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Calcium Signaling and Ca^{2+}/Calmodulin-Dependent Kinase

II Activity in Epithelial to Mesenchymal Transition

Melissa Ann McNeil

A thesis submitted to the faculty of
Brigham Young University
in partial fulfillment of the requirements for the degree of

Master of Science

Marc D. Hansen, Chair
Juan A. Arroyo
Kelly S. Weber

Department of Physiology and Developmental Biology

Brigham Young University

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ABSTRACT

Calcium Signaling and Ca^{2+}/Calmodulin-Dependent Kinase
II Activity in Epithelial to Mesenchymal Transition

Melissa Ann McNeil
Department of Physiology and Developmental Biology, BYU
Masters of Science

Epithelial to mesenchymal transition (EMT) is an important process in embryonic development, tissue repair, inflammation, and cancer. During EMT, epithelial cells disassemble cell-cell adhesions, lose apicobasal polarity, and initiate migratory and invasive processes that allow individual cells to colonize distant sites. It is the means by which non-invasive tumors progress into malignant, metastatic carcinomas. In vitro, EMT occurs in two steps. First, cells spread out, increasing in surface area and pushing the colony borders out. Then cells contract, pulling away from neighboring cells and rupturing cell-cell junctions, resulting in individual highly migratory cells. Recent discoveries indicate that calcium signaling is central in EMT. Both previous data with patch clamping and new calcium imaging data show a series of calcium influxes in cells induced to undergo EMT with hepatocyte growth factor (HGF). It has also been shown that blocking calcium signaling prevents EMT from progressing normally. However, it is not known if calcium alone is sufficient to drive EMT behaviors. By experimentally triggering calcium influxes with an optigenetic cation channel, the behaviors that calcium influxes induce can be determined noninvasively, without use of drugs that may have secondary effects.

The results of using the optigenetic set up along with live cell imaging are that cells become more motile and disrupt normal epithelial cell-cell adhesions. This behavior is believed to be due to increased cell contractility downstream of calcium signaling, and is dependent on Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII). When cells are pre-treated with CaMKII inhibitor before HGF addition, they undergo the spreading step of EMT without subsequent cellular contraction and rupture of cell-cell junctions. CaMKII is a protein kinase that is activated by binding Ca^{2+}/calmodulin, and is a known downstream component of calcium signaling. CaMKII is known to affect the actin cytoskeleton by both physically bundling actin filaments to increase their rigidity, and through signaling by activation of myosin light chain kinase (MLCK), which has a role in stress fiber formation. Immunofluorescence did not show colocalization of CaMKII with actin, ruling out regulation through actin bundling. However, CaMKII does appear to have a role in stress fiber formation. EMT induced with HGF treatment results in increased numbers of stress fibers as well as trans-cellular actin network formation, both actin structures decorated with non-muscle myosin II (NMII). CaMKII inhibition not only blocks these actin formations, but it also decreases stress fiber levels below basal unstimulated levels in cells that have not been treated with HGF. This suggests that CaMKII has a role in regulating contractility through cellular actin networks, indicating a mechanism for calcium's role in cellular contractility in EMT.

Keywords: calcium signaling, epithelial to mesenchymal transition, CaMKII, actin dynamics, cellular contractility

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CHAPTER 1. Introduction

Epithelial to mesenchymal transition (EMT) is a highly organized process that completely changes the morphology and function of the cell (Kalluri and Weinberg 2009, Polyak and Weinberg 2009). Epithelial cells are normally organized in a sheet that behaves as a single unit with basement membrane interactions. In EMT, cells lose epithelial differentiation and become fibroblast-like and mesenchymal by reducing E-cadherin expression and losing both apical-basal polarity, and cell-cell junctions. Cells undergoing EMT become motile and begin to detach from each other, express vimentin and mesenchymal cadherins (such as N-cadherin and cadherin-11), and activate cellular signaling networks, such as pathways through ERK1/2 (Blick, Widodo et al. 2008, Fendrich, Waldmann et al. 2009, Kalluri and Weinberg 2009, Hu, Qin et al. 2011). EMT, also referred to as cell scattering when observed in vitro with cultured epithelial cells, occurs in two major steps. First, within 15 minutes of induction of EMT with hepatocyte growth factor (HGF), cells undergo cell spreading such that the colony area increases by roughly 100% without an increase in cell number. Second, and peaking at about 5 hours after stimulation, cells contract and start to pull apart cell-cell adhesions and eventually detach from the colony (Behrens, Birchmeier et al. 1985, Perryman 1985, Hoj, Davis et al. 2014). This process is dependent on dramatic actin cytoskeleton rearrangements to generate stress fibers and transcellular actin networks through which actomyosin contractility bursts cell-cell junctions and increases motility (Sperry, Bishop et al. 2010, Hoj, Davis et al. 2014). This last step can be chemically recreated by adding and then washing out the myosin II inhibitor blebbistatin. When blebbistatin is washed out and myosin is no longer inhibited, the cells begin to contract and cells pull apart in a manner that is highly reminiscent of HGF induced EMT, though without an

increase in cell migration. This suggests that contractility is sufficient to drive cell-cell detachment during epithelial scattering in vitro.

Cells undergoing EMT produce proteolytic enzymes to facilitate degradation of the basement membrane. Individual cells can then locally invade and initiate more distant migration to new tissues. Here they can undergo mesenchymal to epithelial transition, returning to an epithelial state to create secondary tissues (Fendrich, Waldmann et al. 2009, Kalluri and Weinberg 2009, Polyak and Weinberg 2009). In the case of epithelial cancers, this process occurs in metastasis and results in the formation of distant metastases. Additional changes in cellular behavior also accompany EMT during cancer progression, such as resistance to anoikis/apoptosis, enhanced survival, genome instability, and resistance to chemotherapy, all of which have negative repercussions in terms of disease progression (Blick, Widodo et al. 2008, Kalluri and Weinberg 2009, Polyak and Weinberg 2009).

EMT is characterized by a decrease in epithelial marker expression, including the most notable marker E-cadherin, as well as an increase in mesenchymal marker expression. After cells have detached from the colony, they can temporarily reform cell-cell contacts if they collide with other cells. To fully transition into a mesenchymal state, the E-cadherin promoter is repressed by specific transcriptional factors including Snail, TWIST1, FOXC2, and ZEB1 (Kalluri and Weinberg 2009, Polyak and Weinberg 2009). SNAIL is rapidly phosphorylated by glycogen synthase kinase 3β (GSK-3β) which leads to its degradation (Fendrich, Waldmann et al. 2009). GSK -3β repression, as occurs by activation of an NF-κB dependent pathway, allows for the increased stability of SNAIL. Increased SNAIL stability causes decreased E-cadherin expression and loss of epithelial cell-cell connections, which leads to increased EMT (Fendrich, Waldmann et al. 2009). It should be noted that NF-κB has been implicated in cancer progression and can be

regulated by calcium signaling (Cheng, Liu et al. 2011, Davis, Peters et al. 2012, Bastian Hoesel 2013). Twist, which activates Snail, is overexpressed in some solid tumors, and correlates with increased invasiveness and metastasis by the same mechanism (Fendrich, Waldmann et al. 2009). Increased expression of mesenchymal markers, including these E-cadherin repressors, is seen at the invasive front of tumors where cells migrate into and invade surrounding tissue as single cells or clusters, after undergoing EMT (Fendrich, Waldmann et al. 2009, Kalluri and Weinberg 2009).

Calcium Influx and Signaling in EMT

Recently, calcium has been identified as a crucial component of signaling to induce EMT. Using a forward chemical screen, our group identified a transient receptor potential (TRP) calcium channel blocker as an inhibitor of EMT (Langford, Keyes et al. 2012). Others have also found that TRP channels are related to EMT (Jin, Defoe et al. 2003, Vriens, Janssens et al. 2004, Boca, D'Amato et al. 2007, Waning, Vriens et al. 2007, Wondergem, Ecay et al. 2008, Wang, Yue et al. 2010, Langford, Keyes et al. 2012). Correlations between other calcium channels and EMT have been discovered, many of which are related to cancer (Garriock and Krieg 2007, Bergamaschi, Kim et al. 2008, Dasgupta, Rizwani et al. 2009, Li, Jiang et al. 2011, Takagi, Yamato et al. 2011, Davis, Peters et al. 2012, Davis, Parsonage et al. 2013, Lai, Liu et al. 2013, Davis, Azimi et al. 2014, Sun, Lu et al. 2014, Aggarwal, Prinz-Wohlgenannt et al. 2015, Casas-Rua, Tomas-Martin et al. 2015). Of interest, intracellular calcium chelation prevents EMT in breast cancer cells (Davis, Azimi et al. 2014).

