EFFECTS OF DE NOVO PROTEIN SYNTHESIS ON CELL SPREADING,

FORMATION OF CELL-CELL CONTACTS, AND CO-LOCALIZATION OF

PROTEINS AT CELL CONTACTS IN IAR-2 CELLS

by

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ABSTRACT OF MASTER THESIS

EFFECTS OF DE NOVO PROTEIN SYNTHESIS ON CELL SPREADING,

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Master Thesis Director:

Alexis J. Rodriguez, Ph. D.

Rat epithelial cells were treated with translational inhibitors in order to study their effect on cell spreading, the cell's ability to form lasting cell-cell contacts, and protein co-localization at cell contacts. When cells were treated with translation inhibitors they were found to have similar cell lengths as wild-type, untreated cells up to the 3 hour time point. After 3 hours the translational-inhibitor-treated cells were found to decrease in size while the untreated cells continued to grow. The co-localization of E-cadherin and actin were studied at cell contacts between two cells and it was found in inhibitor-treated cells the co-localization of the two proteins actually increased compared to wild-type cells. Lastly, a 30-fold increase in the ability to make lasting cell contacts was found in wild-type, untreated cells compared to inhibitor treated cells. Altogether these data demonstrate that *de novo* protein synthesis is needed in order for cells to continue to properly grow and spread, for proteins to be synthesized and co-localize properly at cell-cell contacts, and for cells to make lasting, non-random, cell contacts.

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Introduction

mRNA localization is important for creating asymmetry within the Metazoan cell as can be seen in growth cones of neurons (1), oocyte development (2), and developing *Drosophila melanogaster* (3). For proper β-actin zipcode mRNA localization and synthesis, specific signaling pathways are required. Upon activation of RhoA, cells have been shown to exhibit an increase in peripheral β-actin mRNA (4) and in order for mRNA translation to take place Src is needed in order to release the translational block (5). Thus areas with high RhoA and Src activity should be where zipcode containing mRNAs are locally synthesized (6). Cells missing the zipcode β-actin mRNA sequence exhibited β-actin mRNA which was most prevalent in the perinuclear region with decreasing fluorescence moving outwards from the nucleus (7).

In high animals, cell-cell adhesions play a very important role in tissue morphogenesis and are necessary for proper development of the cell (8). Adherens junctions progress through the stages of initiation, expansion, and maturation. During initiation, when two cells first make contact through the protrusions of their lamellipodia, E-cadherin begins to accumulate at this site of contact. As the length of the contact site expands, E-cadherin migrates along the contact site to accumulate at the periphery of the cell contact (8). When cells were treated with low Ca^{2+} medium and then followed by regular medium, areas with E-cadherin adhesion were found to co-localize with active c-Src (9). Furthermore, when treated with an E-cadherin blocking antibody cells were found to have decreased levels of active c-Src (9) thus E-cadherin adhesion activates c-Src signaling. RhoA has been found to drive the latter stages of expansion and maturation as it is present only during these stages and at the most distal leading edge of the contact site

and along the actual contact site itself (8). Therefore, epithelial contact leads to the up-regulation of Src and RhoA.

Cells adhere to their substrates through complexes known as focal adhesions (10). Their adherence to the substrate, be it an inert one (such as glass) or a biological substrate, is necessary for the cell to make contact with the extracellular matrix during cell spreading (11, 10). Focal adhesions are important in many cell processes, including tumor cell metastasis, where the cells are able to detach from the substrate and migrate as can be seen in the more aggressive cancers (10, 12). Upon RhoA activation, focal adhesions are formed, indicating the role of this protein in the process (13).

During cell spreading, focal adhesions form, and the cells progress through stages of development which involve circular patches at the cell periphery called spreading initiation centers (SICs) (10). In the first stage of cell spreading, SICs are found profusely along the cell periphery but as the cell proceeds to the second stage they are less abundant until the third and final stage, when they are no longer found, and the cell has formed mature focal adhesions. Furthermore, ribosomal RNA was found at these SICs, indicating protein translation is occurring there. To determine the effect of the formation of these SICs on cell spreading, the global translation inhibitors cycloheximide and puromycin were used in order to stop the formation of SICs and study the effect of SIC eliminitation on the cell's growth and spreading. Two different inhibitors were used because they both inhibit translation differently. Cycloheximide is considered a "leaky" translational

inhibitor as it incompletely stops translation by slowing down elongation, whereas puromycin stops translation completely by preventing initiation from taking place.

In the epithelial-to-mesenchymal transition (EMT), cells lose their adherens junctions, contributing to their metastatic property. The ability of cells to make contacts that can potentially become adherens junctions is essential to proper cell development and functional maintenance (14). Observation of cells *in vivo* has allowed us to study cells in their natural state, taking into consideration the cells' intrinsic ability to form cell contacts. Different conditions were used to determine their effect on the cells' ability to make a contact, namely substrates coated with Poly-D-lysine, cells lifted using cell dissociation solution, cells incubated in low Ca^{2+} medium, and treatment with cycloheximide. Cells must first attach themselves to their substrate before spreading and migrating (10). To study the effects of the substrate on a cell's ability to migrate and make cell-cell contacts, regular glass substrates versus glass coated with Poly-D-lysine were used. Poly-D-lysine is a cell adhesion molecule that enhances the attachment of cells to its surface due to its positively charged nature and the negative charge of the cell membranes (15). Cells attached more efficiently to their substrate can subsequently begin to spread and form cell contacts. Trypsin is a digestive enzyme that is used to remove cells from their culture substrate by cleaving the integrins attaching the cell to the substrate (16). Cell Dissociation Solution (CDS) was an alternative way of displacing cells from their substrate without cleaving their integrin receptors, thus leaving them intact and able to re-attach to their newly seeded substrate at a faster pace. Calcium is needed in adherens junctions, and its removal subsequently leads to the rapid dissociation

of proteins necessary for an adherens junction. The effect of Ca^{2+} on the formation of cell-cell contacts was further studied with the use of low Ca^{2+} medium. Treatment with cycloheximide enabled us to determine what effect *de novo* protein synthesis has on a cell's ability to form necessary cell-cell contacts. We hypothesized that treatment with translational inhibitors prevents cells from spreading, thus decreasing the co-localization of E-cadherin and actin at cell-cell contacts, and preventing the formation of a cell-cell contact *in vivo*.

