Utilizing CRISPR/Cas9 to Investigate Connexin43 Under Hypoxic Stress

Abstract

Gap junctions, composed of connexin proteins, facilitate intercellular communication. Altered expression and localization of connexin43 (Cx43) in response to cellular stressors results in disrupted intercellular communication and has been implicated to play a role in disease. GJA1, the gene encoding Cx43, undergoes internal translation to produce several small protein isoforms. The GJA1-20k isoform has been speculated to promote Cx43 trafficking to the cell border, and reduction of GJA1-20k expression correlates with reduced gap junction formation during hypoxic stress. The objective was to utilize CRISPR/Cas9 to generate cells expressing connexin43-green fluorescent protein (Cx43-GFP) under control of the endogenous *GJA1* promoter to visualize Cx43 under hypoxic stress, and to compare with traditional immunofluorescence detection of Cx43. Immunofluorescence labeling was performed to identify changes in Cx43 localization in mammary epithelial cells expressing lacZ (NMuMG lacZ), as a control, and GJA1-20k (NMuMG 20k), and in lung epithelial tumor A549 cells. To visualize Cx43 in living cells, guide RNAs (gRNA) were designed to target Cas9 nuclease to the 3' end of the coding region of the *GJA1* gene. Plasmids encoding the CRISPR/Cas9 enzyme, gRNAs, and a donor GFP-puromycin sequence were transfected into A549 cells. Flow cytometry and puromycin were used to screen for cells incorporating the GFP-puromycin DNA under control of the *GJA1* promoter. Quantitative analysis of Cx43 following 0, 24, and 48 hours of hypoxic stress revealed increased accumulation of Cx43 at the cell border. These results suggest expression of GJA1-20k promotes trafficking of Cx43 and increases gap junction formation.

Introduction

The primary means by which cells communicate directly is via gap junctions comprised of connexin proteins. Of the 21 known human connexins, connexin43 (Cx43) is the most ubiquitously expressed, and dysregulation of Cx43 has been implicated to play a role in ailments including heart disease and cancer. Despite decades of research, significant gaps in our knowledge exist regarding cellular regulation of Cx43 during stress, and no therapeutics exist that are capable of restoring normal gap junction function. Cx43 has a surprisingly short half-life of less than 3 hours in the cell, meaning that any change in how efficiently de novo gap junction channels are transported to the cell surface has rapid consequences on cellular communication (Smyth, Shaw, 2012). GJA1, the gene encoding Cx43, undergoes internal translation to produce several small protein isoforms. The GJA1-20k isoform has been speculated to promote Cx43 trafficking to the cell border, as reduction of GJA1-20k expression correlates with reduced gap junction formation during hypoxic stress. This conjecture led to the design of this research – to determine how the presence of forced GJA1-20K expression alters the localization of connexin43 proteins and gap junction formation at the cell border. Cloning of the green fluorescent protein (GFP) revolutionized the field of intracellular protein trafficking as it enables microscopic visualization of protein localization and movement in real time. This gene has been isolated such that it may be placed within a living cell as an in vivo protein attached to a protein of interest (Goodsell, 2013). Generation of Cx43-GFP fusion proteins has revealed valuable details regarding Cx43 transport to and from the plasma membrane and the effects of stressors such as oxidative stress on this process. These fusion proteins can be transferred and become operational gap junctions (Laird, et al., 2001) in the Cx43-GFP. A major caveat to this

approach, however, is that the DNA encoding the Cx43-GFP fusion protein is introduced ectopically with expression driven by a constitutive promoter such as CMV, a viral promoter, and generates super-physiological protein levels, which can lead to aggregation and potential artifacts. In addition, liposomes or viral vectors must be used to introduce the DNA to the cell's nucleus which in itself can be toxic and generate off-target effects on the cell. Recently, CRISPR/Cas9 technology has been adopted by the cell biology community in generation of genetically modified cell lines. Using CRISPR/Cas9, the genome of the cell can be edited very specifically to introduce mutations, deletions, or entire regions of exogenous DNA. This is a unique tool in which every crRNA, a CRISPR specific RNA, consists of a nucleotide repeat and a spacer portion. Cas9 then binds to a crRNA and tracrRNA, or trans-activating crRNA, which are able to locate and lead it to the space in which the cut will be made on each side of the indicated DNA sequence within the cell (Vidyasagar, 2017). Importantly, edited genes remain under control of their natural promotor, ensuring protein levels remain physiological, whereas before CRISPR, GFP-tagged Cx43 was exclusively introduced on exogenous DNA, which results in super-physiological expression levels. It is also possible to place coding sequences under temporal regulation using inducible promotors in cases where edited genetic information may be toxic in the long term.

