Cell coupling mediated by connexin 26 selectively contributes to reduced adhesivity and increased migration


ABSTRACT

Gap junction proteins (connexins) have crucial effects on cell motility in many systems, from migration of neural crest cells to promotion of metastatic invasiveness. Here, we show that expression of Cx26 (also known as GJB2) in HeLa cells specifically enhances cell motility in scrape wounding and sparse culture models. This effect is dependent on gap junction channels and is isotype specific [Cx26 enhances motility, whereas Cx43 (also known as GJA1) does not and Cx32 (also known as GJB1) has an intermediate effect]. The increased motility is associated with reduced cell adhesiveness, caused by loss of N-cadherin protein and RNA at the wound edge. This in turn causes a redistribution of N-cadherin-binding proteins (p120 catenin and β-catenin) to the cytosol and nucleus, respectively. The former activates Rac-1, which mediates cytoskeletal rearrangements needed for filopod extension. The latter is associated with increased expression of urokinase plasminogen activating receptor (an activator of extracellular proteases) and secretion of extracellular matrix components like collagen. Although these effects were dependent on Cx26-mediated coupling of the cells, they are not mediated by the same signal (i.e. cAMP) through which Cx26 has been shown to suppress proliferation in the same system.

KEY WORDS: Gap junction, Connexin, Cell motility, Cell adhesion

INTRODUCTION

The coordination of cell communities is essential for the normal development and function of tissues in complex eukaryotes. Complementing the myriad of endocrine signaling mechanisms that help to achieve this, direct exchange of ions, signals and metabolites between cells through gap junctions is a central component of the integration of cell behavior. Like extracellular signaling pathways, there is evidence that exchanges through gap junctions shows surprising selectivity. The connexin composition of the channels [there are 21 connexin genes in humans – (Willecke et al., 2002)] imparts a surprising diversity in structure and properties (Sosinsky and Nicholson, 2005), that include single-channel conductance [ranging from 15 to over 300pS (Bukauskas and Verselis, 2004; Ek-Vitorin and Burt, 2013)], permeability to larger molecules [e.g. nucleotide variants, IP3, sugars, glutathione, etc. (Bevans et al., 1998; Sneyd et al., 1998; Goldberg et al., 1999)], gating responses (Harris and Contreras, 2014), regulation by different kinases (Lampe and Lau, 2000; Mitra et al., 2012; Solan and Lampe, 2014) and association with various cytoplasmic and membrane proteins (Giepmans, 2004; Laird, 2010). In addition, connexins form hexameric hemichannels (connexons) that, prior to docking, form intercellular gap junction channels and can mediate release of larger molecules to the extracellular space (Goodenough and Paul, 2003; Sáez and Leybaert, 2014).

Integration of cell behavior is particularly crucial during cell migration, whether it is associated with normal developmental processes or pathological conditions such as metastasis. Cx43 (also known as GJA1) expression and coupling of neural crest cells has been shown to facilitate the timing of their migration, which is crucial to the normal development of the heart (Huang et al., 1998; Francis et al., 2011). In this case, the C-terminal domain and its interaction with N-cadherin and p120 catenin (hereafter p120ctn, encoded by CTNNBD1) signaling was implicated, rather than the intercellular coupling function of Cx43 (Xu et al., 2001). Both Cx43 and Cx26 (also known as GJB2) expression have been shown to be important for migration of cortical neurons along radial glia during the development of the cortex, although in this case the adhesive role of the connexins was implicated (Elías et al., 2007, 2010; Valiente et al., 2011). Changes in connexin expression have been reported during wound healing of the skin (Coutinho et al., 2003; Churko and Laird, 2013), where expression of Cx43 was shown to be downregulated while Cx26 was shown to be upregulated in the epidermal layers during wound healing (Kretz et al., 2003; Goliger and Paul, 1995).

Connexin expression has also been associated with increased motility and invasiveness of tumor cells (reviewed in Defamie et al., 2014; Kotini and Mayor, 2015). The invasion of HeLa cells into heart fragments was enhanced by expression of Cx43, Cx31 (also known as GJB3) or Cx40 (also known as GJA5) (Graeber and Hülsér, 1998). Several in vivo assays have also linked connexin expression with increased invasiveness of malignant glioma cells (Lin et al., 2002) and hepatocellular carcinomas (Ogawa et al., 2012). In malignant melanoma cells, a change in cadherin expression during tumor progression helped promote coupling with dermal fibroblasts and increased invasiveness (Hsu et al., 2000). Expression of Cx43, and its associated coupling, increased the migration rate two-fold in an assay of breast cancer cell invasion into underlying endothelial cells (Pollmann et al., 2005). Homotypic Cx43-mediated gap junctional intercellular communication (GJIC) in early and advanced metastatic prostate carcinoma cells also enhanced their migration rate (Miekus et al., 2005). There have been some contrary findings where Cx43 or Cx26 (Defamie et al., 2014) expression has been linked to suppressed motility or metastasis. However, most studies, including clinical correlations of multiple cancers [skin
(Kamibayashi et al., 1995), breast (Kanczuga-Koda et al., 2006), prostate (Tate et al., 2006) and colorectal (Ezumi et al., 2008), support a role for increased Cx43 or Cx26 functional expression being linked to enhanced motility and invasiveness of tumor cells.

The current work addresses the issue of the underlying mechanisms by which gap junctions can enhance motility in a well-established cancer cell line (HeLa cells) where the connexin composition can be precisely controlled. This allowed us to assess whether the effects on cell motility are specific to the type of connexin, as well as to rigorously test which specific functions of connexins are important in regulating motility. This strategy has allowed us to identify a signaling cascade that links enhanced gap junction coupling to facilitation of the motile phenotype that is likely to be useful in understanding the role of gap junctions in metastasis and other processes in development.

RESULTS

In order to distinguish the mechanisms by which connexins regulate proliferation and motility, we utilized the same HeLa cell lines described in Chandrasekhar et al. (2013). Non-clonal, and in some cases clonal, populations of HeLa cells were prepared expressing Cx26, Cx43 and Cx32 (also known as GJB1), as well as variants of Cx26 that form normal gap junction structures, but fail to form functional channels (Cx26T135A; Beahm et al., 2006) or only form functional hemichannels (Cx26R75Y; Deng et al., 2006). These cell lines are extensively characterized in Chandrasekhar et al. (2013) for comparable levels of protein expression and its appropriate cellular localization, as well as both gap junction and hemichannel function. The former was assessed using Calcein dye transfer in a parachuting assay and the latter assessed by uptake of Lucifer Yellow (Table 1). Note that dye uptake was relatively low, as experiments were performed in normal extracellular Ca\(^{2+}\) (a known blocker of hemichannels) to emulate the conditions of the subsequent motility assays.