Methods

Cell Culture

IAR-2, a strain of rat liver epithelial cells, were grown on 100 X 20 mm diameter dishes using Alpha-MEM medium containing 1% penicillin-streptomycin and 10% fetal bovine serum (FBS). Once the cells were ~80% confluent, the medium was removed and the plate was washed with 2 mL of 0.25% Trypsin-EDTA, pH 8. On the third wash the trypsin was left in the plate and incubation at room temperature was continued for about 20 minutes. When the cells were fully lifted from the plate, 8 mL of Alpha-MEM medium (containing serum and antibiotics) was applied and the cells were transferred to a 15-mL Falcon tube. The cells were centrifuged for 8 minutes at 800 rpm. The supernatant was discarded and the cell pellet was re-suspended in 1 mL of Alpha-MEM medium containing serum and antibiotics. Ten μL of the re-suspended pellet was pipetted into a hemocytometer and cells counted separately from five sections and then averaged to get

the total number. The number of cells to be used was determined using the equation below:

Number of cells X 10,000 = number of cells/mL

$$\frac{\text{number of cells wanted to plate}}{\text{number of cells per mL}} = \text{mL per cover slip}$$

2 mL – mL per cover slip = mL of medium to add to each well.

Cells were then plated onto chromic-acid washed cover slips in 6-well plates (total volume 2mL) and incubated at 37°C with CO_2 for various time points.

Sparse Plating Assay – Fixed Cell Imaging and Indirect Immunofluorescence Staining

Fixed Cell Imaging:

Cells were immersed in regular Alpha-MEM medium or Alpha-MEM containing 1µL/mL of cycloheximide or puromycin to test the dependence of translation on cell spreading. A total of 100,000 cells were seeded onto cover slips and incubated for 1, 2, 3, 4, 5, and 24h. After fixing for 30 minutes in 4% Paraformaldehyde in (1X) PBS, pH 7.4, cells were washed with (1X) PBS, pH 7.4, 3 times for 5 minutes and then mounted onto slides using ProLong Gold Antifade Reagent (Invitrogen). The cover slips were imaged, looking specifically for single cells and were measured lengthwise using AxioVision.

Indirect Immunofluorescence Staining:

Cells used in immunofluorescence staining were fixed, washed, and permeabilized with 0.5% TritonX for 1 minute. The samples were washed 3 times for 5 minutes with (1X) PBS pH 7.4 and blocked with 1% blocking solution ((1X) BSA, (1X) PBS pH 7.4, and diH$_2$O) for 1 hour. Using a humidified chamber (150 x 25 mm plate with a Parafilm-

coated bottom), cover-slips containing cells were flipped onto 20 μL of primary monoclonal adherens junction antibodies, anti-E-cadherin (1:250; BD) and incubated overnight at 4°C. The following day, cover slips were washed 3 times for 5 minutes in (1X) PBS. Cover slips were flipped onto 20 μL of goat anti-mouse Cy3 secondary antibody (1:1000, Invitrogen) for 1.5 hours at room temperature in the dark. Cover slips were washed in the dark 3 times for 5 minutes with 1x PBS pH 7.4. The cells were then stained with Alexa488 phalloidin (Invitrogen) for 20 minutes in the dark at room temperature, followed by washs with (1X) PBS pH 7.4, 3 times, for 5 minutes each. The cover slips were then mounted onto slides using ProLong Gold Antifade Reagent (Invitrogen).

Sparse Plating Assay – Live Cell Imaging

IAR-2 cells were cultured in 150 X 25 mm diameter dishes using Alpha-MEM medium containing 1% penicillin-streptomycin and 10% fetal bovine serum (FBS) (Alpha-MEM). When cells were confluent they were lifted off the dish using either 0.25% Trypsin EDTA, pH 8, or (1X) Cell Dissociation Solution Non-Enzymatic (Sigma) for about 20 minutes. The cells were spun down and the pellet was re-suspended in Alpha-MEM. Five hundred thousand cells, either in regular medium or low Ca^{2+} medium (defined as Alpha-MEM without any serum or antibiotics, added with 2 mM EGTA) were then plated directly into 35-mm Mat-Tek dishes that were either uncoated or coated with Poly-D-lysine. The cells were allowed to settle to the bottom of the dish before commencing time lapse imaging. The time lapse movies were made at a rate of one image per minute using DIC optics with a 63X oil immersion objective, for 12 consecutive hours. Cells

were immersed in regular Alpha-MEM medium or Alpha-MEM containing 1μL/mL of cycloheximide to test the dependence of translation on cell contact.

Image Processing

Images were taken on an inverted Zeiss microscope using Axiovision 4.8.2 software (Zeiss). Using a 63x oil immersion objective, 30 slices were taken for each slide with a step size of 0.24 μm. Binning was set to 2 x 2 and the digital gain was 2. Deconvolution was iterative for all images and arrangements were done using Adobe Photoshop CS5 and ImageJ (NIH).

Pearson's Correlation

De-convolved Z-stacks were imported into Volocity 6.0 to calculate the Pearson's correlation values for cells (co-localization of adheren junction complex proteins and actin) at the various time-points of the calcium switch. Free-hand drawn regions on the junction or the cytoplasm of the cells were used to collected the Pearson's correlation values for each region. Data calculations and graphs were made using Microsoft Excel.

Results

Continous Protein Translation is Needed for Cell Spreading

IAR-2 cells were grown in Alpha-MEM medium in a petri-dish and when they were confluent were trypsinized and then spun down. The cell pellet was then re-suspended and cells were counted using a hemocytometer. One hundred thousand cells were seeded onto cover slips in six-well plates and incubated for 30 min, 1, 2, 3, 4, 5, and 24h. Cells were either untreated or treated with cycloheximide or puromycin (1 μL/1mL). These cells were then fixed and mounted with ProLong Gold antifade reagent and left overnight.