Many cancers exhibit altered calcium influx, and store operated calcium entry (SOCE) is the main calcium entry in non-excitable cells (Shenyuan L. Zhang 2005, Hu, Qin et al. 2011, Davis, Peters et al. 2012). SOCE requires the CRAC (calcium release activated calcium)

channel, which is composed of Orai1 and Stim1 (Shenyuan L. Zhang 2005, Cheng, Liu et al. 2011, Hu, Qin et al. 2011). Stim1 is a transmembrane protein with an EF hand motif that binds calcium in the lumen of the ER (Shenyuan L. Zhang 2005). When calcium levels in the ER drop and the EF hand is no longer bound to calcium, a conformational change causes STIM1 to oligomerize and move to PM/ER junctions. Here it recruits and gates Orai1, the pore forming subunit of the CRAC channel, allowing for Orai1 mediated calcium entry (Shenyuan L. Zhang 2005, Cheng, Liu et al. 2011, Hu, Qin et al. 2011, Tojyo, Morita et al. 2014). Orai1 calcium influx activates insertion of TRP channel containing vesicles into the plasma membrane where TRP is recruited and gated by STIM1, allowing for additional calcium entry through these channels (Cheng, Liu et al. 2011).

Calcium influxes are seen as oscillations, with signals from different sources having distinct spatiotemporal profiles, oscillation frequencies, and amplitudes resulting in different cellular responses (Davis, Parsonage et al. 2013, Sun, Lu et al. 2014). For example, Orai1 influx alone is sufficient for NFAT activation, while Orai1 and TRPC1 influx is needed for NF-κB and calcium stimulated potassium channel activation (Cheng, Liu et al. 2011, Davis, Peters et al. 2012). Calcium influx from the plasma membrane can even induce a secondary calcium influx from the ER in calcium-induced calcium release (CICR) (Davis, Parsonage et al. 2013). Oscillations have been shown to have a stronger effect within the cell than steady signals. This reduces the need for high cytoplasmic calcium concentrations, which causes cytotoxicity (Sun, Lu et al. 2014).

SOCE is a regulator of intracellular signaling processes in epithelial cells (Davis, Peters et al. 2012). Calcium controls cell morphology and movement by remodeling the cytoskeleton, and regulating focal adhesion turnover and cell polarity (Hu, Qin et al. 2011). EMT is associated

with altered SOCE as well as changes in calcium signaling through cell surface purinergic receptors, and is also increased by CRAC-mediated calcium influx (Hu, Qin et al. 2011, Davis, Peters et al. 2012, Davis, Parsonage et al. 2013). There are significant increases in ER calcium ATPases, IP_3R, and ryanodine receptor expression levels in cells that have undergone EMT (Davis, Parsonage et al. 2013). Ryanodine receptor levels have been seen to correlate with tumor grade, and can be increased with EMT causing agents such as epidermal growth factor (EGF), transforming growth factor β (TGFβ), and hypoxia (Davis, Parsonage et al. 2013). TRPC3, a TRP calcium channel family member, is increased in ovarian cancer (Davis, Peters et al. 2012). SOCE increases melanoma progression and invasion through facilitating ECM degradation by invadopodia. STIM1 knockdown decreases lung metastasis in zenographs while overexpression of STIM1 promotes invasion and metastasis in melanoma (Sun, Lu et al. 2014). ORAI1 regulates processes important in carcinogenesis and is increased in many breast cancer cell lines (Davis, Peters et al. 2012). Knockdown of ORAI1 is antiproliferative and results in decreased ERK1/2 phosphorylation, less migration in vitro, lower metastasis in vivo, and inhibits various forms of calcium influx (Davis, Peters et al. 2012). TRPC1 knockdown also decreases ERK1/2 phosphorylation and decreases ER calcium release by inhibiting the sarco/endoplasmic reticulum calcium ATPase (Davis, Peters et al. 2012).

Ca^{2+}/Calmodulin-Dependent Protein Kinase II

One common downstream effector of calcium is Ca^{2+}/calmodulin-dependent protein kinase II, or CaMKII. Calmodulin (CaM) directly binds calcium using its four EF-hand motifs, causing a conformational change (Black, Leonard et al. 2006). The change in shape increases the binding affinity of CaM for CaMKII, resulting in binding and activation of the protein.

Calmodulin thus acts as a calcium sensor that directs changes in protein activity in response to changes in cytosolic calcium ion concentrations.

CaMKII holoenzymes contain 8-14 subunits, each with a catalytic domain, a regulatory domain, and an association domain, all organized so that catalytic domains are placed in pairs (Hudmon and Schulman 2002, Hoelz, Nairn et al. 2003, Nguyen, Sarkar et al. 2015, Wang, Zhao et al. 2015). Ca^{2+}/CaM binds to the regulatory domain causing a conformational change to remove an auto inhibitory pseudo-substrate segment from the active site. This allows for ATP binding and also exposes Threonine-286 of the auto inhibitory pseudo-substrate segment for phosphorylation (Colbran, Fong et al. 1988, Mukherji and Soderling 1995, Yang and Schulman 1999, Hudmon and Schulman 2002, Rellos, Pike et al. 2010, Hoffman, Stein et al. 2011) which sterically prevents the inhibitory segment from returning to the active site again once Ca^{2+}/CaM has dissociated, resulting in continued activation independent of calcium (Hanson and Schulman 1992, Yang and Schulman 1999). CaMKII subunits undergo cis-phosphorylation; pairing of subunit's catalytic domains allows for phosphorylation in trans, but this only occurs if both subunits are activated through Ca^{2+}/CaM binding (Mukherji and Soderling 1994, Mukherji and Soderling 1995, Bradshaw, Hudmon et al. 2002, Lucic, Greif et al. 2008).

CaMKII in Cytoskeletal Regulation: Actin Bundling

CaMKII is important in regulation of actin dynamics, which are dramatically altered during EMT and cellular migration (Daft, Yuan et al. 2013). CaMKII's effect on the cytoskeleton has been most extensively studied in neurons. In these cells, dendritic stability is effected by CaMKII, which alters filopodia formation on dendrites by modulating cytoskeletal dynamics and stability (Andersen, Li et al. 2005).

In dendritic spines, it was shown that CaMKIIβ binds and bundles actin in a stoichiometric manner. Since CaMKII is an oligomer, multiple subunits can bind individual F-actin filaments, resulting in bundles that are seen with electron microscopy after F-actin and CaMKIIβ were combined in vitro (Okamoto, Narayanan et al. 2007, Lin and Redmond 2008, Okamoto, Bosch et al. 2009). With calcium present, CaMKIIβ no longer binds F-actin, allowing for actin rearrangements (Okamoto, Narayanan et al. 2007, Lin and Redmond 2008, Okamoto, Bosch et al. 2009). This same activity was later seen with CaMKIIδ, and γ isoforms, the latter of which creates a novel layered bundle structure (Hoffman, Farley et al. 2013).

All isoforms of CaMKII, with β having the greatest activity and α having the least, sequester G-actin monomers when not bound to Ca^{2+}/CaM, releasing them to generate a large increase in actin polymerization when activated by Ca^{2+}/CaM binding (Hoffman, Farley et al. 2013). The G-actin sequestering activity of CaMKII may be part of how calcium influx at the dendritic spine results in rapid and significant changes in spine morphology. Whether CaMKII activities occurring in the remodeling of dendritic spines are important in cytoskeletal changes that occur during EMT and cancer metastasis remains unclear.

Calcium and RhoA Activity in Actin Cytoskeletal Regulation

In addition to releasing CaMKII from bundling actin, calcium influxes can alter actin dynamics by modulating other regulatory pathways. One well studied mechanism is the activation of small GTPases, specifically RhoA, a Ras homolog gene family member (Schubert, Da Silva et al. 2006, Okamoto, Bosch et al. 2009, Fonseca 2012).