Expression of Cx26 in HeLa cells selectively enhances wound healing in a manner that is dependent on intercellular coupling

The effect of connexin expression on cell motility was assessed using a scrape wounding assay of the cell monolayer. Fig. 1A shows the time course of ‘wound healing’ in wild-type HeLa cells (HeLaWT), and representative clones of each connexin transfectant (denoted HeLa26, HeLa32, HeLa43 and HeLa26 R75Y, for Cx26, Cx32, Cx43 and Cx26 R75Y, respectively). Although the cell densities of the monolayers and width of the initial streaks were comparable in all cultures, rates of wound healing differed dramatically. After 48 h, the HeLaWT and HeLa43 cells had only minimally filled the wound, whereas the HeLa26 cells had almost completely filled in the wounded area. HeLa32 cells had an intermediate phenotype. Cell expressing point mutants of Cx26 that have lost gap junction (Cx26 R75Y) or both gap junction and hemichannel activity (Cx26 T135A) failed to show any enhancement of migration over the WT HeLa cells (Fig. 1A,C,E), demonstrating the requirement for intercellular coupling.

Metamorph software allows tracking of individual cell movements over time (Fig. 1B), which confirmed the dramatic differences in migration rates of Cx26-, Cx32- and Cx43-expressing cells. Cx26-expressing cells moved two times faster into the wound (defined as direction ‘X’) than HeLaWT, or cells expressing Cx43 and non-functional Cx26, and 1.5 times faster than Cx32-expressing cells (Fig. 1C). The directionality of movement was enhanced to an even greater degree, with a three-fold increase in the ratio of movement into the wound (X direction) compared to movement parallel to the wound edge (Y direction) in HeLa26 cells compared to all other transfectants other than HeLa32 (where the increase was only two-fold). Real-time video imaging of the wound healing (Movie 1) reveals that HeLaWT and HeLa43 cells migrate as sheets, whereas HeLa26 cells tend to separate out and migrate as single cells, or clusters of cells, at the wound edge, and show much greater filopodia-extending activity. Cells expressing the channel-impaired Cx26 mutants migrate as sheets, like HeLaWT cells.

Using several individual HeLa26 transfected clones, we further compared the rates of wound healing with the levels of Cx26 expression and coupling. Three clones (C, M and 11) showed similar coupling levels and wound healing rates, measured as days to heal the wound (Fig. 1E, black and white bars, respectively). However, in two clones (12 and 16) with notably lower coupling levels, the days taken to heal the wound increased as coupling decreased. This was also true for the two Cx26 mutants that fail to form gap junctions (Cx26 R75Y and T135A). Although there was a correlation between level of Cx26 coupling and rate of wound healing, there was no correlation with levels of protein expression (data not shown).

### Table 1. Gap junction and hemichannel function in HeLa cell transfectants

<table>
<thead>
<tr>
<th>Cell type</th>
<th>% coupled</th>
<th>% First-order coupling</th>
<th>% Dye uptake</th>
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<tbody>
<tr>
<td>HeLaWT</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>HeLa43</td>
<td>95±1.3</td>
<td>73±10</td>
<td>ND</td>
</tr>
<tr>
<td>HeLa32</td>
<td>96±2.0</td>
<td>80±9</td>
<td>ND</td>
</tr>
<tr>
<td>HeLa26</td>
<td>93±0.7</td>
<td>83±8</td>
<td>2±1.7(^a)</td>
</tr>
<tr>
<td>HeLa26 T135A</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>HeLa26 R75Y</td>
<td>0</td>
<td>0</td>
<td>6±1.2</td>
</tr>
</tbody>
</table>

\(^a\)Primary uptake only measured (not spread through gap junctions). ND, not determined. Results are mean±s.e.m. (n=10 or more).

Increased wound healing in HeLa26 cells is not a product of enhanced mitosis

Increased rates of wound closure could also be influenced by rates of cell division, although this could not explain the tracking data we obtained. Nonetheless, this was directly tested by measuring levels of bromodeoxyuridine (BrdU) incorporation at both the leading edge of the wound as well as in the confluent monolayer distal to the wound in each HeLa culture (Fig. 1D). The percentage of mitotic cells (BrdU-positive cells) distal to the wound site was similar for all cells studied, consistent with the similar growth of the different connexin transfectants in 10% serum [Cx26 only affected growth in 1% serum (Chandrasekhar et al., 2013)]. By contrast, at the leading edge, HeLa26 cells showed a decrease the proportion of mitotic cells compared to for those in the distal region, whereas both HeLa43 and HeLaWT showed an increase in BrdU labeling. The mitotic index of HeLa26 cells at the leading edge was approximately two-thirds of that measured for HeLa43 and HeLaWT cells, indicating motility and mitotic activity at the wound edge were inversely correlated.

Co-cultures of connexin-expressing cells show the influence of heterotypic coupling

The differential effects of Cx26, Cx32 and Cx43 on the migration of HeLa cells indicates that the intercellular channels composed of the different connexin isoforms might preferentially pass distinct signals, with Cx26 most effective, and Cx43 least effective in enhancing motility. This leads to a testable prediction that, should heterotypic Cx26–Cx32 channels be intermediate in permeability...
between Cx26 and Cx32 homotypic channels, then in mixed cultures, HeLa26 cells might be able to influence the motility of co-cultured HeLa32. In addition, given that Cx26 and Cx43 cannot form heterotypic gap junctions, one would predict that these cells would have no influence on one another’s motility.

To test this, we performed migration assays on co-cultures of HeLa26 and HeLa43, and HeLa26 and HeLa32 cells plated at 1:1 ratios, where each cell type was differentially labeled with a lipophilic green or red fluorophore. As predicted above, when HeLa26 and HeLa43 cells were mixed and subjected to wounding, the HeLa26 cells filled the wound first, leaving the HeLa43 cells behind (Fig. 2A, top). In contrast, when monolayers of mixed HeLa26 and HeLa32 cells were wounded, HeLa32 cells migrated along with the HeLa26 cells, filling the wound equally (Fig. 2A, bottom). This is most evident in the time-lapse videos (Movies 2 and 3).