The first time point (30 min) showed very few cells and was eliminated from further experimentation. This was expected as trypsin cleaves integrins, which takes about 30 minutes to reassemble (16). Some time is necessary for cells to properly settle down and form focal adhesions to the substrate before they can begin to spread and migrate (10). The results from the sparse plating assay indicate that this period is about 3 hours, as the untreated cells and those that have been treated by cycloheximide or puromycin have relatively the same length up to this point (Table 1, Figures 1A, 1B).

Table 1. Sparse Plating Assay measuring cell spreading in wild type and cycloheximide and puromycin treated cells. One hundred thousand untreated cells or treated with cycloheximide or puromycin and seeded onto cover slips and were incubated for various time points. Cell length was then quantified using AxioVision and the averages for the various incubations and treatments were obtained. In each trial ten cells were measured and shown is the average. *No cells were found for this period in this trial.

Trial 1			
Time (h)	Control (µm)	Cycloheximide (µm)	Puromycin (µm)
1	10.968	10.3	8.579
2	10.214	7.96	13.16
3	10.695	9.892	12.835
4	10.902	8.67	7.46
5	11.871	8.517	7.615
24	25.757	7.489	7.838
Trial 2			
Time (h)	Control (µm)	Cycloheximide (µm)	Puromycin (µm)
1	8.115	8.413	12.239
2	11.009	7.821	11.007
3	9.1945	9.995	16.7566
4	10.377	6.232	7.238
5	11.6652	6.977	6.447
24	24.561	6.356	7.238
Trial 3			
Time (h)	Control (µm)	Cycloheximide (µm)	Puromycin (µm)
1	25.157	15.873	21.687
2	20.929	19.777	14.374
3	10.498	13.354	9.263
4	25.295	13.877	7.592
5	10.305	11.306	7.116
24	23.827	14.469	6.92
Trial 4			
Time (h)	Control (µm)	Cycloheximide (µm)	Puromycin (µm)
1	10.823	13.784	11.823
2	12.431	12.039	13.1
3	14.547	13.98	14.01
4	14.281	16.224	12.396
5	10.734	10.02	11.007
24	18.561	14.23	9.695
Trial 5			
Time (h)	Control (µm)	Cycloheximide (µm)	Puromycin (µm)
1	17.056	18.482	16.309

Time (h)	Control (μm)	Cycloheximide (μm)	Puromycin (μm)
2	20.654	12.902	13.426
3	19.634	14.988	16.358
4	17.851	17.853	14.236
5	19.991	20.38	21.238
24	33.168	19.477	12.256
Trial 6			
Time (h)	Control (μm)	Cycloheximide (μm)	Puromycin (μm)
1	14.671	13.422	12.884
2	12.394	10.381	11.024
3	14.18	14.473	12.845
4	11.256	11.316	13.895
5	11.358	12.831	12.357
24	37.177	17.374	*
Trial 7			
Time (h)	Control (μm)	Cycloheximide (μm)	Puromycin (μm)
1	11.139	15.465	12.453
2	16.369	14.509	11.897
3	15.164	15.344	11.451
4	11.1636	10.823	11.955
5	10.0727	14.867	13.348
24	27.186	11.6845	6.552
Trial 8			
Time (h)	Control (μm)	Cycloheximide (μm)	Puromycin (μm)
1	11.937	10.754	10.236
2	10.973	11.003	10.937
3	10.044	11.754	10.264
4	10.398	10.436	11.903
5	14.001	7.639	9.729
24	21.595	7.825	6.758
Trial 9			
Time (h)	Control (μm)	Cycloheximide (μm)	Puromycin (μm)
1	12.583	13.125	12.293
2	11.537	11.347	10.221
3	11.532	9.435	10.398
4	11.603	8.513	11.645
5	10.948	9.689	8.113
24	23.462	7.766	7.162
Time (h)	Average Control (μm)	Average Cycloheximide (μm)	Average Puromycin (μm)
1	13.6054	13.291	13.167
2	14.057	11.971	12.127
3	12.832	12.579	12.687
4	13.681	11.549	10.924
5	12.327	11.358	10.774
24	26.144	11.852	8.052