RhoA is important in cell constriction, which is necessary for EMT (Ying, Giachini et al. 2009). Our lab has shown that blocking RhoA activity with a dominant negative form of RhoA prevents the cellular contractility that pulls apart cell-cell junctions (Hoj, Davis et al. 2014). Rho

activation results in stress fiber formation, cell contractility, and assembly of contractile actin-myosin filaments (Mariette H.E. Driessensa and Collarda 2002). RhoA also acts on cofilin (Ying, Giachini et al. 2009) and profilin II, which binds G-actin to help add it to the growing end of F-actin (Maekawa, Ishizaki et al. 1999, Ackermann and Matus 2003). ROCK is a downstream effector of Rho implicated in smooth muscle contraction by phosphorylating and inactivating myosin light chain (MLC) phosphatase to increase MLC phosphorylation. This increases actomyosin based contractility and causes the formation of stress fibers (Maekawa, Ishizaki et al. 1999).

RhoA is a small GTPase protein, meaning that it is active when bound to GTP and inactive when bound to GDP. Guanine exchange factors (GEF's) activate RhoA by exchanging the bound GDP for GTP. GTPase activating proteins (GAPs) inactivate RhoA by hydrolyzing GTP to GDP (Mariette H.E. Driessensa and Collarda 2002, Oinuma, Katoh et al. 2003, Ying, Giachini et al. 2009). PDZ-RhoGEF and LARG are examples of some Rho specific GEFs that are expressed in MDCK cells (Mariette H.E. Driessensa and Collarda 2002, Oinuma, Katoh et al. 2003). Overexpression of PDZ-RhoGEF enhances RhoA activation by angiotensin II, while its knockdown decreases angiotensin II mediated RhoA activation (Ying, Giachini et al. 2009). Calcium is necessary and sufficient to activate PYKII, which activates these RhoGEFs by phosphporylation. AM-BAPTA, a calcium chelator, abolishes RhoA activation through angiotensin II by blocking PYKII activity (Ying, Giachini et al. 2009).

Rho-family small GTPases, the subgroup of the Ras homolog gene family that contains RhoA, are key regulators of the actin cytoskeleton and have been shown to cause growth cone collapse and to regulate cell and axon growth downstream of signaling pathways from guidance molecule receptors (Mariette H.E. Driessensa and Collarda 2002, Oinuma, Katoh et al. 2003,

Ying, Giachini et al. 2009). Dominant negative PDZ-RhoGEF suppresses Sema4D-induced cellular contraction (Oinuma, Katoh et al. 2003). Some data indicate that p38 MAPK and RhoA mediate an autocrine TGF-β induced EMT in mammary cells (Kalluri and Weinberg 2009). Semaphorins, one type of guidance molecule that acts upstream of RhoA, are thought to mediate tumor growth and metastasis (Oinuma, Katoh et al. 2003). Rnd GTPases are a distinct branch of Rho family GTPases, which in MDCK cells regulate cell migration speed and are involved in alteration of the actin cytoskeleton associated with oncogenic transformation. Rnd1 directly interacts with Plexin-B1, a semaphorin receptor, to potentiate RhoA activation and induce contraction of COS-7 cells (Oinuma, Katoh et al. 2003).

CHAPTER 2. Results

Calcium Influxes in EMT

It has been previously shown that there is a calcium influx at the beginning of EMT (Davis, Peters et al. 2012, Langford, Keyes et al. 2012, Davis, Azimi et al. 2014). This was shown both in breast cancer MDA-MB-468 cells through whole cell calcium level analysis, and through patch clamping in MDCK cells (Davis, Peters et al. 2012, Langford, Keyes et al. 2012). Patch clamping only shows influx through the cell membrane, and since calcium influxes can come from both the cell membrane and the ER, another method was necessary to determine if the ER is playing a role in HGF induced calcium influx. Calcium imaging using Fura2-AM, which shows whole cell calcium levels, was performed to see the total calcium influxes in untreated and HGF treated MDCK cells.

Fura-2 AM is a well-documented and commonly used fluorescent calcium probe based off of the structure of EGTA and BAPTA (Grzegorz Grynkiewicz 1985, Paredes, Etzler et al. 2008). Fura-2 is a ratiometric dye, meaning that its excitation wavelength changes from 380 nm to 340 nm on binding calcium, while its emission wavelength stays around 500nm (Grzegorz Grynkiewicz 1985, Paredes, Etzler et al. 2008, Udagawa, Hanaoka et al. 2012). Using the ratio of calcium bound fluorescence intensity to calcium free fluorescence intensity corrects for differences in dye loading, tissue thickness, cell size changes during imaging, and photobleaching (Grzegorz Grynkiewicz 1985, Paredes, Etzler et al. 2008).

Calcium Imaging After HGF Stimulation

Previously reported calcium imaging of MDCK cells done with perfusion at a constant 37°C results in calcium oscillations with no change in the overall direction of calcium levels (Udagawa, Hanaoka et al. 2012). However, for these experiments, calcium levels dramatically

plummet as soon as imaging begins, then bottom out within twenty minutes and rise again to levels close to starting calcium levels. This increase reaches its peak and begins to slowly taper off within an hour, though a couple of colonies reached their peak closer to an hour and a half later (Figure 2.1 b-e). Both HGF stimulated and unstimulated cells show these features. This is most likely due to the microscope not being fitted with a perfusion system or a heater, leaving the cells to cool from thirty seven degrees to room temperature over the course of imaging. This decrease in temperature should be responsible for these consistent calcium fluctuations because other settings were taken from the previously mentioned paper (Udagawa, Hanaoka et al. 2012).

While both stimulated and unstimulated cells follow a similar trend in overall calcium levels, unstimulated cells (Figure 2.1 b-c) have a relatively smooth curve (oscillations were observed as previously reported) and stimulated cells have several calcium peaks on top of this baseline trend (Figure 2.1 d-e). The first peak in a colony is seen sometime between five and ten minutes post HGF. There is always a second peak in every colony, and many have three or more calcium peaks as well. These are all of a variable size, duration, and length of delay after the first peak. While there is no predictability to how large, long, or how many peaks there are, there are always at least two peaks. This is consistent with previous patch clamp data showing two consistent peaks coming from the cell membrane alone. Calcium imaging could show more but not fewer peaks than observed by patch clamping since calcium imaging measures total cellular calcium levels. These results suggests that the ER is playing a role in calcium signaling since only two peaks are coming from the membrane. Calcium influx from the plasma membrane can result in calcium influx from the ER in a process called calcium induced calcium release (CICR).

Effects of Calcium Influx

In order to determine the downstream results of calcium signaling in EMT, I have transfected MDCK cells with a channelrhodopsin gene. Channelrhodopsin is a light sensitive cation channel that opens rapidly when stimulated with blue light (Nagel, Szellas et al. 2003). This channel has been used extensively in neurons to stimulate action potentials non-invasively, but has not been heavily utilized outside of the neuron. This protein is an excellent choice to study calcium influx because it can be activated quickly and non-invasively by shining a blue LED on the cells (Campagnola, Wang et al. 2008). The current standard for studying calcium signaling is to treat with drugs such as thapsigargin, and then to wash them out, which does not allow for high temporal resolution, and does not conclusively determine if the results seen are due to calcium influx or a secondary action of the drug. Channelrhodopsin proteins are stimulated using a bright blue LED, which can also be used on control cells to determine if any changes in behavior are due to light exposure alone. The LED is connected to a computer through a simple program that allows control of the frequency and duration of flashes on a millisecond time scale. By placing the LED above cells, transfected and untransfected cells can be stimulated during live cell imaging to monitor behavioral changes.

Calcium is known to activate several signaling pathways. Two likely candidates for EMT are PYKII and CaMKII. RhoA is activated downstream of the calcium sensitive protein PYKII (Ying, Giachini et al. 2009). RhoA has been shown to be important in the cellular contractility stage of EMT through use of a dominant negative mutant (Hoj, Davis et al. 2014). CaMKII is activated by binding Ca^{2+}/CaM and affects the actin cytoskeleton by directly bundling F-actin, as well as activating myosin light chain kinase (MLCK) through phosphorylation (Tansey, Word et al. 1992). MLCK has an important role in stress fiber formation and cellular contractility

(Takashima 2009, Ebrahim, Fujita et al. 2013). Because both of these proteins' roles are related to contractility, calcium influx may induce the cellular contractility phase of EMT without previous cellular spreading. This may be sufficient to tear apart cell-cell junctions and may result in single cells leaving the colony.