By tracking the motility of individual cells as shown above, we can quantitatively compare the rates of movement of each cell type into the wound (Fig. 2B). This clearly demonstrates that the rate of movement of HeLa26 cells remained constant under homotypic or either heterotypic scenario (green bars). Similarly, HeLa43 cell motility was much lower, and was not influenced by heterotypic culture with HeLa26 cells (red bars, right-hand row), consistent with the inability to form Cx43–Cx26 heterotypic channels. In contrast, the motility of HeLa32 cells almost doubled in heterotypic cultures (Fig. 2C, middle row), with increased time to heal the wound (5 days), Cx43 caused some decrease (to 3 days) and Cx26 maximally reduced the time to close the wound to 2 days. A comparison of several HeLa26 clones (right side of graph) that show variable levels of coupling (E) The coupling levels (percentage of first-order neighbors coupled) of various HeLa clones (black bars) were compared to the rate of wound healing (days to heal wound). On the left, a comparison of different connexins showed that all induce similar levels of coupling, but Cx43 did not influence the time to close the wound (5 days), Cx32 caused some decrease (to 3 days) and Cx26 maximally reduced the time to close the wound to 2 days. A comparison of several HeLa26 clones (right side of graph) that show variable levels of coupling (C, M, 11, 12, 16), as well as two Cx26 mutants (T135A and R75Y) which fail to form channels, showed that reduced coupling levels correlate with increased time to heal the wound.
Fig. 2. Selective heterotypic junctional coupling can also enhance motility. (A) Scrape wounding of 1:1 co-cultures of HeLa26 cells (labeled green) with HeLa43 or HeLa32 cells (labeled red) were imaged at 0 (left) and 36 h after wounding (right). In the case of the HeLa26–HeLa43 co-cultures (upper right), where Cx26 and Cx43 do not form heterotypic gap junctions, the HeLa26 cells (green) clearly separate and migrate more rapidly into the wound than the HeLa43 cells (red) (also see Movie 2). By contrast, HeLa32 cells (lower right) migrate at similar rates to HeLa26 cells, such that both green and red cells fill the wound equally, presumably due to Cx32–Cx26 heterotypic coupling (Movie 3). (B) Tracking the motility rates of individual cells into the wound [distance moved into the wound (X direction) in pixels] in homologous (front row) and heterologous cultures confirms that HeLa26 cells migrate at similar rates in homotypic and both heterotypic cultures (green bars). HeLa43 cells migrate at the same rate when alone as when mixed with HeLa26 (red bars in right row), but HeLa32 cells show enhanced migration compared to homotypic cultures when in co-culture with HeLa26, moving at rates similar to the HeLa26 cells (red bars – middle row). Results are means; small boxes on top of each bar represent standard deviations (10 cells were tracked in three independent experiments). *P<0.001 (Student’s t-test).

exchanged with its neighbors. The failure of HeLa26 cells to influence HeLa43 cell motility also demonstrates that the signals are not transmitted extracellularly through the medium.

Cx26-induced increased migration is not just a response to wounding, but a general enhancement of motility

Although the wound healing assay is a useful method to quantify motility, it can include specific responses to signals associated with the wounding process itself. To determine whether the effects of Cx26 on motility are specific to this condition, we also compared motility of HeLaWT, HeLa26 and HeLa26R75Y in sparse cultures by tracking individual cell movements. Although under these conditions, there was no directionality to cell movement, the HeLa26 cells clearly showed much more motility and extension of filopodia (Fig. 3A; also see Movie 4). When the tracks of multiple cells were followed, HeLa26 cells traveled approximately seven-fold further in a given timeframe than HeLaWT or HeLaR75Y cells (Fig. 3B). This dramatic increase in the movement in sparse cultures was surprising given our demonstration above that the enhanced motility in the wound healing assay was dependent on GJIC. It had generally been assumed that intercellular coupling is minimal in sparse cultures, conditions in which connexins predominately form hemichannels. However, in this case the effects on motility were not mediated through hemichannels, as the Cx26R75Y mutant, which forms hemichannels as, or more efficiently than WT, had no effect on motility. Thus, we directly assessed coupling in these sparse cultures by labeling a subpopulation of HeLaWT or HeLa26 cells with Dil and also pre-loading them with a Calcein ester, before mixing them with an excess of unlabeled cells and plating under sparse conditions. After allowing time for the cells to adhere (5–6 h), cells were sorted and the percentage of cells now showing Calcein labeling alone, with no Dil label (i.e. they received calcein through gap junctions from the original double-labeled cells) was determined (Fig. 3C, lower right quadrant). Surprisingly, the data revealed that the sparse cultures show a high degree of cell communication. HeLaWT cells showed 2.5% and 1.2% of Dil-negative cells picking up Calcein in confluent and sparse cultures, respectively. In contrast, HeLa26 cells showed that 15.6% and 8.8% of Dil-negative cells were Calcein positive under the same confluent and sparse conditions, respectively. This result demonstrates that the transient cell contacts that are seen between these mobile cells in the time-lapse movies (Movie 4) allow cell communication to occur 50% as effectively as happens in confluent monolayers. This also demonstrates that increased migration of HeLa26 is an inherent property of the cells and not a product of wounding.

