Glucose-limiting conditions induce an invasive population of MDA-MB-231 breast cancer cells with increased connexin 43 expression and membrane localization

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Abstract

Gap junctional intercellular communication (GJIC) is a homeostatic process mediated by membrane channels composed of a protein family known as connexins. Alterations to channel activity can modulate suppression or facilitation of cancer progression. These varying roles are influenced by the cancer cell genetic profile and the context-dependent mechanisms of a dynamic extracellular environment that encompasses fluctuations to nutrient availability. To better explore the effects of altered cellular metabolism on GJIC in breast cancer, we generated a derivative of the triple-negative breast cancer cell line MDA-MB-231 optimized for growth in low-glucose. Reduced availability of glucose is commonly encountered during tumor development and leads to metabolic reprogramming in cancer cells. MDA-MB-231 low-glucose adapted cells exhibited a larger size with improved cell–cell contact and upregulation of cadherin-11. Additionally, increased protein levels of connexin 43 and greater plasma membrane localization were observed with a corresponding improvement in GJIC activity compared to the parental cell line. Since GJIC has been shown to affect cellular invasion in multiple cancer cell types, we evaluated the invasive qualities of these cells using multiple three-dimensional Matrigel growth models. Results of these experiments demonstrated a significantly more invasive phenotype. Moreover, a decrease in invasion was noted when GJIC was inhibited. Our results indicate a potential response of triple-negative breast cancer cells to reduced glucose availability that results in changes to GJIC and invasiveness. Delineation of this relationship may help elucidate mechanisms by which altered cancer cell metabolism affects GJIC and how cancer cells respond to nutrient availability in this regard.

Keywords: Connexin · Gap junction · Breast cancer

Abbreviations

C×43 Connexin 43
GJIC Gap junctional intercellular communication

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Introduction

The development and progression of cancer is affected by genetic alterations within cancer cells and an ever-changing microenvironment that influences their behavior. Limitations in nutrient availability are common during tumor growth in hypoxic regions of primary tumors and colonization at metastatic sites as cancer cells encounter new tissue microenvironments (Simoes et al. 2015; DeBerardinis and Chandel 2016). In particular, heterogeneous blood perfusion within tumors leads to decreased oxygen and glucose levels and a reprogramming of cellular signaling which creates new cellular phenotypes optimized for growth in these conditions (Vaupel et al. 1989; Gillelis et al. 1999; Garcia-Jimenez and Goding 2019). The ability of cancer cells to adapt to these changing conditions increases their potential to survive and proliferate. This often requires utilization of secondary fuel sources for nitrogen and carbon and necessitates modifications to biochemical pathways (DeBerardinis and Chandel 2016; Pavlova and Thompson 2016).

Gap junctions are intercellular channels mediated by a family of proteins known as connexins and support cellular homeostasis by allowing the regulated passage of ions, nutrients and signaling molecules (Nielsen et al. 2012). This gap junctional intercellular communication (GJIC) is often dysregulated in cancer where it is frequently decreased due to downregulation of connexin genes, mislocalization of connexin proteins and changes to cellular adhesion junctions (Aasen et al. 2016). Numerous reports have demonstrated a causative relationship between the loss of GJIC and acquisition of cancer cell hallmarks or suppression of these features following restoration of GJIC (Mehta et al. 1991; King and Lampe 2004; Shao et al. 2005; Wang et al. 2014). However, other data have established that GJIC can also promote aggressive qualities in cancer cells (Bates et al. 2007; Ghosh et al. 2014; Hong et al. 2015; Zibara et al. 2015). Multiple models have demonstrated connexin expression and/or GJIC to be correlated with metastasis (Lamiche et al. 2012; Ogawa et al. 2012; Tang et al. 2013). Moreover, cancer cell gap junctions form between endothelial cells during metastasis, as well as stromal cells at secondary metastatic sites (el-Sabban and Pauli 1994; Chen et al. 2016). Thus, GJIC remains a dynamic process in the cancer cell, consequences of which are influenced by context-dependent mechanisms that may suppress or promote cancer cell survival and function.