Live cell imaging shows that unstimulated, untransfected cells move around slightly within the colony during the course of imaging, but the colony stays together and will only slightly change shape or size (Figure 2.2 a). Cells never disassemble cell-cell junctions within in the colony. When treated with the blue LED, untransfected cells act just like unstimulated, untransfected cells (Figure 2.2 b). MDCK cells are not photosensitive, so light should not affect their behavior. When treated with HGF, the cells undergo normal scattering, with the colony spreading out, and then the cells contracting and pulling apart from each other, disrupting cell-cell junctions to create large tears in the colony and single cells (Figure 2.2 c).

Unstimulated, transfected cells are more motile during live cell imaging than their untransfected counterparts (Figure 2.2 e). A few colonies even develop large tears between cells. This is likely due to the light from the microscope activating the channelrhodopsin channel with the blue light contained within the white light spectra. To overcome this, a filter was obtained that only allows red light through, preventing the activation of channelrhodopsin channels while imaging with bright light from the microscope. This dramatically decreases the motility of transfected cells, almost down to the level of untransfected cells (Figure 2.2 f). The frequency and size of the tears dropped dramatically as well. These behaviors may not have reached baseline due to ambient light while transferring cells from the incubator to the microscope for imaging. When treated with HGF, the transfected cells scatter, very similarly to the nontransfected cells (Figure 2.2 d).

Because the colony tearing and increased cellular motility only occurred in the presence of white light, and were minimized when the filter was used to remove all blue light from the microscope's light, these scattering behaviors are most likely due to calcium influx from the presence of the blue light in the white light, which supports my aim. To verify this, transfected cells were stimulated with the blue LED while leaving the red filter in place. Most of these cells develop small tears in the colonies with higher frequency than unstimulated cells imaged with the red filter (Figure 2.2 g). These tears increase in size as LED intensity is turned up, although there are always colonies that do not respond to the light (Figure 2.2 h). It is likely that the amount of blue wavelength light is still higher in the white light on the microscope than in the blue LED, resulting in the largest and most frequent tears with white microscope imaging light alone.

Determination of the Signaling Pathway Downstream of Calcium

Calcium in known to activate several cellular signaling pathways with a wide range of effects. For my dissertation, I chose to focus on kinases that are known to be regulated by calcium influxes and play some role in cell spreading, cell contractility, or cell migration. Kinases that could act as downstream effectors of HGF include PYKII and CamKII. PYKII is directly activated by calcium binding (Ying, Giachini et al. 2009). When active it phosphorylates PDZ-RhoGEF and LARG, which in turn will activate RhoA through replacing GDP with GTP (Mariette H.E. Driessensa and Collarda 2002, Ying, Giachini et al. 2009, Marechal and Zou 2014, Osborne, Li et al. 2014). RhoA has an important role in myosin-based cellular contractility, and has been directly linked to EMT in MDCK cells. Dominant negative RhoA mutant MDCK cells fail to rupture cell-cell junctions after treatment with HGF, although they undergo the spreading phase of EMT (Hoj, Davis et al. 2014). CaMKII is activated by binding

Ca^{2+}/CaM, and it phosphorylates many downstream proteins, including MLCK. MLCK phosphorylates myosin to activate it, allowing for increased cellular contractility (Tansey, Word et al. 1992). Either, or possibly both of these pathways may be the intermediate step between the calcium influx induced with channelrhodopsin and the resultant increase in cellular contractility.

PYKII

In order to determine the molecular machinery transducing calcium influxes into cellular behavior changes during epithelial scattering, we performed live cell imaging of MDCK cells responding to HGF after treatment with small molecule inhibitors of either CaMKII or PYKII. The optigenetics experiments showed that calcium was increasing cellular contractility, so blocking the appropriate signaling pathway should decrease cellular contractility when exposed to HGF as well. PF-431396 is an inhibitor of PYKII, which is activated by calcium and is known to activate LARG and PDZ-RhoGEF that in turn generate GTPbound RhoA and RhoA-dependent contractility. When MDCK cells are treated with PF-431396, cells increase motility and contractility and some cell-cell detachment is observed (Figure 2.3 b). When MDCK cells are treated with PYKII inhibitor PF-431396 and then stimulated with HGF, they undergo a process of cell death preceded by a reduction in cell area (Figure 2.3 a). Taken together, this suggests that PYKII activity opposes cellular contractility, initiation of migration, and cell-cell detachment. If PYKII activation plays any role in epithelial scattering, it would likely participate in the early cellular spreading phase. This shows that PYKII is not part of the calcium to contractility signaling pathway, though it does appear to be important and effected by HGF.

While reports indicate that PYKII can drive cellular contractility through Rho GEFs, our observation that PYKII inhibition drives contractile behavior reminiscent of EMT suggests that Rho GEF activation by PYKII is not a key factor during epithelial scattering. We therefore

sought to rule out Rho GEF activation during EMT. To test whether Rho GEF activation occurs in calcium-induced contractility during EMT, the localization of PDZ-RhoGEF, LARG, and GEF H1 was assessed for alterations during HGF-induced EMT. Since these GEFs are all reportedly activated by PYKII, we expected PYKII activation to generate changes in localization of these proteins, particularly recruitment to cellular membranes. MDCK cells were grown on collagen-coated coverslips, fixed at various times before or after HGF stimulation, and then stained with antibodies against Rho GEFs. None of the GEF proteins analyzed showed any change in subcellular distribution over several time points after HGF addition (data not shown). This result suggests that activation of RhoGEFs targeted by PYKII are not being activated during EMT.

CaMKII

KN-93 is an inhibitor of CaMKII that competes with CaM for binding the active site, preventing CaMKII from being activated. When treating with KN-93 alone, cells stay still and do not wiggle slightly like untreated cells or exhibit any other behaviors (Figure 2.4). When cells are pretreated with KN-93 before adding HGF, they spread like they do at the beginning of typical EMT, but then they stay spread out and do not contract and pull apart (Figure 2.4). This behavior is exactly what is expected when blocking cellular contractility. Since this does not occur with drug alone but is an HGF dependent behavior suggests that cellular contraction after stimulation with HGF is CaMKII dependent. Also, cell spreading must be a CaMKII independent behavior since this was unaltered. In summary, when cells are treated with KN-93 and HGF, the HGF causes the cells to spread but the KN-93 prevents the mechanism that causes cellular contractility and the cells are stuck in a spread state. This shows that CaMKII is likely

the signaling molecule that is initiating cellular contraction after calcium influxes with HGF addition.

CaMKII FRET Activation Assay

To test CaMKII activation after addition of HGF, the FRET Camui biosensor was utilized. Camui has a CFP protein fused to one side of the active site, and a YFP protein on the other side. When the active site of the biosensor is closed and the enzyme is inactive, there is positive FRET, meaning stimulation of CFP will result in YFP emission (Erickson, Nichols et al. 2015). This is because the emission wavelength of CFP is the same as the excitation wavelength for YFP, so exciting CFP results in YFP getting excited as well, but only when the two fluorophores are incredibly close together. When Camui is active and open, this FRET transfer does not happen. The ratio of YFP to CFP fluorescence intensity is used to determine if there is more or less activity of the enzyme in the cell. This technique was used along with live cell imaging. A microscope cube was made that allowed excitation of CFP with detection of YFP. Fluorescent live cell imaging with this FRET cube and a regular CFP cube allowed for obtaining FRET levels and control measurements. The computer program slidebook was then used to analyze the colonies and background for fluorescent intensity. The background for both CFP and FRET was subtracted from their respective colony fluorescent intensity values. Then the FRET measurement was divided by the CFP measurement.