N-cadherin is necessary and sufficient to modify migration of HeLa cells

In searching for clues as to what the downstream targets of these intercellular signals might be, a comparison of cell behaviors during both wound healing and sparse culture assays (see Movies 1 and 4) revealed that there was much reduced cell–cell adhesiveness among HeLa26 cells compared to parental and other transfectants. Not only did HeLa26 cells separate out from the advancing wound edge much more frequently, but in sparse cultures, HeLa26 cells did not form colonies and contact after cell division was very short lived. Hence, we examined expression of N-cadherin, the primary adhesion molecule expressed in HeLa cells. Immunofluorescence showed a loss of N-cadherin and its relocation to the cytosol in cells at the wound edges of HeLa26 compared to HeLaWT or all other HeLa transfectants (HeLa43 shown, Fig. 4A). This phenotype was evident in all individual HeLa26 and HeLa43 cells (Fig. S1). The reduction of N-cadherin levels in HeLa26 cells was also seen in western blots (Fig. 4B), and at the RNA level by quantitative real-time PCR (qRT-PCR) (Fig. 4C) from cultures that were ‘stamp wounded’ to create multiple wounding interfaces (see Materials and Methods). Comparisons of N-cadherin levels in unwounded and wounded cultures demonstrated that Cx26 decreased N-cadherin expression compared to that in HeLa43 and WT cells by three- to four-fold in confluent cultures, and to a much greater extent in wounded cultures. HeLa32 cells also showed a similar pattern of decreases, but to a lesser extent than in HeLa26 cells (Fig. S2).
The functional importance of N-cadherin in regards to motility was demonstrated by small interference RNA (siRNA)-mediated knockdown in HeLa43 (Fig. 4D) and HeLaWT cell lines (Fig. S2B) to levels similar to those seen in HeLa26 cells. This resulted in a significant increase in the migration of these cells to rates close to that of HeLa26 cells (Fig. 4E). Scrambled siRNAs had no effect on motility. Conversely, exogenous expression of N-cadherin in HeLa26 to levels comparable to that seen in HeLaWT (Fig. 4F) significantly decreased the rate of migration of these cells compared to cells transfected with empty-vector alone, although not to the level of HeLaWT cells (Fig. 4G). These studies demonstrate that N-cadherin is both necessary and sufficient to modify the migration of HeLa cells, although it appears that it cannot fully account for the Cx26 motility phenotype.

**Redistribution of N-cadherin-binding proteins in HeLa26 cells is also linked to increased migration**

The cytosolic domains of cadherins have been shown to associate with the armadillo family proteins, including β-catenin, γ-catenin and p120ctn (Reynolds et al., 1994). Loss of N-cadherin would result in the release of these components into the cytoplasm, where they have been shown to play other roles. Specifically, a p120ctn–RhoGTPase pathway has been implicated in the modulation of the actin cytoskeleton, leading to branching that increases filopodial or lamellipodial activity and migration (Anastasiadis and Reynolds, 2000; Noren et al., 2000; Reynolds et al., 1996; Grosheva et al., 2001). By contrast, β-catenin has been extensively studied as a transcription factor that typically stimulates oncogenic gene expression (Behrens, 2000).

Although N-cadherin levels are much reduced in HeLa26 cells, total p120ctn levels, which show multiple bands on gels due to posttranslational modification, were similar in all the clones (Fig. 5A). However, with the loss of the N-cadherin tethering it to the membrane in HeLa26 cells, the levels of cytosolic p120ctn were significantly elevated (Fig. 5A). This cytosolic displacement of p120ctn caused activation of Rac1 into its GTP-bound form in HeLa26 cells, as shown by immunoblotting (Fig. 5B). The functional importance of Rac1 activation in migration was tested by siRNA knockdown of Rac1 in HeLa26 cells (Fig. 5C), which resulted in a significant decrease in migration rates to levels indistinguishable from HeLa43 (Fig. 5D).

We also looked at the localization of β-catenin, another major N-cadherin-binding protein, at the regions distal to, and at, the edge of the wound. HeLa and HeLa43 cells showed β-catenin colocalization with N-cadherin at the cell membrane, both distal and proximal to the wound (Fig. 4A). In contrast, HeLa26 cells showed this same colocalization in the monolayer distal to the wounds, but a redistribution of β-catenin to the nucleus at the wound edges (Figs 4A, 5E; Fig. S1). Given that β-catenin is a major transcriptional co-activator, we tested several known transcriptional targets of β-catenin. Two such targets associated with the motile phenotype, the urokinase plasminogen activator receptor (uPAR, also known as PLAUR) (Fig. 5F) and collagen IV (Fig. 5G), showed selective increases at both the RNA and protein level in HeLa26 wounded cultures. Another extracellular matrix protein (ECM), fibronectin, also showed an increase in HeLa26 cells, but in this case HeLa43 cells also showed an increase.

uPAR plays a central role in tumor development through control of cytoskeletal dynamics, cell adhesion and ECM integrity (Blasi and Carmeliet, 2002), whereas collagen IV and fibronectin are important components of the ECM needed to support cell motility in culture. Consistent with the latter, the migration rate of HeLaWT...
Fig. 4. N-cadherin is necessary and sufficient to modify HeLa26 cell migration. (A) HeLa WT (left), HeLa43 (center) and HeLa26 (right) scrape-wounded monolayers were stained for N-cadherin (green), β-catenin (red) and with DAPI (blue). All showed colocalization of N-cadherin and β-catenin at cell interfaces in the monolayer distal to the wound (top row), although HeLa26 cells appeared to have lower levels of N-cadherin. This colocalization was preserved, albeit at lower levels, at the wound edge in HeLaWT and HeLa43 cells, but HeLa26 cells showed reduced levels and cytosolic distributions of N-cadherin, and nuclear localization of β-catenin (see Fig. S1 for images of individual clones). (B) Western blots confirm that HeLa26 cells have reduced levels of N-cadherin compared to HeLa43 and WT cells. (C) qRT-PCR demonstrated that the reduction in N-cadherin in HeLa26 compared to WT and HeLa43 cells was also seen at the RNA level (also see Fig. S2A for levels in wounded and unwounded cultures). Results are mean±s.e.m. (n=3). *P<0.0001 (Student’s t-test). (D) Treatment of HeLa43 cells with an siRNA targeted to N-cadherin reduced protein expression to 50% of the levels of scrambled siRNA-treated controls (although still three times higher than HeLa26). (E) Treatment with N-cadherin siRNA, but not a scrambled form, increased the degree of wound closure seen in 2 days to close to that of HeLa26 cells (three independent experiments) (see Fig. S2B for parallel experiments with HeLaWT). Results are mean±s.e.m. (n=3). *P<0.0001 (Student’s t-test). (F) Transient overexpression of N-cadherin in HeLa26 raises the levels of N-cadherin protein to a level similar to that in HeLa WT cells. (G) The overexpression of N-cadherin in HeLa26 decreases the percentage of wound closure significantly over 2 days although not to the level of HeLa WT. Results are mean±s.e.m. (n=3). *P<0.005 (Student’s t-test).
and HeLa32 cells in the wound healing assay increased on plates pre-coated with collagen, whereas this had little effect on the HeLa26 cells (Fig. S4), presumably because they make their own collagen.