An increased understanding of the role GJIC plays in cancer cell metabolism is providing important information for how gap junctions mediate the transfer of metabolites in the tumor microenvironment (Contreras et al. 2002; Dovmark et al. 2017, 2018). Conversely, to better understand the role of metabolism in regulation of GJIC, we evaluated changes in the triple-negative breast cancer cell line MDA-MB-231 when these cells adapted to low-glucose availability, a variant we refer to as MDA-MB-231LG. This derivative exhibited increased connexin protein expression and GJIC. Additionally, these cells exhibited highly invasive qualities that were affected, in part, by inhibition of GJIC.

Materials and methods

Antibodies

Antibodies targeting connexin 43, actin, vimentin, N-cadherin, E-cadherin, claudin-1, cadherin-11 (immunofluorescence), β-catenin, LC3 and horse radish peroxidase conjugated secondary antibodies were purchased from Cell Signaling (Danvers, MA, USA). Cadherin-11 antibody (western blot) and Alexa fluor conjugated secondary antibodies for immunofluorescence were purchased from Thermo Fisher Scientific (Waltham, MA, USA). GAPDH antibody was purchased from EMD Millipore. HIF1α antibody was purchased from EMD Millipore.

Cell lines and culture

MDA-MB-231 (HTB-26), Hs578T (HTB-126), MCF-7 (HTB-22) and T47D (HTB-133) cell lines were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA) and maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS). Cells were grown in a humidified incubator at 37 °C, 5% CO₂ and passaged using trypsin–EDTA. Routine screening for mycoplasma was performed via PCR detection using the Promokine PCR Mycoplasma Test Kit I/C (PromoCell, Heidelberg, Germany). MDA-MB-231LG were grown in RPMI 1640 glucose-free media supplemented with 10% fetal bovine serum (FBS). All experiments involving parental and LG cells were performed in the respective media unless otherwise indicated. All cells were validated by short tandem repeat (STR)-analysis at the Northwestern University NUSeq Core Facility (Chicago, IL, USA) and compared to ATCC cell identification data for verification (Table 1, Supp. Table 1).

Proliferation and viability

Cellular proliferation and viability were measured by use of CellTiter 96 AQuueous One Solution reagent (EMD Millipore) on a PerkinElmer Enspire Multimode plate reader (Waltham, MA, USA) according to manufacturer’s protocols. CellEvent Green Caspase 3/7 and SYTOX-AADvanced
reagents (Invitrogen, Carlsbad, CA, USA) were used to assess caspase activity and cell integrity using a Beckman Coulter (Brea, CA, USA) CytoFLEX flow cytometer with CytExpert analysis software. Positive controls for CellEvent reagent were demonstrated with staurosporine (Caspase 3/7) and heat killing (SYTOX) (Supp. Fig. 1).

**pH readings**

Cells were plated at equal densities and allowed to grow for 96 h without media changes. At 96 h media from wells was removed and filtered through a 0.22 µm sterile filter and read on a Mettler-Toledo SevenEasy pH meter.

**Scanning electron microscopy**

Cells were grown on glass coverslips and fixed with 4% paraformaldehyde at 4 °C for 24 h. Cells were then washed with phosphate-buffered saline (PBS), dehydrated with increasing concentrations of ethanol (30%, 50%, 70%, 80%, 95% and 100% for 10 min each at 4 °C), and dried in a Leica (Wetzlar, Germany) EM CPD300 critical point dryer. Samples were then sputter-coated with 10 nm silver using a Leica EM ACE600. Cells were visualized with a JEOL (Akishima, Tokyo, Japan) JCM-6000Plus scanning electron microscope at 5 kV and 10 kV.