As a control, I added KN-93 to cells during live cell imaging with FRET analysis (Figure 2.5). Because KN-93 blocks Ca^{2+}/CaM from binding, CaMKII is inactivated and the active site is closed. This puts the two fluorophores in close proximity and should allow for FRET as measured by YFP emission. Before drug addition, the FRET/CFP ratio was primarily below 1 and averaged 0.95. After drug addition, the FRET/CFP ratio was primarily above 1 and averaged

1.16. This shows that the biosensor works, and that the change in values tends to be small, which is consistent with other biosensors and the creator of Camui's data (Erickson, Nichols et al. 2015). To show that changes in activation are not due to photobleaching or other experimental artifacts, I did FRET analysis on unstimulated cells. This showed a very minimal decrease in FRET/CFP intensity over an hour, showing that the KN-93 data is not due to photobleaching (Figure 2.5).

If the calcium influxes at the beginning of EMT are activating CaMKII, FRET analysis after HGF addition should show a decrease in FRET at about twenty to thirty minutes. However, FRET analysis shows that CaMKII is not being activated within an hour of stimulation with HGF (Figure 2.5). This was surprising considering the calcium influxes occurring during this time frame and the live cell imaging data with CaMKII inhibition. However, calcium influxes can occur at different frequencies and amplitudes, and the proteins they activate depends on these characteristics. It is possible that the oscillations of the calcium influx at the beginning of EMT are not compatible with CaMKII, so it is not activated during this time. There may be additional calcium influxes at a later point in EMT that have the correct frequency and amplitude to activate CaMKII, and these later calcium influxes would be directly responsible for cellular contractility. Considering that cellular contractility occurs around five hours after HGF treatment, this hypothesis seems very likely and is worth studying further.

CaMKII Downstream Effects

Previously, it has been shown that treatment of MDCK cells with HGF results in increased stress fibers and actin trans-cellular network formation (Sperry, Bishop et al. 2010). These actin structures colocalize with non-muscle myosin II (NMII) and are contractile. They are believed to be the physical structures that cause the increase in contractility during EMT.

CaMKII has a known function in phosphorylating and activating myosin light chain kinase (MLCK), which in turn phosphorylates and activates myosin, effecting stress fiber regulation and function (Tansey, Word et al. 1992, Ebrahim, Fujita et al. 2013).

Actin and Non-Muscle Myosin II

To see if CaMKII has a role in the formation of these structures, immunofluorescence staining for NMII and actin with and without KN-93 pre-treatment was done. Staining cells that have not been treated with either HGF or KN-93 results in many cells with only a few stress fibers, since MDCK cells are slightly motile (Figure 2.6 a). Treatment with HGF alone for four hours results in increased stress fiber formation as well as trans-cellular actin networks and star shaped actin-myosin structures, as previously reported (Sperry, Bishop et al. 2010) (Figure 2.6 b). Pretreatment with KN-93 and HGF treatment for four hours results in no trans-cellular actin structure formation and an absence of stress fibers (Figure 2.6 c). Pretreatment with KN-93 alone for twenty minutes results in a slight decrease in stress fibers (Figure 2.6 d). These data suggest that CaMKII is involved in the regulation and maintenance of these contractile actin fibers since its inhibition decreases these structures. CaMKII inhibition likely results in cell spreading with no subsequent contraction because the contractile units are no longer present.

CaMKII Actin Bundling

CaMKII also has a known role in neuron dendrites as a structural protein, where it bundles actin filaments into various layered structures depending on the isoform. MDCK cells express the gamma and delta isoforms of CaMKII, which have bundling ability but to a weaker extent than the neuronal beta isoform. CaMKII binds and bundles actin when inactive, and when calcium influx activates CaMKII it releases the actin, resulting in a loss of rigidity and increased synaptic plasticity in neurons. If CaMKII were to bundle actin similarly in MDCK cells, the

timing of the larger calcium influx corresponds with the beginning of actin rearrangements at the start of spreading, creating a potential argument for a secondary role for CaMKII in the spreading step of EMT in addition to its contractile role. Immunofluorescence of CaMKII and actin however did not show colocalization with actin for either the gamma or delta CaMKII isoform, disproving this hypothesis (Figure 2.7).

CHAPTER 3. Discussion

These studies suggest that HGF stimulates an increase in total cytoplasmic calcium, resulting in the creation of contractile stress fibers and trans-cellular actin networks. These contractile actin structures increase cellular contractility and disassemble cell-cell junctions, resulting in individual, highly migratory mesenchymal cells that can move to new tissues and form metastases. This process is dependent on CaMKII despite its not being activated during the calcium influxes at the beginning of EMT.

Calcium Imaging

Previous patch clamping showed two highly reproducible calcium peaks at eight and thirty minutes after HGF addition. Calcium imaging data showed more peaks with more variability, which is consistent with patch clamping only measuring influxes at the membrane and calcium imaging measuring the whole cell's calcium levels. This suggests that the ER is playing a role in post HGF calcium signaling. The average first peak is around ten minutes, and the biggest additional peak is around twenty two minutes. However, there were sometimes as many as four peaks in a single colony within an hour after addition of HGF.

Optigenetics

Optigenetic analysis allows for determining the impact that calcium influxes have on cells without adding and washing out drugs that may have secondary effects. Use of a blue LED allows for high temporal resolution and reproducibility. Light treated, untransfected MDCK cells do not exhibit any tears in the colonies, and behave essentially the same as untreated MDCK cells. Imaging with transfected MDCK cells without HGF or exposure to blue light, less than half the colonies will develop a small tear between only two cells. This is likely due to ambient light from transporting the cells to the microscope from the incubator. Treating with the blue

LED at high brightness causes colonies to tear open cell-cell junctions, and sometimes the behavior resembles scattering. A notable trait of these colony tears and contractility is that there is no cellular spreading before the creation of large tears with calcium alone. These experiments show that calcium influx alone is enough to induce cells to contract and disrupt cell-cell junctions in the colony.

Determination of Downstream Signaling Pathways

To determine the factors downstream of calcium that are mediating cellular contraction, small molecule inhibitors for signaling molecules that are known to be activated by calcium were utilized. Blocking the correct pathway would result in HGF induced spreading, which is calcium independent according to the optigenetics experiments, but not contraction. PYKII inhibition resulted in cells shriveling up and dying when added with HGF. However, addition of KN-93, an inhibitor of CaMKII, resulted in the correct behavioral changes when HGF was present, but not when added alone.

CaMKII Activation

To determine that CaMKII is being activated after HGF stimulation, the FRET biosensor Camui was used to monitor CaMKII activation. CaMKII was not seen to be activated within an hour after HGF stimulation. This may actually be expected if CaMKII is regulating the cellular contractility phase of EMT which happens around five hours after HGF stimulation. Further analysis of CaMKII activation looking over a larger time course is necessary to determine if CaMKII is playing an active role in EMT, or if it is essential in maintaining stress fibers, which are essential for cellular contractility.

CaMKII and Actin Dynamics

To assay for CaMKII's effect on the actin cytoskeleton, immunofluorescence was performed with staining for non-muscle myosin II and actin, colocalization of which shows contractile cytoskeletal elements. Untreated cells have some stress fibers but very rarely have transcellular actin networks. When treating with HGF alone, increased stress fibers and transcellular actin networks reported previously by Sperry et al. could be seen. When cells were pre-treated with KN-93, not only were the transcellular actin networks gone, but all stress fibers as well. This shows that CaMKII is important in forming and maintaining contractile cytoskeletal structures, which is consistent with CaMKII having a role in the contraction and not the spreading phase of EMT.

CaMKII and Actin Bundling

In the dendrites of neurons, CaMKII has a documented role in bundling actin fibers which is released when calcium is present in the cell, allowing for increased actin reorganization and synaptic plasticity. If CaMKII were to bundle actin in MDCK cells, and since calcium influxes occur around the time that actin begins to be released from the actin ring that supports the cell, it could be argued that CaMKII plays a role in cell spreading by bundling and then releasing actin. Immunofluorescence of both the gamma and delta isoforms of CaMKII did not show any colocalization with actin, ruling out a role for CaMKII as a structural protein that may play a role in spreading.

This data shows that EMT causes calcium signaling through CaMKII to reorganize the actin cytoskeleton and allow the cell to become more contractile so it can pull away from neighboring cells and become mesenchymal.