**DISCUSSION**

There have been several reports linking connexin expression to modulation of cell motility (Huang et al., 1998; Xu et al., 2001; Francis et al., 2011; Churko and Laird, 2013; Elias et al., 2007, 2010; Valiente et al., 2011). In cancer, enhanced connexin expression, or specifically surface localization, has been correlated with increased metastasis (Defamie et al., 2014; Kotini and Mayor, 2015), which typically also involves an enhanced migratory phenotype. Using a controlled exogenous expression system, we now show that the motility of HeLa cancer cells is enhanced when the cells couple through Cx26. This effect required formation of intercellular channels, unlike some neuronal systems reported previously, where the mechanisms are linked to the structural characteristics of gap junctions or their role in adhesion (Xu et al., 2001; Elias et al., 2007). It was further shown to be dependent on the connexin isoform, as Cx43 coupling did not change motility and Cx32 had an intermediate effect.

A second major finding is that Cx26 intercellular coupling mediates enhanced motility through a significant loss of cell...
adhesion. Several studies in the past have reported that cell adhesion enhances, or is required for, intercellular coupling (Keane et al., 1988; Jongen et al., 1991; Musil et al., 1990; Zhu et al., 2010). However, this is the first study to show that gap junctional coupling can have a reciprocal effect on adhesion. It is particularly intriguing that this is not due to the close physical associations between the components, but is caused by exchanges of intercellular signals specific to Cx26 that cause a loss of N-cadherin transcription (or a destabilization of its mRNA).

The HeLa system recapitulates many of the early steps of metastasis, with an upregulation of coupling (induced exogenously in this case), a drop in cellular adhesiveness and enhanced motility. As HeLa cells have lost the E-cadherin gene, adhesion is mediated by N-cadherin. Fig. 6 summarizes the mechanism whereby the downregulation of N-cadherin in HeLa26 cells leads to decreased adhesivity, causing release of the N-cadherin-binding proteins p120ctn and β-catenin to the cytosol and nucleus, respectively, where they activate targets to promote enhanced migration.

In terms of these downstream effects, p120ctn overexpression in fibroblasts has previously been shown to induce a dramatic actin-branching phenotype, with increased contractility and focal adhesion formation, and augmented migratory ability (Grosheva et al., 2001). This has been attributed to potent inactivation of RhoA (Anastasiadis and Reynolds, 2000; Noren et al., 2000) and/or activation of Rac1, which we directly demonstrate in this study, and Cdc42 (Noren et al., 2000; Grosheva et al., 2001). p120ctn has also been shown to stabilize microtubules important for directional cell migration in a cadherin-independent manner (Ichii and Takeichi, 2007).

One interesting consequence of the loss of N-cadherin induced by Cx26 coupling is that, as Cx26 transfectants migrate, the cells at the leading edge are sparsely populated and are often not in direct contact with adjacent cells (Movie 1). This can lead to loss of coupling at the leading edge of cell migration, accompanied by a loss of connexin plaques. It seems paradoxical that GJIC is necessary for cell migration, yet it might be disrupted among the most motile cells. Two possibilities exist to explain this observation. First, that Cx26 GJIC might serve to ‘prime’ the actions of the cells at the wound edge by inducing the initial loss of N-cadherin. This triggers the cascade of events that promote motility, after which continued coupling might not be needed. Second, despite the general lack of contact, some coupling might still be retained. This is consistent with our observations in sparse cultures, where we demonstrated that, even in highly motile cells that show minimal adhesion and only transient contacts, coupling can still be quite effective (Fig. 3C). This observation is highly important, as it suggests that during cell migration in development and tumorigenesis, transient contacts with the surrounding tissue might be crucial for guiding motility or for the extravasation of tumor cells that make transient contacts with endothelial cells of blood vessels or lymphatics.

As yet, we have not identified the initial intercellular signal that mediates the loss of N-cadherin, and ultimately enhanced modulation, induced by Cx26 coupling. The challenge, like all gap-junction-regulated processes, is the diversity of possible candidates, notably all signaling molecules under 1000 Da. Some notable candidates could be: inositol 1,4,5-trisphosphate (IP3), a known junctional permeant (Sneyd et al., 1998), which affects migration by activating the Rac–Cdc42 pathway (Coon and Herrera, 2002) or Ca2+, another junctional permeant that has been shown to regulate motility through protein kinase C (Etienne-Manneville and Hall, 2001). Even larger molecules, like microRNAs, have also shown to pass through gap junctions (Zong et al., 2016), and can regulate both motility and adhesion [e.g. miR-124 (Zhang et al., 2015)]. Of particular interest is cAMP, the intercellular signal that mediates growth suppression in HeLa cells, which has also been shown to be specific to Cx26 (Chandrasekhar et al., 2013). cAMP is also known as a regulator of cadherin expression through cAMP response elements (Pon et al., 2005).

To test this, we monitored the spatial distribution of cAMP by antibodies directed to the activated form of protein kinase A (PKA), which we showed to reflect the actual concentration of cAMP in the cells. This revealed a marked upregulation of PKA activity at the wound edge in HeLa cells (Fig. 7A) that was eliminated in HeLa26 cultures (Fig. 7B), consistent with a redistribution of cAMP. The distribution of PKA activity is broadened in HeLa26 cells at the wound edge, presumably because these motile cells are minimally in contact (Fig. 7B, yellow bars). However, the difference in PKA activities at the wound edge and the monolayer distal to the wound is also eliminated in HeLa43 cultures (Fig. 7C), although in this case the distribution in each population remains tight as all the cells retain coupling. However, given that migration and N-cadherin levels are not affected in HeLa43 cells, the redistribution of cAMP cannot be the determining signal for motility. Hence, the suppression of cell
growth and promotion of motility, both induced selectively by Cx26, are mediated by different signals. This opens possibilities for targeting connexins therapeutically in cancer, and other areas, by differentially affecting growth and motility effects.