**Fluorescence microscopy and GJIC analysis**

For immunofluorescence, cells were grown on glass coverslips in 24-well plates, washed with ice cold PBS and fixed in ice cold 100% methanol at −20 °C for 20 min. Cells were blocked in 2% bovine serum albumin (BSA) in PBS and incubated with indicated antibodies. For GJIC analysis, cells were analyzed by the method of Goldberg and quantified by flow cytometry (Goldberg et al. 1995; Czyz et al. 2000). Briefly, donor cells were labeled with 5 µM of the lipophilic permanent dye CM-DiI (Invitrogen) and 10 µM calcein-AM (Invitrogen) and cultured with non-labeled cells. Spread of calcein from donor cells (CM-DiI/calcein positive) to acceptor cells (calcein only) indicated GJIC activity as demonstrated with the highly gap junction coupled Hs578T breast cancer cell line (Supp. Fig. 2a). Images for immunofluorescence and GJIC analysis were obtained with a Leica Dmi8 fluorescent microscope equipped with a Leica DFC 9000GT camera and X-Cite XLED1 fluorescence source (Lumen Dynamics, Mississauga, Ontario, Canada). Quantification of GJIC was performed using a BD Biosciences (San Jose, CA, USA) FACSCalibur flow cytometer with CellQuest Pro software using the assay described above with 0.5 µM Cell Tracker Deep Red (Invitrogen) and 0.75 µM calcein-AM (Supp. Fig. 2b). Coupling efficiency was represented as the level of GJIC activity and calculated as acceptor cells/donor cells and normalized to control.

**Matrigel invasion assays**

Growth factor reduced (GFR) Matrigel basement membrane matrix (Corning, NY, USA) was utilized for two different three-dimensional cell culture assays. For the embedded assay, Matrigel was diluted from 8 to 5 mg/ml using RPMI 1640 with or without glucose with 10% FBS before 300 µl was added to each well. 30 µl of 1 × 106 cells/ml were added to 270 µl of diluted Matrigel and added on top of the first layer. After approximately 30–45 min of incubation, 500 µl of appropriate medium was added to the Matrigel layers. For the on-top assay, 300 µl of Matrigel was added directly to the wells without dilution. The Matrigel layer was allowed to solidify at 37 °C and 5% CO2 for 45 min and 250 µl of 25,000 cells/ml were plated directly on top of the first Matrigel layer and allowed to incubate for 45 min. 25 µl of Matrigel was mixed with 225 µl of the appropriate medium and added to the wells. Cells were then incubated for 6 days for each assay with medium changed at 48 h.

Growth factor reduced Matrigel invasion chambers (Corning) were used for the Matrigel invasion assay. 500 µl of warm serum-free medium (± 2 mM glucose) was added to

### Table 1 STR analysis of MDA-MB-231 and MDA-MB-231LG

| Marker        | MDA-MB-231 | MDA-MDB-231LG |
|---------------|------------|---------------|
| Amelogenin*   | X          | X             |
| D3S1358       | 16         | 16            |
| D1S1656       | 15, 17     | 15, 17        |
| D2S441        | 14, 15     | 14, 15        |
| D10S1248      | 14, 16     | 14, 16        |
| D13S317*      | 13         | 13            |
| Penta E       | 11         | 11            |
| D16S539*      | 12         | 12            |
| D18S51        | 11, 16     | 11            |
| D2S1338       | 20, 21     | 20, 21        |
| CSF1PO*       | 12, 13     | 12, 13        |
| Penta D       | 11, 14     | 11, 14        |
| TH01*         | 7, 9, 3    | 7, 9, 3       |
| vWA*          | 15, 18     | 15, 18        |
| D21S11        | 30, 33.2   | 30, 33.2      |
| D7S820*       | 8, 9       | 8, 9          |
| D5S818*       | 12         | 12            |
| TPOX*         | 8, 9       | 8, 9          |
| D8S1179       | 13         | 13            |
| D12S391       | 17, 18     | 17, 18        |
| D19S433       | 11, 14     | 11, 14        |
| FGA           | 22, 23     | 22, 23        |
| D22S1045      | 16         | 16            |

Asterisk indicate 9 markers defined by ATCC criteria for 100% match.
were considered statistically significant at \( p < 0.05 \) (*).