Figure 1A. Sparse Plating Assay measuring cell spreading in wild type and cycloheximide and puromycin treated cells. Untreated cells or cells treated with cycloheximide or puromycin and seeded onto cover slips and were incubated for the various hours indicated in the images. (A) Cells were untreated (control). (B) Cells treated with cycloheximide. (C) Cells treated with puromycin. Cell length was quantified using AxioVision and the averages for the various incubations and treatments were obtained. Ten cells were measured in each trial.
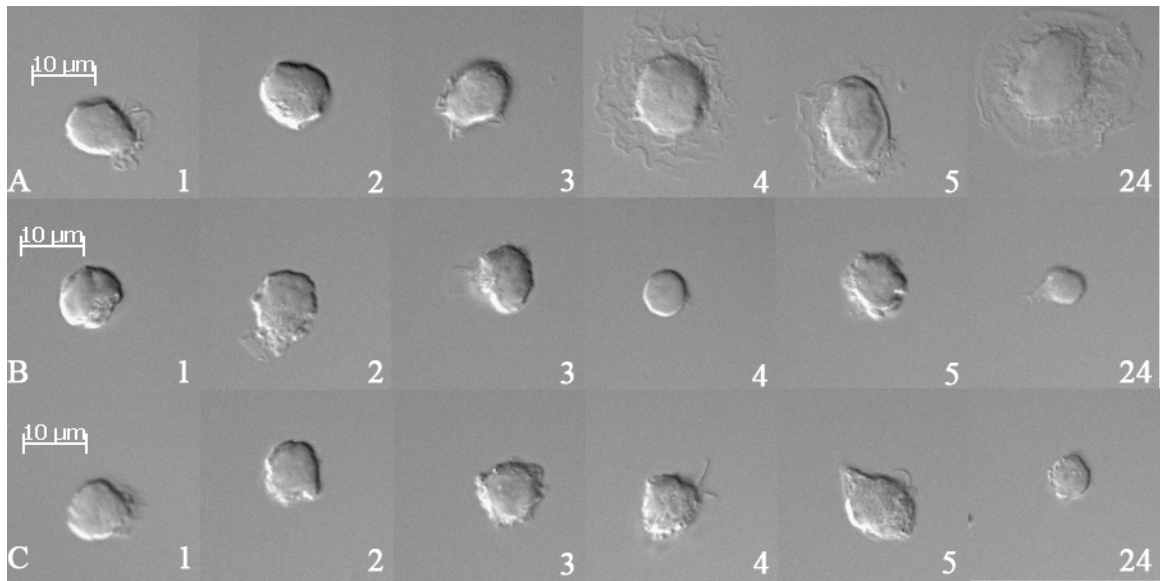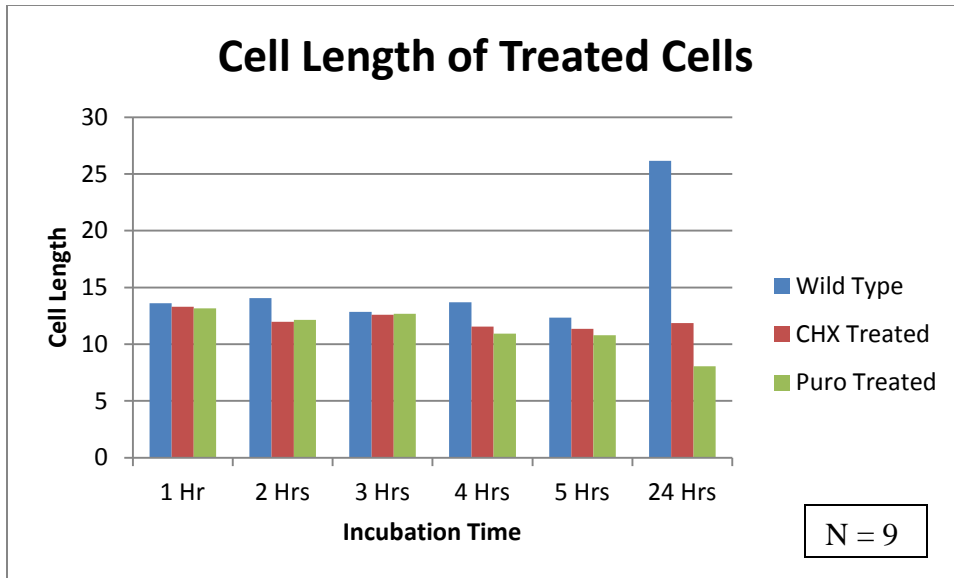
Figure 1B. Sparse Plating Assay measuring cell spreading in wild type and cycloheximide and puromycin treated cells. One hundred thousand untreated cells or treated with cycloheximide or puromycin and seeded onto cover slips and were incubated for various time points. Cell length was then quantified using AxioVision and the averages for the various incubations and treatments were obtained.

After 30 min, the untreated cells are able to start to spread while the translational-inhibitor-treated cells continue to stay relatively at the same size, but eventually round up and die (Table 1, Figures 1A, 1B). Spreading cells go through stages that involve the formation of SICs (10). Protein biosynthesis takes place at the SICs and by treating with translational inhibitors their formation is impeded, thus preventing the cell from being able to proceed through spreading. Similar results were obtained when this experiment was performed using human umbilical vein endothelial cells (HUVECs), where up to 3 hours there is no significant difference in cell spreading between untreated and cycloheximide-treated cells (16). The effect of cycloheximide and puromycin on cell spreading was also tested in malignant mesothelioma (MM) cells, resulting in the inhibition of cell spreading (17). Cumulatively, these results from the literature indicated that continuous protein translation is needed for cell spreading. From our data that shows up until the 3-hour time point, untreated cells and translational-inhibitor-treated cells have relatively the same cell lengths, I conclude that up until this point, the translational-inhibitor-treated cells require previously translated proteins in order to form SICs, which in turn allow them to attach to the substrate and subsequently spread. However, as indicated by other workers (17), continuous translation is needed in order for cells to continue to spread. Thus, after the 3 hour time point, new proteins are not being made and the cell loses the ability to spread, as seen in the inhibitor treated cells.

Protein Translation is Necessary for Lasting Cell-Cell Contacts

IAR-2 cells were grown with Alpha-MEM medium in a Petri dish and when they were confluent were trypsinized or lifted off the dish with cell dissociation solution and then spun down. The pellet was then re-suspended and cells were counted using a

hemocytometer. Batches of 500,000 cells were either untreated, treated with cycloheximide, or treated with low calcium medium, and plated directly onto a Mat-Tek dish, which was either uncoated or coated with Poly-D-Lysine. Cells were allowed to settle down to the bottom of the dish before the time lapse began. The time lapse consisted of one picture taken per minute in DIC for 12 consecutive hours. For cycloheximide treatment, 500,000 cells were plated directly into a Mat-Tek dish with 1 μL/1mL of medium. Cells lifted using CDS often came off attached as sheets and the chelating agent EGTA was used to break up the adherens junctions formed between cells. After the different time lapse videos were obtained (data not shown) they were analyzed. Cells were studied in time lapse videos in order to observe when two cells made contact and the time in which they stayed in contact was recorded (Tables 2A – 2F).

Table 2A. Contact time of wild type, untreated cells. 500,000 cells were plated directly onto a Mat-Tek dish and the length of two cells making contact was recorded. "+" signs indicate the cells were making contact up until the end of the video.

122210 Control	011911 Control	020711 Control
1	3	102
1	6	1
14	463+	83
1	369+	1
3		2
1		1
4		583+
1		2
628		1
8		1
		1
		1
		3
		4

Table 2B. Contact time of cycloheximide, treated cells. 500,000 cells treated with cycloheximide, 1 μL cycloheximide/1mL of media, were plated directly onto a Mat-Tek dish and the length of two cells making contact was recorded.