CHAPTER 4. Review

CaMKII

Evidence of a Role for CaMKII in EMT

CaMKII has been implicated in EMT and cancer progression. (Sun, Zhao et al. 2013). In zenopus development, CaMKII is seen in the EMT and subsequent migration of fin core cells (Garriock and Krieg 2007). CaMKII is deregulated in both thyroid and prostate cancers and is implicated in the progression of human glioblastoma multiform (HGM). α-CaMKII, one of the four isoforms of CaMKII, specifically has been implicated in osteosarcoma, colon, prostate, brain, and breast cancers (Rusciano, Salzano et al. 2010, Wang, Symes et al. 2010, Cuddapah, Turner et al. 2013, Daft, Yuan et al. 2013, Sun, Zhao et al. 2013, Rokhlin, Taghiyev et al. 2014).

CaMKII is seen to effect EMT by interacting with Siva1, a protein that stabilizes microtubules by inhibiting stathmin, which inhibits microtubule formation by binding to tubulin monomers (Li, Jiang et al. 2011). In U2OS cells, Siva1 assists CaMKII in phosphorylating and inactivating stathmin, resulting in growth and polymerization of microtubules. ShRNA of Siva1 results in a dramatic increase in the number of migratory cells, increased vimentin and decreased E-Cadherin expression, and more tumors after zenograft of MCF7 cells in mice (Li, Jiang et al. 2011). This is believed to be through loss of Siva1 mediating CaMKII phosphorylation of stathmin,

In HGM cells that have been treated with autocamtide-2-related inhibitory peptide (AIP), a CaMKII specific inhibitor, cell migratory ability is decreased. This was found to be mediated through the acid sensing ion channel 1 (ASIC1), a nonspecific cation channel which complexes with and is phosphorylated and activated by CaMKII (Sun, Zhao et al. 2013). In glioblastoma cells, it was discovered that CaMKII is necessary for cell migration along with ClC-3, a voltage

gated chloride channel that is activated by CaMKII phosphorylation (Cuddapah, Turner et al. 2013). ClC-3 is upregulated in glioma samples taken from patients and immunoprecipitates with CaMKII. Both proteins localize to the leading edge of glioma cells (Cuddapah and Sontheimer 2010, Cuddapah, Turner et al. 2013).

In osteosarcoma samples taken from patients, α-CaMKII is shown to be highly expressed and phosphorylated. Overexpression of α-CaMKII in several osteosarcoma cell lines results in an increase in migration, proliferation, and a dramatic increase in invasion, while knock-down results in a significant reduction of these measurements (Daft, Yuan et al. 2013). Overexpression of α-CaMKII in non-tumor forming HOS cells results in cells gaining the ability to form tumors, and deletion in MG-63 cells results in less tumor forming ability in vivo (Daft, Yuan et al. 2013).

CaMKID, a homologue of CaMKII, is overexpressed in invasive carcinomas and leads to EMT, increased cell proliferation, loss of cell-cell adhesions, and increases migration and invasion (Bergamaschi, Kim et al. 2008). Specifically, CaMKID was shown to modulate cell proliferation in MCF7 cells, and in MCF10A cells it activates the transcription factor CREB which activates genes involved in cell proliferation and differentiation (Bergamaschi, Kim et al. 2008).

CaMKII and Wnts in EMT

CaMKII and Wnts are known to interact to alter cell morphology and cell-cell connections in the progression of cancer. Early work in xenopus showed that different types of Wnts act on cell adhesion differently, with Xwnt-5A decreasing cadherin based cell-cell adhesion, and Xwnt-1 increasing cell-cell adhesion (Tortes, Yang-Snyder et al. 1996). Later it was elucidated that these opposing effects result from Xwnt-5a antagonizing downstream effects of Xwnt-8 (which acts similarly to Xwnt-1) through a non-canonical calcium pathway that

activates CaMKII, allowing remodeling of both cadherin-based cell-cell adhesions and alterations in cell morphology (Kuhl, Geis et al. 2001).

In prostate cancer, the non-canonical Wnt5a calcium pathway was shown to be important in wound closure and cell migration. Inhibition of CaMKII with AIP prevents wound closure in scratched confluent sheets and decreases cell motility while altering cellular structure by increasing the length and number of filopodia as imaged by scanning electron microscopy (Wang, Symes et al. 2010). In melanoma, Wnt5a correlates with high-grade invasive tumors and promotes invasiveness in melanoma cell lines (Witze, Litman et al. 2008). Treatment of WM239A melanoma cells with Wnt5a and a chemokine gradient was shown to cause uniformly distributed F-actin to relocate to form a dense band near the back of polarized cells. Myosin IIB relocalizes to overlap both F-actin and a multivesicular body structure consisting of the vesicles being endocytosed from the constricting membrane during migration (Witze, Litman et al. 2008).

Wnt11-R also elicits intracellular calcium fluctuations that result in activation of CaMKII which cause the cell to undergo EMT. The CaMKII inhibitor KN-93 prevents Wnt11-R positive cells in xenopus somites from undergoing EMT, showing that this process works through CaMKII activation. Calcium influx due to thapsigargin can rescue EMT in Wnt11-R knockout frog embryos (Garriock and Krieg 2007).

CaMKII Regulation of Actin Bundling Proteins

CaMKII has been reported to phosphorylate filamin, decreasing its affinity for F-actin. Filamin normally loosely cross links F-actin, causing the cytosol to become gel-like, an association that must be antagonized to change cell morphology and increase motility (Bourguignon, Gilad et al. 2006). Increasing cytosolic calcium in head and neck squamous cell carcinoma cells increases filamin phosphorylation and decreases filamin actin association, while

adding the CaMKII inhibitor KN-93 returns both phosphorylation and actin binding to baseline (Bourguignon, Gilad et al. 2006). Spinophilin binds to and bundles F-actin, but when phosphorylated by CaMKII, its affinity for F-actin is reduced (Grossman, Futter et al. 2004). Spinophilin also binds protein phosphatase 1 (PP1), which dephosphorylates autophosphorylated CaMKII, and may also bind to kalirin-7 (Ryan, Alldritt et al. 2005).

S100 Proteins

S100 proteins are a family of 25 known members that are widely expressed in vertebrates (Emberley, Murphy et al. 2004, Donato, Cannon et al. 2013, Chen, Xu et al. 2014, Bresnick, Weber et al. 2015). They were named because of their ability to dissolve in 100% saturated ammonium sulphate at a neutral pH (Salama, Malone et al. 2008). While the majority of cell types express at least one type of S100 protein, their expression pattern is cell specific (Donato 1999, Donato, Cannon et al. 2013). S100 proteins exist as homodimers and occasionally heterodimers or calcium induced oligomers (Donato 1999, Emberley, Murphy et al. 2004, Salama, Malone et al. 2008, Lukanidin and Sleeman 2012).

All S100 proteins have two calcium binding EF-hands that cause a conformational change in the protein upon calcium binding, which exposes protein binding surfaces and allows S100 proteins to act as calcium sensors that modulate a range of downstream behaviors through binding partners (Donato 1999, Emberley, Murphy et al. 2004, Bresnick, Weber et al. 2015). Between the two EF-hand motifs is a motif called the hinge which, along with the C-terminal region, comprises the majority of the variability in the family (Gross, Sin et al. 2014). These variable regions are believed to be responsible for associations with a wide variety of binding partners (Salama, Malone et al. 2008). S100 protein variable region binding partners include

enzymes, cytoskeletal proteins, receptors, and transcription factors, to name a few (Donato, Cannon et al. 2013).

While S100 proteins have no intrinsic enzymatic activity, the cellular functions of these proteins are extensive, and include proliferation, differentiation, inflammation, migration and invasion, and intracellular calcium homeostasis (Donato 1999, Emberley, Murphy et al. 2004, Salama, Malone et al. 2008, Lukanidin and Sleeman 2012, Donato, Cannon et al. 2013, Bresnick, Weber et al. 2015). At the molecular level, S100 proteins' functions include regulating enzymatic activity and protein phosphorylation by steric interference, calcium homeostasis, regulation of cytoskeletal components, and regulation of transcription factors, such as p53 and NF-κB (Donato 1999, Gross, Sin et al. 2014, Ji, Huang et al. 2014). S100 proteins are not functionally interchangeable. However, a single S100 protein may have many functions that depend on cell type and cellular context (Bresnick, Weber et al. 2015). Due to the ubiquitous functional roles of S100 proteins, it is not surprising that many have been implicated in EMT and cancer metastasis.