**Results**

**Characterization of MDA-MB-231\(^{LG}\)**

To examine the effects of metabolic changes on GJIC in breast cancer cells, we grew the MDA-MB-231 cell line in RPMI 1640 without glucose and supplemented with 10% FBS. Because trace amounts of glucose are present within FBS, we did not consider this media glucose-free. However, based on manufacturer’s specifications, final glucose concentration in the prepared media were below 0.130 mM. This accounted for a greater than 15x fold reduction in available glucose compared to control RPMI 1640 containing 2 mM glucose. Growth in this media decreased viability within 2–4 days of culture when evaluated using an MTT assay (Fig. 1a). To generate a population of cells optimized for growth in these conditions, we plated MDA-MB-231 at low density (20–30% confluence) and maintained surviving cells for 4–6 weeks. Media was replaced every 2 days to remove apoptotic cells and cellular debris. Small populations of surviving cells initially exhibited quiescence followed by restoration of cellular proliferation. To avoid clonal selection, surviving populations from multiple plates were combined and propagated. The growth pattern of these cells normalized and regular maintenance was initiated using continued culture in low-glucose media. The surviving population exhibited morphology that was noticeably different from the parental MDA-MB-231 (Fig. 1b). Cells were larger with rounded membranes, distinctly different from the mesenchymal morphology of the parental MDA-MB-231. These cells were designated MDA-MB-231\(^{LG}\) for their ability to grow in low glucose conditions. MDA-MB-231\(^{LG}\) did not acidify low-glucose culture media compared to parental cells grown under standard conditions as evidenced by the effects on phenol-red (Fig. 1c). Analysis of cellular proliferation showed a similar growth pattern (Fig. 1d). Cells appeared healthy with minimal signs of cellular stress. To confirm this, we assessed markers for apoptosis (caspase-3/7 cleavage) and overall cellular integrity (SYTOX) by flow cytometry demonstrating greater than 90% viability in low-glucose media similar to that of MDA-MB-231 grown in control media (Fig. 1e). As our growth conditions involved the reduction of glucose but did not affect oxygenation, we evaluated levels of LC3II as a marker of autophagy and hypoxia inducible factor α (HIF1α) as an indicator of response to hypoxic conditions. Indeed, greater levels of LC3II were present in the MDA-MB-231\(^{LG}\) while HIF1α remained relatively unchanged (Fig. 1f).

Since the MDA-MB-231\(^{LG}\) displayed considerably different morphology, we verified that the MDA-MB-231\(^{LG}\) were a true derivative of the parental MDA-MB-231. STR-analysis was performed to validate each cell line. Both the MDA-MB-231 and MDA-MB-231\(^{LG}\) resulted in a 9/9 match for markers established by ATCC to confirm identity of cell lines corresponding to a 100% match by these criteria (Table 1). An additional 14 markers were also examined and demonstrated > 96% similarity with
Glucose-limiting conditions induce an invasive population of MDA-MB-231 breast cancer cells…

the only difference at marker D18S51 corresponding to a heterozygous versus homozygous result in parental MDA-MB-231 and MDA-MB-231\(^{LG}\) respectively.

**Increased membrane contact in MDA-MB-231\(^{LG}\)**

When observed by phase microscopy, MDA-MB-231\(^{LG}\) appeared to exhibit greater contact at the plasma membrane between cells. To examine this in more detail, we performed scanning electron microscopy of both the MDA-MB-231 and MDA-MB-231\(^{LG}\). Results demonstrated an expected mesenchymal phenotype in MDA-MB-231 showing an elongated appearance with overlapping membranes and minimal cell–cell junctions (Fig. 2a). In contrast, MDA-MB-231\(^{LG}\) clearly showed an increased ability to form cell–cell junctions with non-overlapping membrane connections along portions of their plasma membranes (Fig. 2a, b). While both cell lines exhibited lamellipodia and filopodia, long pseudopodia were noted in the MDA-MB-231\(^{LG}\) (Fig. 2a). High
power analysis allowed for the observation of membrane contact between MDA-MB-231 LG cells (Fig. 2b).