021011 Cycloheximide	021511 Cycloheximide	022511 Cycloheximide
12	1	4
2	1	2
12	1	3
1	1	2
4	1	8
6	3	1
6	1	4
5	1	3
2	8	1
	1	1
	5	1
	1	1
	1	1
	1	1
	2	1
	12	2
	1	2
	7	3
		17
		3
		2
		6
		2
		4
		1
		1
		1
		1
		4
		3
		4
		5
		2
		4

Table 2C. Contact time of wild type, untreated cells on Poly-D Lysine coated cover slips. 500,000 cells were plated directly onto a Mat-Tek dish that was coated with Poly-D Lysine and the length of two cells making contact was recorded. "+" signs indicate the cells were making contact up until the end of the video.

031011 Poly-D Control	032211 Poly-D Control	072111 Poly-D Control
2	1	5
4	12	11
4	5	4
61	3	1
10	1	7
7	3	4
3	2	5
2	2	149
3	1	5
2	1	2
1	1	643+
3	3	31
1	11	11
6	2	11
8	2	7
9	1	1
1	1	1
8	22	2
20	7	3
2	3	4
33	3	12
18	1	1
2	1	2
1	10	1
5	1	3
1	4	1
1	5	2
49+	1	9
	1	9
	1	3
	6	
	2	3
	3	1
	6	5
	4	2
	1	9
	2	10
	7	2
	464+	8
	68	279

		97		10
		8	296+	
		22	5+	
		1		
		119+		
		6		
		281+		
		2		
		1		
031011 Poly-D Control		032211 Poly-D Control		072111 Poly-D Control

Table 2D. Contact time of cycloheximide, treated cells on Poly-D Lysine coated cover slips. 500,000 cells treated with cycloheximide, 1 μL cycloheximide/1mL of media, were plated directly onto a Mat-Tek dish that was coated with Poly-D Lysine and the length of two cells making contact was recorded.

033011 Poly-D Cycloheximide	040311 Poly-D Cycloheximide	072711 Poly-D Cycloheximide
14	1	1
9	5	7
1	1	3
3	2	3
15	1	4
1	2	1
2	3	1
1	2	1
1	3	3
7	4	1
2	5	1
2		1
1		1
2		14
1		1
1		1
1		5
7		7
2		12
4		1
5		1
4		10
2		
13		
22		
1		
6		
3		
7		
5		
9		
3		
15		
11		
1		
1		

7		
16		
4		
5		
25		
1		
5		
11		
1		
1		
2		
2		
1		
3		
7		
5		
7		
1		
18		
2		
3		
3		
3		

Table 2E. Contact time of wild type, untreated cells treated with cell dissociation solution. 500,000 cells were lifted off the petri-dish with cell dissociation solution (CDS) and plated directly onto a Mat-Tek dish and the length of two cells making contact was recorded. "+" indicates the cells were making contact up until the end of the video.

091911 Poly-D Control CDS	092811 Poly-D Control CDS	101411 Poly-D Control CDS
1	1	1
1	1	4
7	206	5
7	29	2
2	1	449+
1	2	2
2	1	5
3	1	6
1	1	3
6	1	113+
2	2	
9	5	
1	65	
2	9	
5	13	
1	1	
315+	3	
2	34	
2	347	
1	2	
1	2	
9	1	
1	1	
4	2	
1	3	
3	7	
2	2	
7	5	
7	5	
1	8	
9	1	
1	12	
1	1	
1	1	
148+	5	
9	4	

21	1	
2	5	
5	2	
17	65	
3	19	
13	395	
2	52	
1	48	
1	474+	
1	2	
1	24	
2	9	
1	3	
19+	6	
2	1	
1	3	
	1	
	1	
	5	
	5	
	2	
	3	

Table 2F. Contact time of wild type, untreated cells treated with Low Calcium media. 500,000 cells were treated with low calcium media and plated directly onto a Mat-Tek dish that was uncoated, coated with Poly-D Lysine, and lifted off the petri-dish with cell dissociation solution (CDS) and then plated directly onto a Mat-Tek dish coated with Poly-D Lysine, respectively, and the length of two cells making contact was recorded. "+" indicates the cells were making contact up until the end of the video.

101811 Control Low Ca	102411 Control Low Ca Poly-D	102711 Control Low Ca Poly-D CDS
1	22	256+
1	6	2
1	2	7
1	15	8
1	2	235+
694	4	249+
1	3	5
1	1	8
1	1	14
2	1	14
1	1	
1	1	
4	55	
2	4	
1		
2		
2		
1		
3		
1		
1		
2		
2		
1		
2		
1		
1		
1		
2		
7		
1		
1		

Any two cells making contact for less than 20 minutes was considered a random event. Thus the percentage of cell contacts considered not random was calculated from time lapse video images. These results were totaled for either control cells or cycloheximide-treated cells and were averaged to determine the extent of not random cell contacts. For control cells it was determined that 18.1% of cells were making non-random contact while for cycloheximide-treated cells only 0.55% of cells made non-random contacts (Figure 2).

Our results from this assay indicate that, although untreated cells also make random contacts, the percentage of the cells that make non-random contacts is more than 30 times greater than that for the cycloheximide-treated (Figure 2, Table 2). The non-random contacts made by the untreated cells often lasted for a few hours, as opposed to the cycloheximide-treated cells, which rarely lasted over 20 minutes.

There are two phases to the formation of a cell-cell adhesion. The first phase is initiation, where contact between the two cells is first made. The second is expansion, where the contact area is enlarged (8). Cycloheximide as a global, nonspecific protein translational inhibitor, prevented the synthesis of a protein or proteins necessary for the cells to make lasting contacts. Otherwise, it appears as if two cells would come into contact and initiate the formation of a cell-cell adhesion, but are not able to continue into the expansion phase and detach from each other. Based on these results, I conclude that *de novo* protein synthesis is necessary in order for cells to make lasting contacts as cell-cell adhesions.

