For quantification of Cx43 expression at cell–cell borders, pan-cadherin images were used to identify cell–cell borders for vehicle and TGF-β–treated cells, respectively. Lines (10 µm) were drawn perpendicular to cell–cell borders every 10 pixels for the length of each border. The plot profile function in ImageJ was used to quantify Cx43 fluorescence intensity from each line and data averaged. Images were acquired and analyzed by separate individuals, with image identity blinded to the analyst.

Separation of soluble (nonjunctional) and insoluble (junctional) Cx43
Cells were harvested in 1% Triton X-100 buffer (50 mM Tris, pH 7.4, 1% Triton X-100, 2 mM EDTA, 2 mM ethylene glycol-bis[b-aminoethyl ether]-N,N',N',N’-tetraacetic acid [EGTA], 250 mM NaCl, 1 mM NaF, 0.1 mM NaVO4) supplemented with HALT Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific). Samples were lysed in 1% Triton X-100 buffer for 1 h at 4°C. After lysing, 25 µl of lysate was removed and snap-frozen to serve as total protein fraction. The remaining lysate was centrifuged at 15,000 × g for 30 min, and supernatant removed to serve as the soluble fraction. Pellets containing insoluble protein were resuspended in 4X Bolt LDS sample buffer (Thermo Scientific). All samples were sonicated and centrifuged for 20 min at 10,000 × g following the addition of 4X Bolt LDS sample buffer (Thermo Scientific) supplemented with DTT (400 mM) and SDS–PAGE Western blotting performed as described above.

Real-time quantitative PCR
RNA was extracted from NMuMG cells using the Purelink RNA Mini Kit (Thermo Scientific) and homogenized by passage 10 times through an 18-gauge needle. DNA was digested on column using PureLink DNase (Thermo Scientific). RNA (1 µg) was reverse transcribed with iScript cDNA Synthesis Kit (Bio-Rad). RT-qPCR was performed using SYBR Select Master Mix for CFX (Thermo Scientific) on a CFX Connect Real-Time System (Bio-Rad). Validated PrimeTime qPCR primers were purchased from Integrated DNA Technologies. Cycling parameters consisted of 50°C, 2 min; 95°C, 2 min; 39 cycles of 95°C, 15 s; 55°C, 15 s; 72°C, 1 min followed by a melt curve.

Click chemistry pulse-chase assay
NMuMG cells were plated in six-well dishes and fully supplemented DMEM and treated with TGF-β or vehicle as described above. After 48 h TGF-β stimulation, cells were rinsed and starved for 1 h in methionine/cysteine-free DMEM (Thermo Scientific) supplemented with 10% dialyzed FBS (GE Healthcare). Cells were then pulsed for 1 h with Click-iT L-Azidohomoalanine (ThermoScientific; 50 µM final concentration) added directly to starvation media. Samples were harvested over an 8 h period at 2 h intervals to 200 µl lysis buffer (1% SDS, 50 mM Tris-HCl) supplemented with HALT Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific). Lysates were incubated on ice for 15 min and sonicated before centrifugation at 10,000 × g for 20 min at 4°C. Protein concentration was quantified using the Bio-Rad DC Protein Assay and all samples normalized to 400 µg/100 µl. The click reaction was performed on 400 µg protein using the Click-IT Protein Reaction Buffer Kit and tetramethylrhodamine (TAMRA) alkyne (ThermoScientific; 40 µM final concentration) as per manufacturer’s instructions. Immunoprecipitation was performed as previously described (Smyth et al., 2014). Briefly, Cx43 was immunoprecipitated from precleared lysates using 1 µg rabbit anti-Cx43 (Sigma) and protein G Dynabeads (Thermo Scientific). Western blotting was performed as described above and pulsed Cx43 detected using antibody directed against TAMRA (ThermoScientific; 1:1000). Blots were stripped using Re-Blot Plus Strong Solution (Millipore) and reprobed for total Cx43 with rabbit anti-Cx43 (Sigma; 1:3000).

Blue NativePAGE
NMuMG cells were washed twice with PBS and harvested by scraping into 1X NativePAGE sample buffer (Thermo Scientific) containing 1% Digitonin and HALT Protease Inhibitor Cocktail (Thermo Scientific). Lysates were centrifuged at 100,000 × g for 15 min at 4°C using a Beckman Coulter Optima TLX ultracentrifuge. G-250 sample additive was added to supernatants to 0.25%. Samples were loaded on a 4–16% Bis-Tris Novex NativePAGE Gel (Thermo Scientific) and electrophoresed at 4°C according to the manufacturer’s instructions. Proteins were transferred to PVDF membranes in Bolt transfer buffer (Thermo Scientific) for 1 h at 25 V. GJA1-43k was detected with rabbit anti-Cx43 (Sigma; 1:3000), and goat anti-rabbit horseradish peroxidase (HRP) (Abcam; 1:5000). Membranes were imaged following the addition of Clarity Western ECL Substrate on a ChemiDoc MP (Bio-Rad).
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