MATERIALS AND METHODS

Cells and transfections

Wild-type HeLa cells were a gift of Klaus Willecke (University of Bonn, Germany). Each WT connexin was inserted into a pIRES hygro vector (Clontech, Palo Alto, CA), and following lipofection (Lipofectamine plus, Invitrogen, Carlsbad, CA) clones were selected using 400 µg/ml hygromycin (Sigma, St. Louis, MO). Cx26 T135A or Cx26 R75Y DNA was inserted into a pIRESpuro3 vector, and pools of transfectants were selected with 1 µg/ml puromycin (Sigma) and maintained in 0.5 µg/ml puromycin. Cells were tested for protein expression (western blotting and immunofluorescence) and functional coupling (dye preloading assays). Cx26 clones C, M and 11 were pooled in equal ratios for functional and migration analyses. siRNA for N-cadherin (Dharmacon, Lafayette, CO), or Rac (Pierce, Rockford, IL) were used to knockdown their respective RNAs by transfection using 100–200 nM siRNA with Dharmafect reagent and Lipofectamine, respectively. N-cadherin was overexpressed in HeLa26 cells by transfection of N-cadherin in a pCMV myc vector (Origene, Rockville, MD) using Lipofectamine (Invitrogen).
**Confocal imaging for gap junctions**

Cells were plated onto 12-mm round cover slips and streaked with a pipette tip after they reached confluence. After 24 h, the cells were fixed (2% paraformaldehyde for 20 min), permeabilized (1% Triton X-100 in PBS for 20 min), and blocked (0.1 M glycine and 0.5% BSA in PBS for 1 h at 37°C) prior to labeling with the relevant primary [Cx43 (cat. no. 13-8300); Cx26 (cat.no. 51-2800), both at 1:100; Thermo-Fisher, Waltham, MA], and secondary antibodies (goat anti-rabbit-IgG conjugated to Alexa Fluor 488, Molecular Probes, Eugene, OR). Slides were viewed with a 60× objective and zoomed three-fold with an Olympus FV-500 Confocal Laser Scanning Microscope in the Core Optical Imaging Facility at UT Southwestern. Confocal images were collected using an Olympus Fluoview 500 laser scanning system mounted on an Olympus IX-81 microscope using a 60× Uplan Apo, NA 1.4. Alexa Fluor 488 was excited by the 488-nm line of an argon laser and imaged through a 505–525-nm bandpass filter. Images shown were assembled from a Z-stack taken at 0.5-mm intervals.

**Dye transfer and uptake assays**

Coupling was assayed using the pre-loading method of fluorescent dye transfer (McNeil et al., 1984). The donor cells were incubated with two fluorescent dyes: calcein AM (5 mM) and Dil (10 mM) (Molecular Probes) for 20 min in isotonic glucose. These pre-loaded donor cells were then plated with unlabeled cells at a ratio of 1:72 and allowed to settle for 5–6 h. The percentage coupling of each clone was determined as the percentage of preloaded cells passing calcein to at least one receiver cell (donors identified by Dil). Data was obtained from at least two independent experiments, with at least 100 donors. For the assay of hemichannel function, cells were grown in 24-well plates, washed with PBS and mechanically stimulated by dropping 0.5 ml of PBS in all four quadrants of the well. Immediately, 2% Lucifer Yellow and 2% Rhodamine-conjugated dextran (Molecular Probes) was added to the medium and incubated for 10 min. The cells were washed five to seven times, and dye uptake was observed. Images were photographed with a Zeiss Axiosert 35 photomicroscope, equipped with UV epifluorescence to measure green (Lucifer Yellow, connexin channel uptake) and red (Dil, uptake by damaged cells) fluorescence. Data was recorded on an AxioCamMR CCD camera and stored digitally using Axiovision software.

**Cell migration assays**

HeLaWT and transfectants were plated in Dulbecco’s modified Eagle’s medium (DMEM)+10% fetal bovine serum (FBS) at 4×10⁵ cells per 35-mm tissue culture dish (Becton Dickinson, Franklin Lakes, NJ) on duplicate plates. At 48 h after plating, a time by which all cell lines achieved confluence, the plates were streaked with a micropipette tip. After rinsing off released cells, photos were taken in three regions along the streak at time 0, and every 24 h for 3 days. For each clone, the time to fill the wound was determined as the point at which the wound was completely filled with cells. The percentage of the wound filled was determined by measuring the area of the wound at any time, and counting the number of cells that filled that area before, and at various times after, wounding. The percentage of the wound filled was the ratio of these numbers. All experiments were done in triplicate. These studies were supplemented with real-time in vivo migration videos taken over the course of a 36–48-h time period using a Nikon TE microscope and processed with Metamorph software.

**Video microscopy and cell tracking**

A confluent monolayer of cells in a 60-mm plate were wounded with a pipette tip and placed in a CO₂, humidity- and temperature-controlled chamber attached to a Nikon TE 2000 microscope. Images of migrating cells were taken over time (one image every 6 min) for 48 h and a user-defined time-lapse stack was created. The cell-tracking app module from the Metamorph software was used to calculate the velocity of migration, distance traveled in a defined direction (X, perpendicular to the wound; Y, parallel to the wound), and the directionality (X/Y) of each cell type. Similarly, the particle track feature of the software was used to track individual cells using the stack of the images generated to calculate the velocity and directionality of movement in sparse cultures. In both wounded and sparse cultures, at least 10 cells were tracked in each field, and all experiments were performed in triplicate.

**Bromodeoxyuridine labeling**

The same wounding experiment described above was performed on cells grown on 22-mm square cover slips (Corning, Acton, MA). At time 0, and at 1 day or 2 days after wounding, the cover slips were incubated in freshly prepared 20 μM BrdU (Sigma) in PBS for 30 min at 37°C. After rinsing, the cells were fixed in 2% paraformaldehyde (Sigma) for 20 min, permeabilized with 0.25% Triton X-100 (Sigma), denatured in 4 M HCl (Fisher Scientific, Springfield, NJ) for 30 min, and after a 5 min block with 2% BSA (Sigma) in PBS, exposed to anti-BrdU primary monoclonal antibody (cat. no. MA3-071, Thermo-Fisher, Waltham, MA) at a 1:50 dilution for 2 h in blocking buffer, followed by anti-mouse-IgG conjugated to Alexa Fluor 488 (Molecular Probes) for 30 min at a dilution of 1:200 in blocking buffer. Coverslips were viewed with a 40× objective on an Olympus BX51 microscope equipped with a Sensicam CCD camera. Five fields were photographed for each coverslip including the leading edge (up to 20 cells in from the edge) and a region distal to the wound (approximately 300 cells from the edge). The percentage of BrdU-labeled cells was obtained from ratios of the number of fluorescent cells per field to the total number of cells per field as determined from phase-contrast micrographs.

**Co-culture migration cell labeling**

HeLa26, HeLa32 and HeLa43 cells were suspended at a density of 1×10⁶ cells/ml in serum-free culture medium. 5 μl of the cell-labeling solution (Vibrant Cell-labeling solutions, Molecular Probes), D0 for HeLa26, and Dil for HeLa32 and HeLa43, was added per ml of cell suspension. The treated cells were incubated for 10 min at 37°C. The labeled cells were centrifuged at 1500 rpm (430 g) for 5 min at 37°C. The cells were resuspended and plated at a density of 1:1, before growing to confluence and performing the wounding assay described above.