The objective was to utilize CRISPR/Cas9 to generate cells expressing connexin43-green fluorescent protein (Cx43-GFP) under control of the endogenous *GJA1* promoter to visualize Cx43 under hypoxic stress and to compare these results with traditional immunofluorescence detection of Cx43. This was performed by immunofluorescent labeling of NMuMG cells which either expressed lacZ as the negative control or GJA1-20K. It was hypothesized that under

hypoxic conditions, the presence of GJA1-20k will maintain Cx43 trafficking and gap junction formation.

Methods and Materials

Vector engineering was performed to create two plasmids: the CRIS-PITCh donor vector and the All-in-one CRISPR/Cas9 vector. *GJA1* microhomology arms were added to the pCRIS-PITChV2-FBL plasmid to create the donor vector. The CRISPR/Cas9 vector was created by means of Golden Gate Assembly, where the PITCh gRNA was excised into the plasmid, such that the vector contained the PITCh gRNA, *GJA1* gRNA, the Cas9 nuclease, and the ampicillin resistance gene. These vectors were transfected into A549 cells and then screening for cells with the incorporated plasmids and GFP was completed. This was done by means of flow cytometry and puromycin selection. Transfected cells were trypsinized and resuspended in sorting buffer. Negative control non-transfected cells were sorted to obtain background fluorescence, and cells with fluoresence greater than background were sorted into a 96 well plate for recovery.

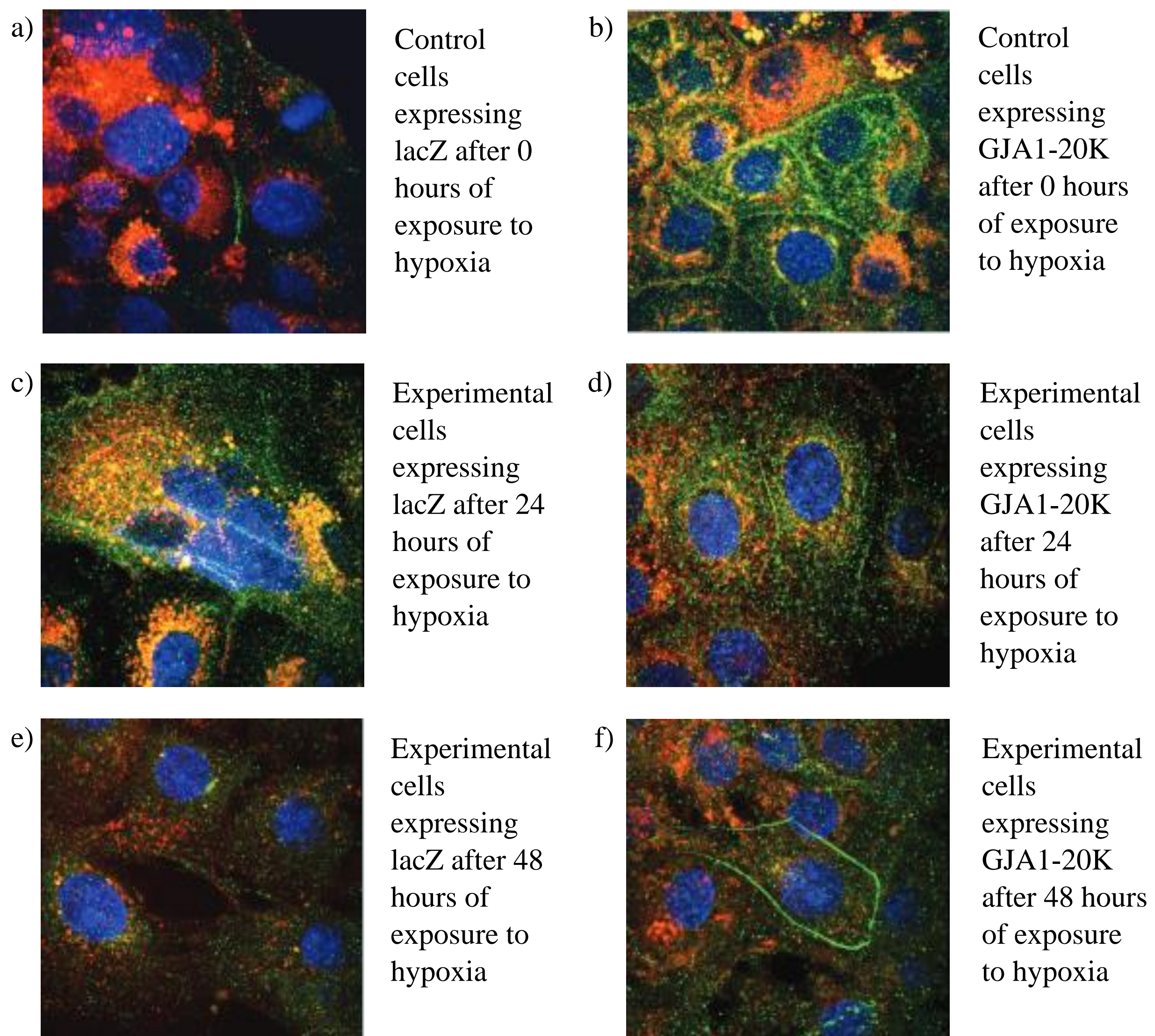
NMuMG cells were plated on glass bottom dishes with two plates of each cell type – one expressing lacZ and the other expressing GJA1-20K. These plates were then exposed to oxidative stress, one of each type of plate in the normoxic (18% oxygen) and hypoxic (1% oxygen) environments for 48 hours. The cells were then washed in PBS and fixed in methanol. Immunofluorescent labeling was then performed. Cells were blocked in 0.1% triton x-100, 5.0% normal goat syrum, and 1x PBS for one hour. Primary antibody dilutions were done in the blocking buffer using 1:2000 mouse Cx43 and 1:500 WGA 647. Cells were then washed and secondary antibody dilutions were done in the blocking buffer using specific antibodies anti rabbit-488, anti mouse-A647, and dapi was added. Z-scan images were then captured on a confocal microscope and analyzed using ImageJ software. Cell-cell borders from different