Furthermore, movement of cycloheximide-treated cells that make contact with another cell does not appear to slow down, when compared to wild type cells (Figure 2, Table 2). They appear to lose their contact inhibition of movement as can be seen by how fast they continue to move and put out lamellipodia in search of another cell, even after they have made contact with another cell. Untreated cells slow down after making contact, possibly because they will eventually settle down and spread (Figure 3). Many cycloheximide-treated cells appear to spin around in circles in place as they lose directionality of movement. Cell motility is dependent on the formation of focal adhesions. When focal adhesions are prevented from being formed, e.g. through treatment with cycloheximide, cells lose their ability to properly spread and move.
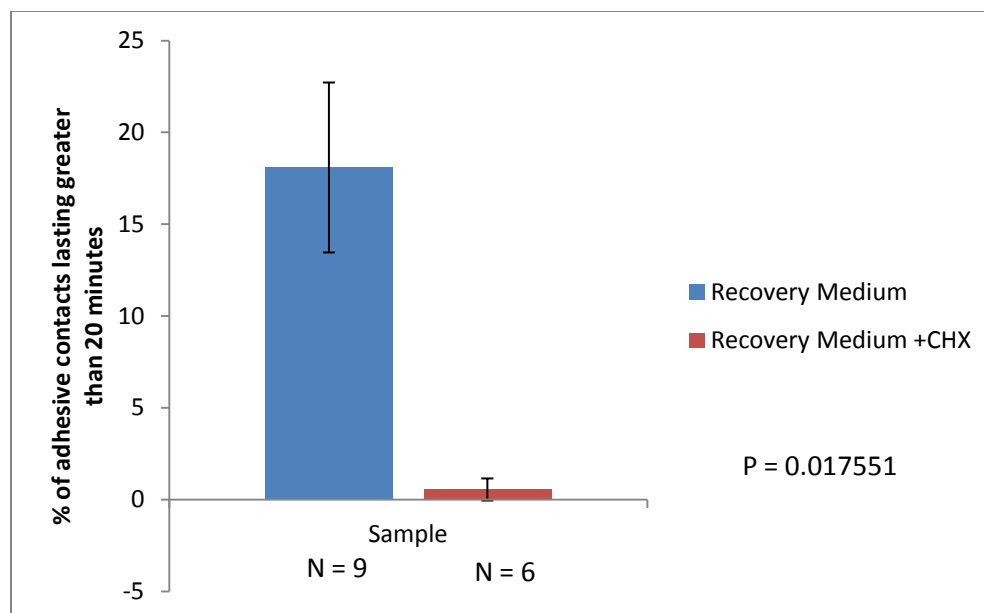
Figure 2. Percentage of adhesive contacts in wild type and cycloheximide-treated cells. 500,000 cells were plated directly onto a Mat-Tek dish and the length of two cells making contact was recorded. The percentage of non-random contacts, lasting for longer than 20 minutes, is shown with cycloheximide-treated cells showing a largely reduced ability to make a contact (P = 0.001). Error bars are ±SEM.

Translational Inhibition Prevents the Organized Recovery of Adherens Junction Assembly

IAR-2 cells were grown in Alpha-MEM in a Petri dish and when they were confluent were trypsinized and then spun down. The pellet was then re-suspended, cells were counted using a hemocytometer, and 100,000 cells were originally seeded. Because there were only a few cells making contact at the various time points, 250,000 cells were seeded onto cover slips in six-well plates and incubated for 1, 2, 3, and 4h. cells were untreated or treated with cycloheximide: 1µL/1mL of media (Figure 3, Supplementary Figure S1). These cells were then fixed with paraformaldehyde, treated with Triton X-100, put in block solution, and then stained for E-cadherin, actin, and nuclei with Alex Fluor 488, rhodamine, and DAPI respectively. The cover slips were then mounted with ProLong Gold antifade reagent and left overnight.
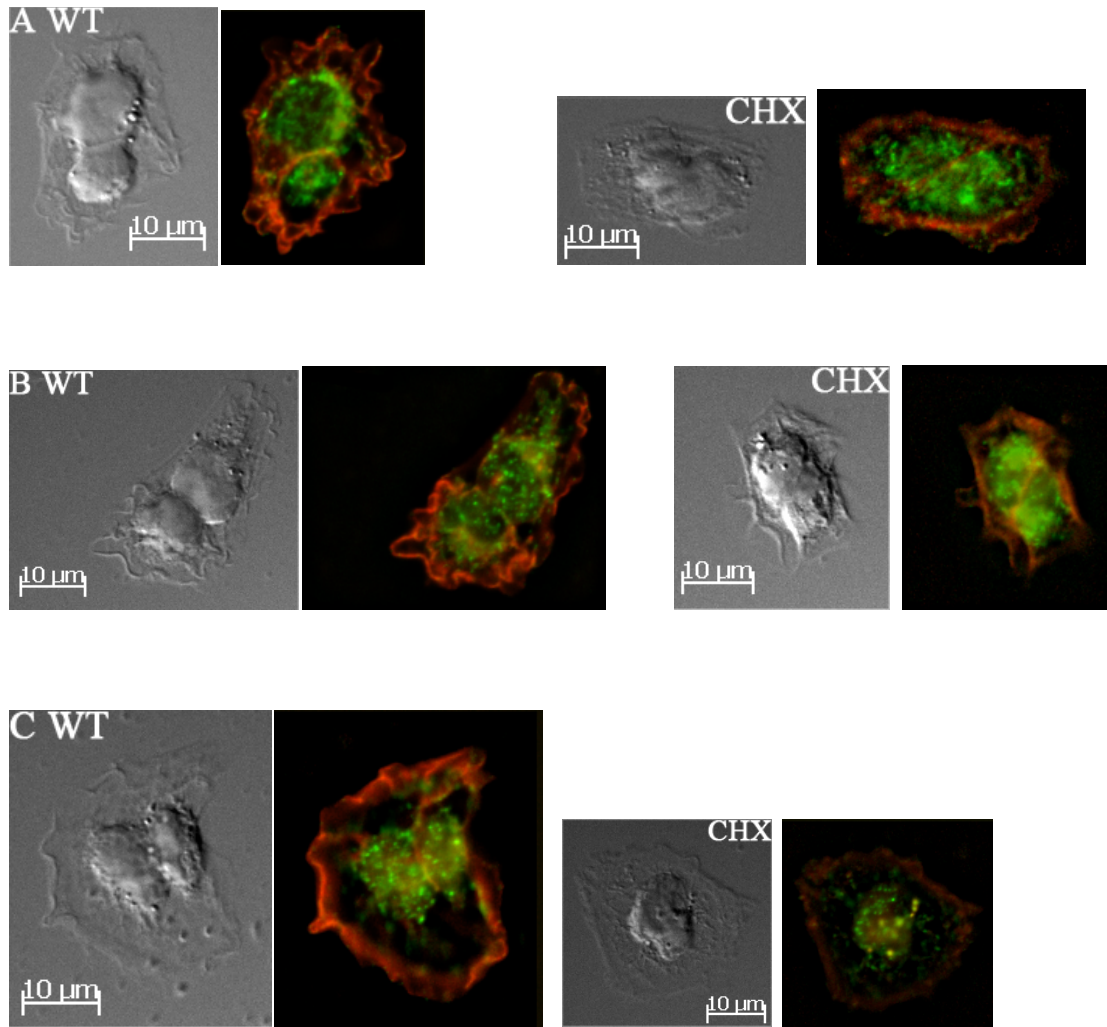
Figure 3A. Sparse Plating Assay for cells making contacts. 250,000 cells were used as control or were treated with cycloheximide and seeded onto cover slips and were incubated for various time points. Cells were stained for E-cadherin (green) and actin (red) and then imaged, looking for cells making contact. (A) Cells were incubated for 2h. (B) 3h. (C) 4h.