**Dye transfer in sparse cultures**

The donor cells were incubated with two fluorescent dyes: calcein AM (5 mM) and Dil (10 mM) (Molecular Probes), for 20 min in isotonic glucose. These pre-loaded donor cells were then plated with unlabeled cells at a ratio of 1:6 and allowed to settle for 5–6 h. The cells were then trypsinized and a FACS sort was performed to separate donor cells (having Dil and Calcein) from acceptor cells having only calcein on a BD FACS Calibur instrument, with analysis by the Cell Quest software.

**Stamp wounding system**

In order to be able to biochemically analyze (by western blotting or qRT-PCR) cells involved in wound healing, we used a stamp-wounding system that generated plates of cells where the majority of the cells are involved in wound healing. The stamp-wounding device is a circular contoured ‘stamp’, the same diameter as a culture plate, that denudes cultured cells in circular patterns, with intervening strips of intact cells. These remaining cells migrate and proliferate in near synchrony to fill the wounds (Lan et al., 2010).

**Western blots**

Total cell lysates were prepared in RIPA buffer (PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 10 mg/ml PMSF, 10 μg/ml Na₃VO₄, aprotinin, leupeptin, pepstatin; 1 ml/100 mm dish). Lysates were passed through a needle 18 times to shear the DNA and were spun at 15,000 rpm (21,700 g) for 20 min. Protein estimations were performed on the supernatant (MicroBCA protein assay kit, Pierce, Rockford, IL) with BSA as a standard. 20 μg of protein per sample was separated on a 12% SDS polyacrylamide gel and electro-blotted onto Immobilon-P membranes. The blots were probed with monoclonal or polyclonal anti-Cx26, -Cx32 and -Cx43 antibodies (Cell Signaling Technology, Danvers, MA), followed by a secondary antibody conjugated to horseradish peroxidase (HRP; Cell Signaling) and then ECL plus reagent (Amersham Biosciences) prior to exposure to X-ray film (Kodak). The blots were stripped (100 mM 2-mercaptoethanol, 2% w/v SDS, 62.4 mM Tris-HCl, pH 6.7) for 30 min at 50°C with mild agitation and re-probed with actin antibody (Sigma). Cell...
lyses from migrating cells were prepared 24 or 48 h after stamp-wounding confluent monolayers. Antibodies for N-cadherin (cat. no. 33-3900), Thermo-Fisher, Waltham, MA), p120 catenin (cat. no sc-23872, Santa Cruz Biotechnology, Santa Cruz, CA) and Rac1 (cat. no. PAI-091, Thermo-Fisher, Waltham, MA) were used according to manufacturer’s instructions.

**qRT-PCR**

Total RNA was extracted using TRizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. 1 µg of total RNA was reverse transcribed using the Taqman reverse transcription reagents from Applied Biosystems (Foster City, CA). 1 µl of the reverse transcription reaction mixture was analyzed by qRT-PCR with SYBR green mix using an ABI Prism 7700 sequence detection system (Applied Bio Systems). The mRNAs measured were normalized with respect to 18 s rRNA. Primers were designed using primerquest software from Integrated technologies as follows: uPAR forward primer, 5′-CATGCTGATGGACGCAAGG-3′, and uPAR reverse primer, 5′-CTGGAGCTGTGGGAAAGAG-3′, N-cadherin forward primer, 5′-CAGGAGATTCCTCCCAAGT-3′ and reverse primer, 5′-CATTGGAGTCACATTGGCAGA-3′.

**Rac1-GTP pulldown assay**

Rac1 GTP was detected using the Active Rac1 Pull-Down and Detection kit (Pierce Biotechnology, Rockland, IL). Briefly, cells were collected 1 day after stamp wounding using the lysis, binding and wash buffer and centrifuged at 16,000 g at 4°C. The supernatant is then placed on immobilized Glutathione swelGel discs containing the GST-tagged human PAKI-PBD in a spin cup. After incubation at 4°C for 1 h, the spin cup with collection tube is centrifuged at 7200 g for 10–20 s, removed and washed before eluting the protein with sample buffer containing β-mercaptoethanol and SDS. The sample is then electrophoresed and gel probed for Rac1-GTP using anti-Rac1 antibody (cat. no. PAI-091, Thermo-Fisher, Waltham, MA).

**Statistics**

Pairwise comparisons to assess significance of differences shown throughout were performed using unpaired, two-tailed Student’s t-tests through Prizm software. Numbers of measurements and experimental repeats are described in each figure or the methods.

**Competing Interests**

The authors declare no competing or financial interests.

**Author contributions**

S.R.P. helped to perform the migration studies, including cell labeling and movies, and developed the procedures for measuring coupling in sparse cultures. He also performed all analyses of signaling pathways, and helped in the writing of the manuscript, contributing to its intellectual content. E.A.K. developed the video procedures for following migration and quantitatively measuring rates, including the labeling procedures for heterologous cultures. He also contributed to some of the signaling analyses and writing of initial drafts of the manuscript, contributing to its intellectual content. A.C. helped to develop and characterize all cell lines used in these studies, and contributed to the early design of the project. S.N.Z. shared major responsibility for the original conception and design of the project, and hence shares credit for senior authorship. She also performed initial measurements using the wound healing assay, and served as the direct supervisor of A.C. B.J.N., in conjunction with S.N.Z., conceived the original design of the project and served as mentor and supervisor to A.C., and S.R.P. and E.A.K. He helped in the design of the signaling studies, and contributed primarily to the intellectual content and editing of the manuscript.

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**Data availability**

Movies are available at: Movie 1, https://figshare.com/s/1bd9eb852b1a06376 (download to see all frames); Movie 2, https://figshare.com/s/cf57df94cbfeddfe077; Movie 3, https://figshare.com/s/0a3a849fd09bce209f9 and Movie 4, https://figshare.com/s/2add823eddf984759a1f (download to see all frames).

**Supplementary information**

Supplementary information available online at http://jcs.biologists.org/lookup doi:10.1242/jcs.185017.supplemental

**References**


VIDEO LINKS for Poulisani et al. (JOCES/2105/185017)

Four Videos showing the various migration assays presented in Fig 1 (Video 1), Fig 3 (Videos 2 and 3) and Fig 4 (Video 4) have been uploaded to Figshare. The links are provided below each caption.