Due to these observations, we examined markers of epithelial-to-mesenchymal transition (EMT) to determine if re-expression of epithelial markers may account for the increased cell–cell junctions. Epithelial-cadherin (E-cadherin, CDH1) and claudin-1 (CLDN1) were not detected in either MDA-MB-231 or MDA-MB-231 LG (Fig. 3a), indicating that upregulation of these markers was not responsible for the increased cell–cell attachment noted in the MDA-MB-231 LG. N-cadherin (CDH2), a mesenchymal-associated cadherin was not detected in the MDA-MB-231 or the MDA-MB-231 LG and levels of the intermediate filament and mesenchymal marker vimentin (VIM) remained relatively unchanged (Fig. 3a). However, MDA-MB-231 have been shown to express cadherin-11 (CDH11) and analysis in the MDA-MB-231 LG demonstrated a significant increase in the levels of this attachment factor (Fig. 3a). When observed by immunofluorescence, strong membrane localization was observed throughout the cell population, particularly at sites of cell–cell contact (Fig. 3b). By comparison, cadherin-11 was observed intermittently throughout MDA-MB-231 populations. Levels of β-catenin (CTNNB1), a multi-function protein involved in both cellular signaling and cell–cell attachment, remained similar between the cell lines (Fig. 3a). However, increased fluorescence overlap of β-catenin with cadherin-11 at the plasma membrane was apparent in the MDA-MB-231 LG (Fig. 3b).

Fig. 2 MDA-MB-231 LG exhibit increased membrane contact. a Analysis of MDA-MB-231 and MDA-MB-231 LG morphology and cell–cell interactions by scanning electron microscopy at indicated magnifications. *Indicates overlapping membranes; arrows indicate sites of cell–cell membrane contact. L: lamellipodia; F: filopodia; P: pseudopodia. b, High magnification images of membrane interactions in MDA-MB-231 LG.
Adaptation to low glucose increases connexin 43 and GJIC

We next set out to determine if the metabolic adaptations in the MDA-MB-231<sup>LG</sup> and alterations to membrane structure affected GJIC. We first examined protein levels of connexin 43 (GJA1, C×43), a major connexin protein expressed in breast tissue and found an increase in proteins levels of this connexin in the MDA-MB-231<sup>LG</sup> (Fig. 4a). C×43 is subject to significant post-translational modification and higher molecular weight species of C×43 can be detected by western blot analysis (Supp. Fig. 3). However, in both the MDA-MB-231 and MDA-MB-231<sup>LG</sup>, we did not detect higher molecular weight species of C×43 (Fig. 4a).

We then determined if membrane localization of C×43 was also affected in the MDA-MB-231<sup>LG</sup>, MDA-MB-231 showed minimal staining for C×43 that was predominantly peri-nuclear with little localization at the membrane (Fig. 4b). In contrast, MDA-MB-231<sup>LG</sup> displayed a higher degree of C×43 localization at the plasma membrane, particularly at cell junctions, indicative of gap junction formation (Fig. 4b and Supp. Fig. 4). To determine if the increase in C×43 membrane localization corresponded to functional gap junctions, a double-label dye transfer technique was performed to assess GJIC with transfer of the fluorescent dye calcein indicating active GJIC. MDA-MB-231 exhibited minimal spread of calcein while a greater number of MDA-MB-231<sup>LG</sup> were capable of transferring this dye to neighboring cells (Fig. 5a). This led to a measurable increase in GJIC when quantitatively assessed by flow cytometry (Fig. 5b).

MDA-MB-231<sup>LG</sup> exhibit increased invasive qualities

Because changes to GJIC have been shown to affect the invasive capabilities of cancer cells, we sought to evaluate if this quality was also altered in the MDA-MB-231<sup>LG</sup>. We examined the morphologic appearance of these cells in three-dimensional culture utilizing Matrigel reconstituted basement membrane matrix. Cells were embedded within...
Matrigel, allowing for growth in three-dimension, a condition which more closely resembles cell growth in vivo and allows for observation of phenotypic characteristics. While both MDA-MB-231 and MDA-MB-231\textsuperscript{LG} formed spheroids, MDA-MB-231\textsuperscript{LG} exhibited greater stellate extensions at 6 days of culture (Fig. 6a). This was confirmed by use of a second assay in which cells were grown on top of a Matrigel matrix and overlaid with additional Matrigel (Fig. 6b). This procedure allows for more clear documentation of cellular morphologies and extensions and demonstrated a more invasive phenotype in MDA-MB-231\textsuperscript{LG}. To determine if these changes to three-dimensional morphology correlated to increased invasive function, Matrigel invasion chamber assays were used to quantitatively assess the ability of each cell line to invade through a three-dimensional matrix and traverse a membrane containing 8 µm pores. Cells were seeded at the top of the matrix in serum-free media and FBS was used as a chemoattractant in the lower chamber of the assay. Invading cells were stained with crystal violet and MDA-MB-231\textsuperscript{LG} demonstrated a clear increase in the number of invading cells (Fig. 6c).