The Pearson's correlation for cycloheximide treated cells was expected to decrease with increased incubation time compared to untreated cells as protein translation is being inhibited and E-cadherin and actin are not synthesized and localize properly at the cell contacts (Table 3).

The Pearson's correlation, on the contrary, shows the opposite, with cycloheximide-treated cells actually increasing in the amount of E-cadherin and actin that are localized at the cell contacts (Figure 3, Supplementary Figure S1, Table 3). Figure 3 illustrates more clearly how control, untreated cells present a linearly increasing co-localization of E-cadherin and actin as time increases.

However, similar Pearson's correlation is observed for cycloheximide-treated cells with respect to untreated cells. Pearson's correlation values increase rapidly up to 3 hours, before somewhat stabilizing at 4 hours. This result may indicate that inhibiting protein translation prevents the organized assembly of adherens junctions. As can be seen from the differential interference contrast (DIC) images control cells display a clear region where two different cells make contact, as opposed to cycloheximide-treated cells, which seem to be on top of each other (11). The latter observation led me to conclude that cycloheximide interferes with a cell's ability to adhere to a substrate and its ability to exhibit proper motility. Cycloheximide treatment in cells model improperly induced epithelial to mesenchymal transition (EMT), a phenomenon displayed in some of the more aggressive cancers.

Table 3. Pearson's Correlation between E-cadherin and actin in untreated and cycloheximide-treated cells at cell contacts. 250,000 cells were used as wild type or treated with cycloheximide and seeded onto cover slips and were incubated for various time points. Pearson's correlation was then quantified for E-cadherin and actin at cell contact sites using the program Volocity.

|	2h Control	2h Cycloheximide	3h Control	3h Cycloheximide	4h Control	4h Cycloheximide
	0.743	0.826	0.675	0.942	0.806	0.261
	0.714	0.734	0.778	0.888	0.785	0.937
	0.7	0.858	0.11	0.926	0.598	0.943
	0.76	0.449	0.897	0.653	0.884	0.93
	0.69	0.271	0.754	0.817	0.76	0.845
	0.175	0.174	0.467	0.776	0.803	0.928
	0.435	0.79	0.743		0.668	0.799
	0.502	0.771	0.748		0.672	0.916
	0.554	0.389	0.44		0.54	
	0.281	0.605	0.7		0.715	
	0.705	0.327	0.574			
	0.581		0.567			
	0.623		0.705			
	0.498					
	0.453					
Average	0.561	0.563	0.628	0.834	0.723	0.820
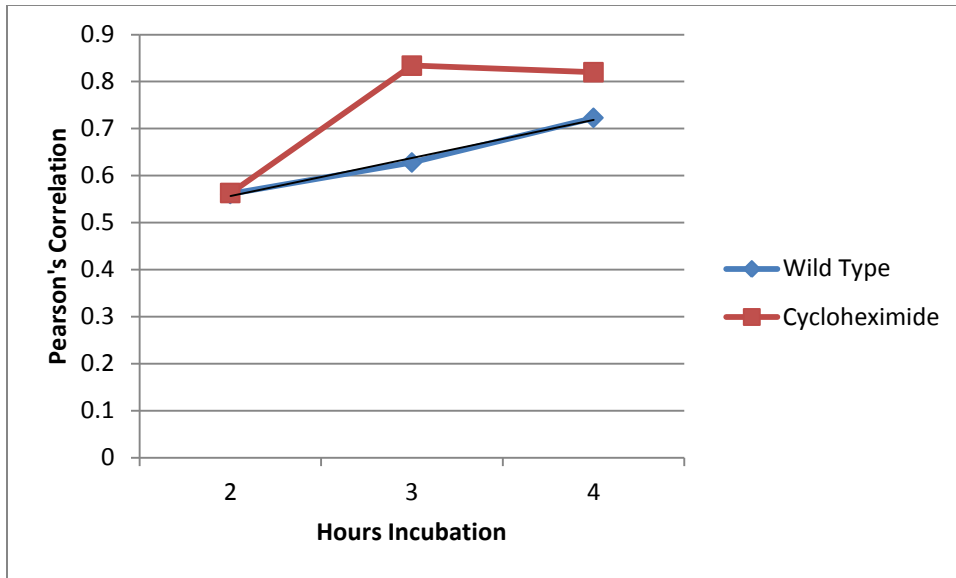
Figure 3B. Pearson's correlation between E-cadherin and actin in control vs. cycloheximide-treated cells at cell contacts. 250,000 cells were used as wild type or treated with cycloheximide and seeded onto cover slips and were incubated for various time points. They were stained for E-cadherin, actin, and DAPI and then imaged, looking for cells making contact. Pearson's correlation was then quantified for E-cadherin and actin at cell contact sites using Volocity.