images were analyzed for each condition and time period and statistics were calculated in graph pad software.

Results

Fixed cell immunofluorescent labeling: confocal immunofluorescence z projection images of NMuMG cells following 0, 24, and 48 hours of exposure to hypoxic environments were taken with Cx43 specific antibody labeling (demonstrated in green), WGA membrane staining (demonstrated in red), and nuclei staining with dapi (demonstrated in blue).

Figure 1



a) Control cells expressing lacZ after 0 hours of exposure to hypoxia

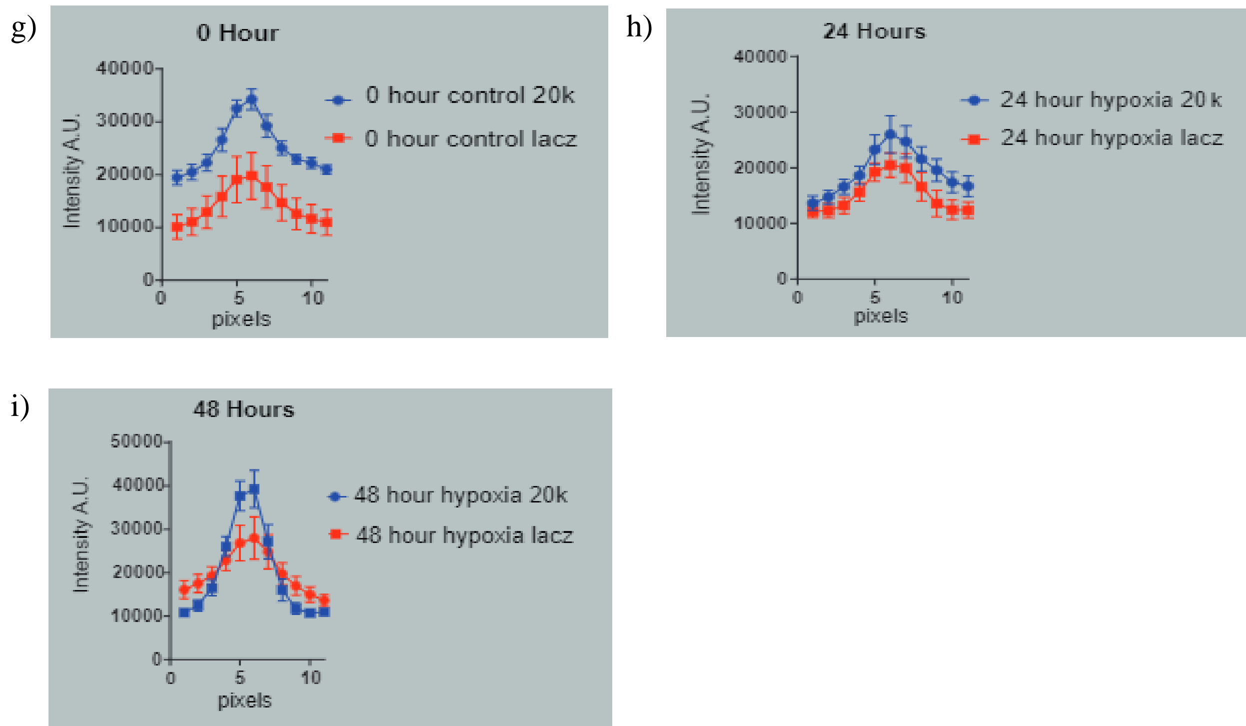
b) Control cells expressing GJA1-20K after 0 hours of exposure to hypoxia

c) Experimental cells expressing lacZ after 24 hours of exposure to hypoxia

d) Experimental cells expressing GJA1-20K after 24 hours of exposure to hypoxia

e) Experimental cells expressing lacZ after 48 hours of exposure to hypoxia

f) Experimental cells expressing GJA1-20K after 48 hours of exposure to hypoxia



Images captured at all time points indicated an increase localization of the connexin43 protein at the cell border. Following zero hours of hypoxic exposure (Figure 1a, 1b), there were higher levels of membrane localization as well as baseline expression of connexin43, demonstrated by quantitative analysis by imageJ (Figure 1g). Following 24 hours of hypoxic exposure (Figure 1c, 1d), the experimental cells expressing GJA1-20K demonstrated increased levels of localization at the cell border (Figure 1h). Following 48 hours of hypoxic exposure (Figure 1e, 1f), the experimental cells stably expressing lacZ showed higher levels of baseline expression. However, the cells expressing GJA1-20K maintained connexin43 localization at the border.

Discussion and Conclusions

Quantitative analysis of Cx43 following 0, 24, and 48 hours of hypoxic stress revealed increased accumulation of Cx43 at the cell border. These results suggest expression of GJA1-20k