( NOTE: As the movies have multiple frames displayed, you need to download the file to view them, as the Preview only shows one frame)

VIDEO DESCRIPTIONS:

VIDEO 1 – HeLa parental and HeLa26 confluent cultures were subjected to a scrape wound and allowed to close the wound over 28 hours. The timelapse video shows how the HeLa parental cells close the wound slowly by progressing into the wounded area as a cohesive “sheet”, maintaining close cell contact. In contrast, the HeLa cells expressing Cx26 move more quickly to fill the wound, frequently breaking contact with the main monolayer and actively migrating independently, showing much higher rates of filopodial extension and activity. Quantification of motility rates shown in Fig 1
https://figshare.com/s/bbd9ddb582b1a0f0637c
VIDEO 2 – Homotypic (top two panels) or Mixed (bottom panel) confluent cultures of HeLa26 (green) and HeLa43 (red) were subjected to a scrape wound and followed as in Video 1, but with longer spacing between frames. Cx26 and Cx43 are known not to form heterotypic channels, so these two cell lines do not couple. Each cell type was labeled by a lipophilic dye (DiI (Cx43) and DiO (Cx26), and the color distribution in each cell averaged by Metamorph software. In the mixed culture (lower panel) the green cells (HeLa26) fill the wound more rapidly than the red ones (HeLa43), each migrating at rates similar to what they do in homotypic cultures (shown in panels above). Quantitation shown in Fig 3B
https://figshare.com/s/c05749d9046bdffdc077
VIDEO 3 - Homotypic (top two panels) or Mixed (bottom panel) confluent cultures of HeLa26 (green) and HeLa32 (red) were subjected to a scrape wound and followed as in Video 2. Cx26 and Cx32 are known to form heterotypic channels and these cell lines do couple. Cells were labeled as in Video 2. In this case, in the mixed culture, the green (HeLa26) and red cells (HeLa32) fill the wound equally, as the HeLa32 cells now show higher motility than in their homotypic culture, approximating the motility rate of HeLa26 cells. Quantitation shown in Fig. 3B
https://figshare.com/s/0aaa849fd009bec0209f
VIDEO 4 – Sparse cultures of HeLa parental (top left), HeLa26 (top right) and HeLa26 R75Y (a mutant that forms hemichannels but not gap junctions – bottom) were monitored over 15 hours by time lapse video. HeLa26 showed dramatically higher motility and activity in filopodial extensions than the other HeLa cells. Since the only property that distinguishes Cx26 and Cx26R75Y is the ability to form intercellular channels, this demonstrated that despite the sparse cultures, intercellular exchange of metabolites is required for the enhanced motility. Quantitation of rates on cell movement are shown in Fig 4.

https://figshare.com/s/addf823e0d98c4759a1f
Fig. 1S: N-cadherin (green), β-catenin (red) and DAPI (blue) staining of two HeLa43 and HeLa26 clones distal to, and at, the wound edge.
Figure 1S: Cadherin and beta-catenin staining of HeLa43 and 26 clones

(linked to Fig 5A)

Cultures of two individual clones of HeLa43 cells (D19 and K7) and two clones of HeLa26 (M and 11) were wounded and stained for N-cadherin (green), β-catenin (red) and DAPI, to highlight the nuclei. Images are taken in the unwounded monolayer distal to the wound (left) and at the edge of the wound (right). The co-localization of N-cadherin and β-catenin that was seen at the interfaces between cells in all clones examined, was largely maintained at the wound edge in HeLa43 cells, but was lost in the HeLa26 cells. Here the cadherin was redistributed to the cytosol (and overall levels appear to decrease), and β-catenin appears in the nucleus (superimposition of red and purple labeling).
Fig 2S: N-cadherin during wound healing of HeLa
Figure 2S: N-cadherin levels and modulation during wound healing

(linked to Figs 5C and E)

(A) N-cadherin mRNA levels, measured by RTqPCR from confluent (clear bars) and stamp wounded cultures, showed that Cx26, and to a lesser extent, Cx32, induced decreased N-cadherin expression in confluent cultures compared to HeLa WT or 43 cells. This was much greater in wounded cultures, where N-cadherin message in HeLa26 was barely detectable. HeLa32 cells also showed a significant drop in N-cadherin in wounded cultures.

(B) In parallel studies to those shown in Figure 5E, siRNA knockdown of N-cadherin in HeLaWT cells induced them to significantly increase their rate of wound healing to close to that seen in HeLa26 cells.
**Fig 3S:** β-catenin and N-cadherin in HeLa WT, 43 and 26
Figure 3S: β-catenin and N-cadherin levels and distribution in unwounded and wounded cultures (linked to Figs 5A and 6E)

(A) Western blots of β-catenin, N-cadherin and Actin (as loading control) from confluent (unwounded) and wounded cultures of different HeLa transfectants show reductions of N-cadherin and β-catenin in HeLa26 unwounded cultures. In wounded cultures, there is a complete loss of N-cadherin protein in HeLa26, and a partial loss in HeLa cells expressing the non-functional Cx26T135A mutant and in HeLa32 cells. However, β-catenin remains present in all cell lines after wounding, although at reduced levels in HeLa26.

(B) The relative localization of N-cadherin and β-catenin immunofluorescent signals with the nuclear compartment (associated with DAPI staining) and with one another was assessed using Metamorph software on a Nikon TE 2000 microscope in HeLaWT, HeLa43 clones (DM19 and K7) and HeLa26 clones (C, M and 11) distal to, and at the edge of the wound, (see images in Fig. 1S). Generally patterns were similar, except for the HeLa26 clones at the wound edge, where an increase in β-catenin (and N-cadherin) nuclear localization was evident, along with a drop in N-cadherin-β-catenin co-localization (in all clones except HeLa26 M).
**Fig 4S:** Effect of different ECM components on mobility of HeLa cells expressing different Cxs.
Figure 4S: Motility of different HeLa cell lines on various extracellular matrices
(linked to Figs 6 F and G)

Since HeLa26 cells express collagen (Fig 6 G) to a greater degree than HeLa WT or Cx32 and 43 transfectants. We examined how each clone migrates (measure as % of wound closure after 48 hours) with or without coating of the plates with extracellular matrix components. HeLa26 shows no difference in migration rate on any matrix compared to plastic, while HeLa32 and WT both showed enhanced migration with collagen and fibronectin, with no, or slight inhibitory affects associated with laminin.