Discussion

Proper cell growth involves cell attachment to a substrate before it is able to spread and grow (10). *De novo* protein synthesis is essential for a cell to begin to spread, as preventing translation does not allow for necessary proteins essential for the formation of focal adhesions to form. Experiments with IAR-2 cells showed treatment with cycloheximide prevented cells from spreading when compared to untreated cells which were able to spread. Studies using human umbilical vein endothelial cells have yield similar results indicating it takes about 3 hours for a cell to begin to produce proteins involved in spreading initiation centers and eventually mature focal adhesions (16, 10). Furthermore, experiments with MM cells indicated continual protein translation is needed for a cell to be able to spread (17). In the formation of cell-cell contacts there is a re-distribution of the proteins E-cadherin and actin, which co-localize at the cell peripheries (8). When cycloheximide is added to the medium the co-localization of these two proteins increases compared to wild-type, untreated cells. The differential interference contrast images show that the translation-inhibitor-treated cells lose contact inhibition of movement and start crawling on top of each other as there is no clear distinction of the two individuals cells compared to the control cells where there is an obvious invagination of where the two cells meet. This is further shown in live cell imaging, when cycloheximide treated cells lose the ability to recognize other cells to make contact, and constantly move through the field of view to find other cells. Untreated cells have an over 30-fold increase in its ability to make lasting contacts which further supports that protein translation is needed in order to synthesize proteins that can co-localize properly in order to make lasting cell contacts. *De novo* protein synthesis is necessary in order for a cell to

spread and form lasting cell-cell contacts while cycloheximide-treated-cells increase the co-localization of E-cadherin and actin at cell-cell contacts.

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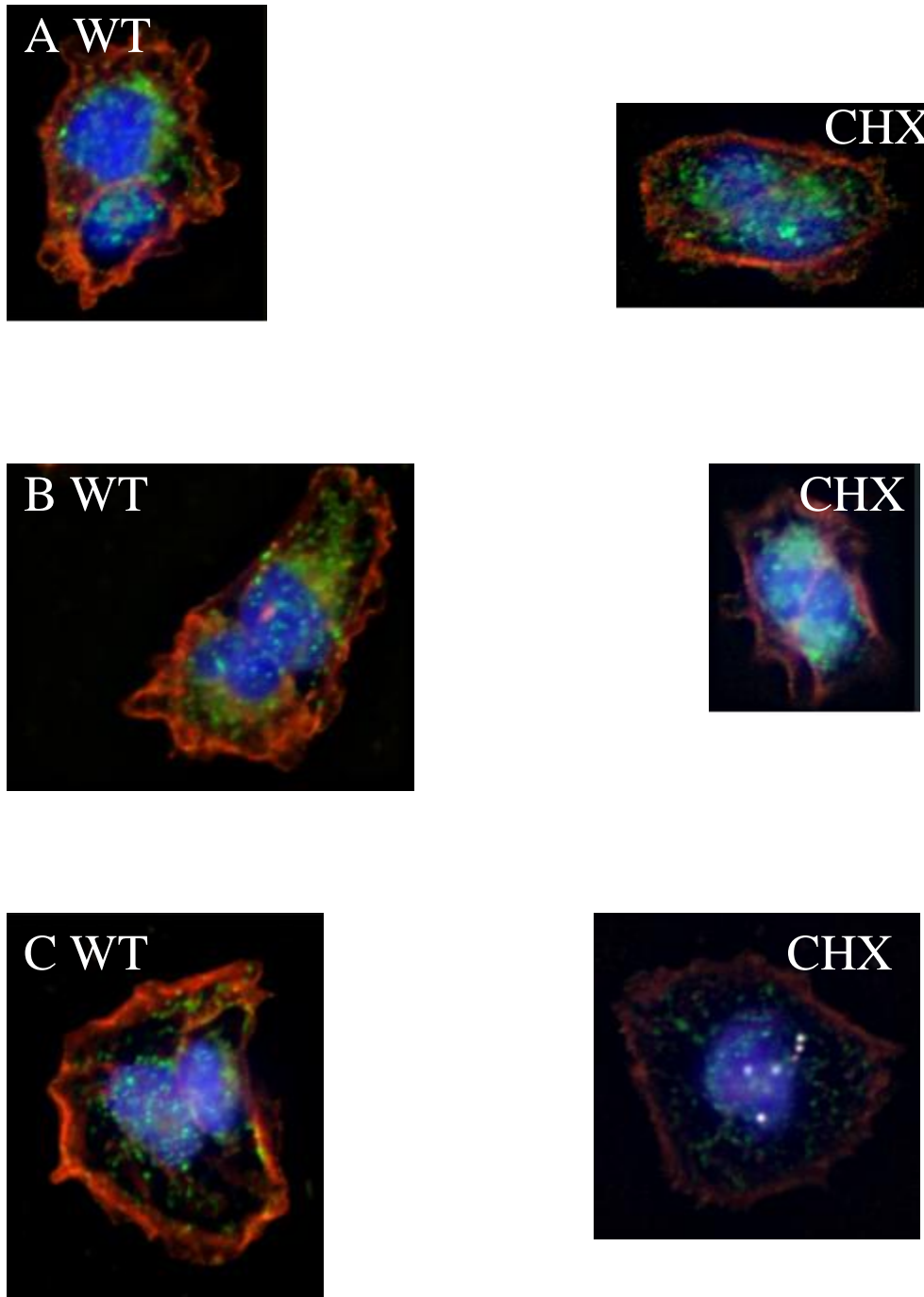
Appendix: Supplementary Figure

Figure S1. Sparse Plating Assay for cells making contacts. 250,000 cells were used as control or were treated with cycloheximide and seeded onto cover slips and were incubated for various time points. Cells were stained for E-cadherin (green) and actin (red) and DAPI (blue) and then imaged, looking for cells making contact. (A) Cells were incubated for 2h. (B) 3h. (C) 4h.

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