To more quantitatively assess the invasive capacity of the MDA-MB-231\textsuperscript{LG} we adapted our invasion chamber conditions to fixation with DAPI and analyzed these images using fluorescence microscopy and computer-based software quantification. Additionally, to evaluate potential contributing effects of glucose availability on each cell line, we conducted these experiments in the presence or absence of glucose for 24 h. The MDA-MB-231\textsuperscript{LG} exhibited a significantly more invasive capacity in each condition compared to MDA-MB-231 (Fig. 7a, b). The overall results with MDA-MB-231\textsuperscript{LG} demonstrated significantly higher invasive qualities in each condition.

Finally, to examine a potential link between the increased GJIC in MDA-MB-231\textsuperscript{LG} and their invasive qualities, we used the gap junction un-coupling agent 18α-glycyrrhetinic acid (18αGA) to reduce GJIC in MDA-MB-231\textsuperscript{LG}. To decrease the possibility of non-specific effects of 18αGA, we used the minimum concentration of 18αGA capable of inhibiting GJIC at 24 h for each condition in the Matrigel invasion chamber assay. This corresponded to 10 µM in serum-free conditions (upper chamber) and 50 µM in 5% FBS (lower chamber) (Fig. 8a). 18αGA under these conditions caused a slight but significant reduction in Matrigel chamber invasion of MDA-MB-231\textsuperscript{LG} when compared to untreated conditions (Fig. 8b, c).
Glucose-limiting conditions induce an invasive population of MDA-MB-231 breast cancer cells...

Discussion

During cancer progression, breast cancer cells exhibit a multitude of responses to the extracellular milieu in which they exist. Among these alterations are changes in GJIC and adaptation to fluctuating metabolic conditions. A better understanding of how these two processes affect each other, and how this contributes to the cancer cell phenotype, will provide important information about this relationship. In this report, we describe the generation of a metabolic variant of the MDA-MB-231 parental cell line adapted for growth in glucose-limiting conditions. These cells maintained a proliferative capacity and high viability despite reduced utilization of this carbon source. The increased levels of autophagy that we observed in the MDA-MB-231LG are likely a contributing factor to their survival, yet not high enough to induce autophagic (type II) cell death. We believe that generation of the MDA-MB-231LG represent a metabolic adaptation to these growth parameters due to their significantly altered morphology as well as the molecular and functional changes we have characterized. However, persistent subpopulations within the MDA-MB-231 have been reported (Louie et al. 2010; Wang et al. 2015; Amaro et al. 2016; Morata-Tarifa et al. 2016). Therefore, the possibility that the MDA-MB-231LG represent isolation of an existing variant through the selective metabolic pressure described in our experiments remains possible. Although these cells displayed significant differences in appearance and function compared to the parental MDA-MB-231, STR analysis validated these cells a true derivative. Thus, we evaluated the characteristics of the MDA-MB-231LG on GJIC and invasion to determine how this metabolic adaptation affected these qualities.

The MDA-MB-231 cell line was originally isolated from a pleural effusion of a patient with an intraductal carcinoma and displayed rounded to spindle-like morphology following establishment in culture (Cailleau et al. 1974). Cells were isolated post-chemotherapeutic treatment and displayed significant aneuploidy. Molecular characterization has revealed a triple-negative profile with mutations in numerous genes including TP53, BRAF and KRAS (Hollestelle et al. 2010). Cells were isolated post-chemotherapeutic treatment and displayed significant aneuploidy. Molecular characterization has revealed a triple-negative profile with mutations in numerous genes including TP53, BRAF and KRAS (Hollestelle et al. 2010). The MDA-MB-231 exhibit a phenotype consistent with EMT and express mesenchymal markers such as the intermediate filament vimentin and lack of epithelial proteins such as E-cadherin (Nieman et al. 1999; Pishvaian et al. 1999; Li
Fig. 6 MDA-MB-231<sup>LG</sup> display greater invasive qualities. a MDA-MB-231 and MDA-MB-231<sup>LG</sup> were embedded in Matrigel membrane and allowed to grow for 6 days. Cells grew in colonies within the matrix and invasive protrusions of cells were indicative of invasive qualities. Two representative fields shown from three independent experiments. Scale bar: 100 µm. b Matrigel on-top procedure involved culture of cells on matrix overlaid with Matrigel and grown for 6 days. Two representative fields shown from three independent experiments. Scale bar: 100 µm. c Matrigel invasion chamber assays were performed in duplicate in three independent experiments for both cell lines with 24 h culture. MDA-MB-231 and MDA-MB-231<sup>LG</sup> were grown in upper chambers in serum free media corresponding to each cell line. Lower chamber of inserts contained 5% FBS as a chemoattractant. Cells invading through the matrix and 8 µm pore inserts indicated invasion and were stained with crystal violet for visualization. Scale bar: 100 µm.