S100 Proteins in Cancer and Cell Migration

Differential expression of S100 proteins between healthy cells and cancer has been well documented, with S100A4, S100A6, S100A7 and S100B being overexpressed and S100A2 being under-expressed in many cancers (Chen, Xu et al. 2014). Most S100 genes are located on human chromosome 1q21 which is prone to genomic rearrangements, and provides a possible mechanism for how S100 genes become misregulated during cancer progression (Chen, Xu et al. 2014). Because S100 proteins' functions are cell and context dependent, the same S100 protein may be upregulated in some cancers, while downregulated in others. For example, S100A2 is a

tumor suppressor in oral cancer, but a tumor promotor in lung cancer (Bresnick, Weber et al. 2015).

Many S100 proteins, including S100A1, S100A4, S100A6, S100A8/A9, S100A11, S100A12, S100A14, S100B and S100P bind to RAGE to induce signaling through MAP Kinase, NF-κB, and phosphatidylinositol 3-kinase (PI3K)/AKT pathways (Chen, Xu et al. 2014). S100's are also involved in the epidermal growth factor (EGF) pathway, with S100A4 interacting with EGFRs and EGF treatment inducing S100A7 expression (Chen, Xu et al. 2014). EGF is a known inducer of EMT in many cell lines (Lu, Ghosh et al. 2003). The effects of many less studied S100 proteins on EMT or EMT related processes are listed in Figure 4.1.

Specific S100 Protein Functions in EMT and Cell Motility

S100A2 is a tumor suppressor in many, but not all cancers. Loss of nuclear expression is associated with poor prognosis (Donato, Cannon et al. 2013). S100A2 binds p53, which may explain its mechanism as a tumor suppressor (Salama, Malone et al. 2008, Donato, Cannon et al. 2013). Experimental reduction of S100A2 expression increases the motility of cells, while addition of S100A2 to cell media reduces rates of migration in squamous carcinoma cell lines (Gross, Sin et al. 2014). Overexpression in NSCLC cell lines, however, increases the rate of migration and invasion using transwell and trans-endothelial assays, and increases metastasis in vivo (Gross, Sin et al. 2014). In A549 lung cancer cells, S100A2 directly induces EMT and increases growth of tumors in immunocompromised mice (Naz, Bashir et al. 2014). S100A2 interacts with tropomyosin in a calcium dependent manner, but other connections to cell motility, as well as an explanation for how it both increases and decreases rates of migration remain elusive (Gross, Sin et al. 2014).

S100A4 overexpression is associated with metastases and decreased survival in many cancers, and it is considered an EMT promoter and marker (Salama, Malone et al. 2008, Chen, Xu et al. 2014, Gross, Sin et al. 2014). Increased expression of S100A4 was found to be correlated with decreased tumor cell differentiation and portal veil invasion in hepatocellular carcinoma. High S100A4 and vimentin expression along with low E-cadherin expression is correlated with an aggressive, malignant phenotype in this cancer as well (Zhai, Zhu et al. 2014). Increased levels of expression of S100A4 in renal proximal tubular epithelial cells, as well as tumor cells, results in a more mesenchymal phenotype (Gross, Sin et al. 2014). Overexpression can also cause increased invasion and motility in cancer cell lines derived from breast, pancreas, colon, and lung (Gross, Sin et al. 2014). In fact, overexpression in non-metastatic tumor cells leads to metastasis in xenograft (LLoyd, Platt-Higgins et al. 1998, Salama, Malone et al. 2008, Lukanidin and Sleeman 2012), whereas knockdown of S100A4 in metastatic tumor cells prevents metastases in animal models (Gunhild Mari Maelandsmo, Hovig et al. 1997, Lukanidin and Sleeman 2012). This is likely because S100A4 interacts with F-actin, non-muscle myosin heavy chain (NMMHC) IIA, tubulin, and non-muscle tropomyosin to increase cell migration through force generation as well as forming and stabilizing lamellipodia (Salama, Malone et al. 2008, Lukanidin and Sleeman 2012, Donato, Cannon et al. 2013, Gross, Sin et al. 2014, Ji, Huang et al. 2014, Bresnick, Weber et al. 2015).

Removal of S100A4 from a cell line results in increased assembly of non-muscle myosin IIA complexes, while overexpression results in large lamellipodia with a loss of focal adhesion maturation and filopodia (Gross, Sin et al. 2014). S100A4 is also involved in the regulation of matrix metalloproteinases (MMPs) and E-cadherin, whose dynamics are crucial in EMT (Salama, Malone et al. 2008, Donato, Cannon et al. 2013). MMPs degrade the extracellular

matrix, allowing metastasizing epithelial cells to move through the basement membrane and surrounding tissues (Ji, Huang et al. 2014). They also impinge on cellular signaling pathways (Lin, Sloniowski et al. 2008, Sugiyama, Gucciardo et al. 2013). Extracellular S100A4 in VMR cells results in increased MMP-13 activity and actin cytoskeleton remodeling (Lukanidin and Sleeman 2012). S100A4 also interacts with Rhotekin, a Rho binding and regulating protein that participates in actin-based contractility during migration and invasion (Gross, Sin et al. 2014).

During EMT, S100A4 is expressed when its promotor is stimulated by epithelial growth factor (EGF) and tumor growth factor β1, which leads to secretion of MMP-2 and MMP-9 (Strutz, Zeisberg et al. 2002, Lukanidin and Sleeman 2012). S100A4 is upregulated by cannonical Wnt signaling, and inhibition of β-catenin decreases tumor cell migration and invasion through downregulation of S100A4 (Donato, Cannon et al. 2013, Chen, Xu et al. 2014). NFAT is expressed in metastatic breast cancer and also transcriptionally activates S100A4 (Li, Wang et al. 2014). We found that NFAT activation is driven by calcium influxes during EMT, indicating that there might be multiple connections between S100A4 and increased intracellular calcium concentration in EMT (Langford, Keyes et al. 2012). In some types of cancer cells, extracellular S100A4 activates NF-κB to induce transcription of EphrinA1 and optineurin among other gene products which may lead to EMT (Chen, Xu et al. 2014).

S100A6 has been connected to cell proliferation, cytoskeletal dynamics, tumorigenesis, and ubiquitination of β-catenin (Donato, Cannon et al. 2013). Since its overexpression leads to cell specific phenotypes, it is difficult to identify a general role in cancer (Gross, Sin et al. 2014). It is known to interact with tropomyosin β and annexin 11 and 2 (Ji, Huang et al. 2014). High nuclear S100A6 levels are related to poor prognosis in pancreatic ductal adenocarcinoma (PDAC) (Chen, Liu et al. 2015). Experimental S100A6 overexpression in PDAC cells induces

increased expression of β-catenin, as well as mesenchymal markers N-cadherin and vimentin, and decreased expression of epithelial marker E-cadherin, which are the hallmarks of EMT (Chen, Liu et al. 2015).

At the cellular level, overexpression increased migration and invasion which is believed to be regulated through β-catenin (Chen, Liu et al. 2015). In fibroblast cells, knockout of S100A6 results in reorganization of the cytoskeleton to produce a cortical actin network, along with tropomyosin structures and an increase in vinculin at the cell periphery (Gross, Sin et al. 2014). Knockdown of S100A6 in the pancreatic cancer cell line Panc-1 leads to increased E-cadherin, and decreases N-cadherin and vimentin (Chen, Liu et al. 2015). These observations directly tie S100A6 to EMT in this cell type. However, in osteosarcoma cells, downregulation of S100A6 was correlated to an increase in cell motility, and upregulation with a decrease in cell motility, underlying the complexity and context dependent activity of S100A6 and S100 proteins in general (Gross, Sin et al. 2014).