promotes trafficking of Cx43 and increases gap junction formation. The hypothesis was accepted, as image analysis demonstrated a greater quantity of Cx43 detectable in cells expressing the GJA1-20k isoform as well as an increase in protein localization at the border in comparison to the lacZ cells. The results were consistent with the findings of (Fu, Y. et. al, 2017) such that overexpression of GJA1-20k assists in the protein's localization to the cell border when placed under hypoxic stress. Aligning with the results were the findings of (Basheer, W. et. al, 2017), which concluded the GJA1-20K isoform upholds localization in ischemic conditions. The experiment was largely successful, both in the construction of specific plasmids using the CRISPR/Cas9 system and the discovery of the role of GJA1-20K in Cx43 trafficking. A novel procedure, the cells containing the CRISPR vectors are showing promising results, with many growing and colonizing at the lab. The procedure for cell transfection could be improved by determining ideal conditions for quantity of puromycin and duration to achieve optimal results. Amount of cells available for observation following cell sorting may be increased by immediately adding media to the cells to prevent them from drying out. In the future, the goal would be to utilize CRISPR to visualize the trafficking of the proteins in real time, such that antibody labeling would no longer be necessary. This would minimize error by immunofluorescense labeling and give a highly accurate depiction of the movement of the proteins from expression to membrane localization.

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I certify that the use of recombinant DNA in the experimentation and observations covered by this paper was in accordance with the revised NIH Guidelines for Research Involving Recombinant DNA Molecules and the rules contained in the latest edition of the VJAS Handbook.