Fig. 7 MDA-MB-231<sup>LG</sup> maintain invasiveness in the presence of glucose. a Matrigel invasion chamber assays were performed in duplicate in three independent experiments in the presence or absence of glucose and invading cells stained with DAPI. Scale bar: 200 µm. b Quantification of results in a. Data represent the mean of three independent experiments normalized to MDA-MB-231 + glucose and shown as fold change ± SD. (*p < 0.05; NS, not significant)
Glucose-limiting conditions induce an invasive population of MDA-MB-231 breast cancer cells (et al. 2011; Liu et al. 2015). MDA-MB-231 also fall in the category of “claudin-low” due to their decreased expression of tight junction proteins such as claudin-1 (Majer et al. 2016; Dias et al. 2017; Chiang et al. 2019; Mattern et al. 2019). Collectively, this promotes a decrease in cell–cell attachment and increase in cellular motility. Because the MDA-MB-231LG exhibited stronger cell–cell contacts and altered morphology, we explored if these cells underwent a reversal of this process known as mesenchymal-to-epithelial transition (MET) by analysis of cytoskeletal and membrane EMT markers (Chao et al. 2010; Liu et al. 2016). No upregulation of E-cadherin or claudin-1 was observed. Additionally, N-cadherin, a mesenchymal-associated cadherin, was not detected in the MDA-MB-231 (consistent with previous reports (Hazan et al. 1997; Nieman et al. 1999; Wang et al. 2002; Shankar and Nabi 2015) and could not be detected in the MDA-MB-231LG. However, MDA-MB-231 express cadherin-11 (Nieman et al. 1999; Wang et al. 2002; Li et al. 2011; Satriyo et al. 2019). Cadherin-11, like other cadherins, promotes cell–cell contact through homomeric extracellular interactions that help to link cytoskeletal proteins on the cytoplasmic side. Cadherin-11 is upregulated in invasive breast cancer (Assefnia et al. 2014; Pohlodek et al. 2016). Moreover, inhibition of cadherin-11 in the MDA-MB-231 decreased migration, growth in soft agar, stem cell marker expression, and in vivo tumor growth (Assefnia et al. 2014; Satriyo et al. 2019). The observation of higher cadherin-11 levels in the MDA-MB-231LG may contribute to the increase in cell–cell contact in these cells as it exhibited a membrane localized pattern, particularly at sites of cell–cell interaction. Interestingly, we observed a corresponding increase of cadherin-11 at sites of β-catenin near the plasma membrane. β-catenin exhibits multiple subcellular localizations within the nucleus, cytosol and plasma membrane corresponding to different functions and activity. β-catenin at the plasma membrane is involved in linking adherens junctions to cytoskeletal proteins such as actin and can contribute to cytoskeletal reorganization dynamics. The markers examined here represent only a small fraction of the many EMT, membrane and cytoskeletal proteins altered in cancer and further characterization is required to confirm a relationship. Nonetheless, these results suggest a potential relationship to the morphologic and membrane changes seen in the MDA-MB-231LG.

Since membrane contact is a fundamental aspect of GJIC, we explored if the plasma membrane interactions observed in the MDA-MB-231LG correlated with the formation of gap junctions. Cx43 is highly expressed in breast tissue and one of the most well studied connexins altered during cancer progression. Similar to previous reports, we observed that parental MDA-MB-231 expressed low levels of Cx43 with the absence of plasma membrane localization and GJIC in these cells (Qin et al. 2001; 2003, Jiang et al. 2017). Remarkably, Cx43 protein was elevated in MDA-MB-231LG. We observed predominantly lower molecular weight forms of Cx43 in each cell line similar to previous reports in MDA-MB-231 (Qin et al. 2002; Talhouk et al. 2013; Ming et al. 2015). However, the increase in Cx43 indicated a potential adaptive response related to glucose levels and Cx43 expression. Conversely, a recent report demonstrated an inverse relationship between high-glucose levels and Cx43 expression in osteocyte-like cells (Yang et al. 2020). Augmented connexin protein levels alone are not capable of increasing GJIC when trafficking and membrane transport of connexin proteins remains dysregulated (Qin et al. 2001, 2003; Tang et al. 2013; Ming et al. 2015).
To demonstrate function, we documented the ability of MDA-MB-231LG to transfer calcein to neighboring cells and noted an increase in GJIC. An important observation was that not all MDA-MB-231LG were coupled via gap junctions, and only a portion of the cells exhibited spread. However, the results described in this report regarding C×43 and GJIC are noteworthy because they developed as a consequence of the metabolic response to growth in glucose-limiting conditions without the influence of exogenous connexin expression or the use of pathway inhibitors or activators. Since this is a metabolic circumstance frequently overcome by cancer cells during tumor growth, it provides insight to a potential reprogramming response involving connexins and GJIC that may affect their ability to survive and adapt. Because the connexin family consists of 21 genes, some of which have been shown to be upregulated in different types of cancers and in response to different stimuli, we cannot exclude the influence of other connexins in the MDA-MB-231LG.

Because cancer cell invasion is a key initial feature of the metastatic process, and because GJIC has been shown to affect this process, we examined if these collective changes affected this aggressive quality. We used Matrigel to evaluate multiple three-dimensional in vitro growth models to characterize cellular invasion. Despite greater membrane contact, increased connexin expression and higher GJIC, all features more similar to normal epithelial cells, the MDA-MB-231LG demonstrated significantly higher invasive qualities in both the presence and absence of glucose, indicating intrinsic qualities in these cells. Previous studies which directly modulated expression of C×43 in the MDA-MB-231 cell line have shown reduction in proliferation, invasive characteristics in three-dimensional culture and an increased state of differentiation in response to C×43 expression while an inverse relationship was observed following reduction of C×43 (Qin et al. 2002; Shao et al. 2005; McLachlan et al. 2006; Talhouk et al. 2013). Some of these effects were attributed to gap junction independent functions of C×43. Other studies using this cell line have demonstrated both suppression and facilitation of aggressive and metastatic qualities in response to C×43 expression (Li et al. 2008; Fu et al. 2015; Lin et al. 2016; Kazan et al. 2019). Because this study examined the effects on C×43 expression as a result of changes to glucose metabolism, our results are therefore set amongst the background of this metabolic adaptation. Collectively, these studies emphasize the complexities related to the role of C×43 in cancer cells that are dependent upon experimental approach and context, highlighting the intricacies related to the study of GJIC and cancer.

To examine if the increase in invasiveness and GJIC in the MDA-MB-231LG were connected, we evaluated the invasive qualities of these cells during the inhibition of GJIC. We utilized 18α-glycerrehetic acid (18αGA), a nonselective gap junction inhibitor which uncouples GJIC (Davidson et al. 1986; Salameh and Dhein 2005). The reduced invasive capacity of these cells in the presence of 18αGA indicated a potential link between these two attributes. The fact that not all MDA-MB-231LG exhibited GJIC must be considered when examining the change in invasiveness during 18αGA treatment. The effects on invasion under these conditions may have been more prominent if the population was more uniformly coupled by GJIC. Thus, careful delineation of these potential relationships will be of importance to our understanding of connexin function in response to metabolic regulation. The results of this study shed light on phenotypic changes that occur in response to nutrient withdrawal and further support a role for cellular metabolism on the regulation of connexins and gap junctional activity in this context.

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