S100A8/S100A9 is a heterodimer complex that promotes malignant progression in cancer. It interacts with the cytoskeleton in a calcium dependent manner that effects migration of monocytes and neutrophils, and also promotes microtubule polymerization and F-actin crosslinking (Lukanidin and Sleeman 2012, Donato, Cannon et al. 2013). S100A8/S100A9 is thought to regulate cell proliferation. Before metastasis, a tumor will secrete VEGF-A, TGFβ, and TNFα to cause lung endothelial cells to secrete S100A8 and S100A9, which may increase the motility of circulating cells and act as a chemoattractant to draw circulating tumor cells to certain tissues to set up metastases (Salama, Malone et al. 2008, Bresnick, Weber et al. 2015). Extracellular S100A8/S100A9 can activate the RAGE receptor to activate MAPK and NF-κB signaling to increase tumor aggressiveness and MMP2 and MMP12 levels (Lukanidin and

Sleeman 2012, Chen, Xu et al. 2014, Bresnick, Weber et al. 2015). S100A9 that is not complexed to S100A8 can reduce the S100A8/S100A9 complex's ability to cross-link F-actin and decrease microtubule polymerization (Donato, Cannon et al. 2013). It can also antagonize TGFβ1 from inducing EMT by binding to it (Basso, Bozzato et al. 2014). Knockout mice for S100A9 have reduced tumor incidence and decreased metastasis of both spontaneous tumors and after injection with colon cancer cells (Gross, Sin et al. 2014). An inhibitor for S100A9 is currently in clinical trials for prostate cancer (Bresnick, Weber et al. 2015).

S100P is currently believed to be upregulated in all cancers (Bresnick, Weber et al. 2015). It is a metastasis inducer and knockdown decreases metastatic potential *in vivo* (Chen, Xu et al. 2014). In HeLa cells, lung squamous carcinoma, pancreatic carcinoma, and breast carcinoma, overexpression leads to cellular motility (Gross, Sin et al. 2014). Knockdown in cell lines from colon cancer and pancreatic cancer, which have significantly high levels of S100P before knockdown, leads to decreased migration and invasion (Gross, Sin et al. 2014). In vivo, high S100P can induce carcinogenesis (Gross, Sin et al. 2014). Effects on the actin cytoskeleton are cell type specific, and in some cell lines there are no changes with overexpression (Gross, Sin et al. 2014). In HeLa cells, overexpression results in disruption of stress fibers, which leads to decreased focal adhesion formation through interaction with non-muscle myosin IIA (Gross, Sin et al. 2014). Overexpression in Panc-1 cells results in downregulation of several cytokeratins and an increase in expression of S1006 and strong phosphorylation of cofilin (Gross, Sin et al. 2014).

Calpains

Calpains are a family of 16 proteins, 14 of which are cysteine proteases and 2 of which are regulatory domains (Franco and Huttenlocher 2005, Xu and Deng 2006, Xu and Deng 2006). Calpains form heterodimers when one of the two regulatory proteins binds to one of the protease

calpains (Franco and Huttenlocher 2005, Xu and Deng 2006, Xu and Deng 2006). Unlike most proteases, calpains do not completely degrade a protein, but cleave proteins in a limited fashion as part of post-translational modification or as regulation of many proteins (Franco and Huttenlocher 2005). Substrates range from transcription factors, signaling enzymes, transmembrane receptors, cytoskeletal proteins, and focal adhesion proteins (Fox 1999, Franco and Huttenlocher 2005, Gressner, Lahme et al. 2008). The two most common and ubiquitously expressed isoforms of calpains are m-calpain and μ-calpain, which are activated with millimolar and micromolar concentrations of calcium respectively (Franco and Huttenlocher 2005). However, since physiological calcium levels do not readily reach the micromolar range, calpain activation is increased by phosphorylation by mitogen activated protein (MAP) kinase, epidermal growth factor (EGF), and PKCι (Franco and Huttenlocher 2005, Xu and Deng 2006, Xu and Deng 2006, Chen, Tang et al. 2013). Calpains have been shown to have a role in apoptosis, proliferation, cell migration, and cancer metastasis (Franco and Huttenlocher 2005, Xu and Deng 2006, Roumes, Leloup et al. 2010, Chen, Tang et al. 2013).

Calpain activity has been linked to tumor aggressiveness. The expression and activity levels of calpains tend to be higher in cancer cells than in control cells (Xu and Deng 2006, Roumes, Leloup et al. 2010, Chen, Tang et al. 2013). In hepatocellular carcinoma cell lines, knockdown of m- and μ-calpains results in decreased invasive, adhesive, and migratory potentials. It also decreases the secretion of MMP-2 and MMP-9, which are important for degrading the ECM so cells can migrate through it (Chen, Tang et al. 2013). In the rhabdomyosarcoma cells line ARMS, calpain activity is directly proportional to cell migration velocity (Roumes, Leloup et al. 2010). In fact, completely inhibiting calpain activity through adding the inhibitor calpeptin drops the invasiveness of ARMS cells down to the level of the

non-cancerous skeletal myoblast cell line LHCN-M2. Reorganization of the cytoskeleton is also seen when calpains are inhibited by calpeptin (Roumes, Leloup et al. 2010). It has been shown in human lung cancer cells calpains are activated by phosphorylation by PKCι, which is activated downstream of the nicotinic acetylcholine receptor (Xu and Deng 2006). This pathway directly increases the invasion and migration of lung cancer cells when treated with nicotine, and may explain some of the metastatic inducing behavior of nicotine (Xu and Deng 2006). Protein phosphastase 2A (PP2A) has been shown to dephosphorylate both m-calpains and μ-calpains, and decrease migration and invasion in lung cancer cells, even after treatment with nicotine (Xu and Deng 2006).

Chapter 5. Materials and Methods

MDCK Cells

MDCK cells have been used as a model for cell scattering and EMT since 1985 when they were shown to have a very rapid and pronounced response to media containing what is now known as HGF [19]. They are better than using a cancer cell line because they do not have mutations and altered physiology which could complicate results, and which are common in cancer cells. Cells were cultured in low glucose DMEM with 10% FBS at 37°C in 5% CO_2. Camui plasmid was obtained as a gift from Dr. Julie Bossuyt from University California Davis, and the Channelrhodopsin plasmid was obtained from Dr. Arminda Suli here at BYU.

Calcium Imaging Protocol

Cells were seeded onto collagenized glass 8-well dishes with 7,000 cells per well at least twenty-four hours before imaging. Wells were washed with PBS to remove any DMEM, which has fluorescent properties. PBS is removed and replaced with 2.25ug/mL fura-2 AM in Ringers solution with 10% FBS and allowed to incubate for a half an hour at 37 degrees Celsius. Cells are then washed and incubated for another thirty minutes in Ringers solution with 10% FBS without fura-2. Imaging was done with excitation at 340 nm/380 nm and emission above 500 nm, with an exposure time of one second at five second intervals. The ratio of the fluorescence intensity minus background at the two excitation wavelengths is calculated and analyzed.

Light Emitting Diode

In order to stimulate the channelrhodopsin transfected cells, a high intensity CREE XLamp XR-E royal blue LED (450-465 nm) controlled in both intensity and duration of light pulses by a custom made amplifier circuit that converts analog voltage input from a computer to a high current for the LED (Campagnola, Wang et al. 2008). The controller has a manual mode

to run simple experiments without computer input, and is connected to the computer through an Arduino. A simple computer program was used to program the Arduino, and through it, control the LED. The LED is soldered onto a heat sink to prevent thermal damage while running at high powers for long periods of time, and was held directly above the dish while stimulating cells. The advantages of using this system are that it works independently of the microscope light train, it is cheap to make, and it is portable for use on different microscope systems.

Live Cell Imaging

Live cell imaging was done with collagen coated DeltaT dishes from Bioptechs. Cells were cultured for at least 24 hours before use. Immediately before imaging, cells were washed and either a HEPES buffered DMEM for non-fluorescent imaging, or non-fluorescent HEPES buffered media for fluorescent imaging was added. The heated microscope stage from Bioptechs kept cells at 36 degrees Celsius. Long, bright light scattering movies had images taken every 2 minutes for 13 hours. Short fluorescent movies had images taken every three minutes for 60-90 minutes. Small molecule inhibitors, when used were added in approximately ten minutes before imaging, and HGF was added approximately five minutes before the start of imaging.

Immunofluorescence

Immunofluorescence was done with cells seeded for at least 24 hour on collagenized glass coverslips. Cells were induced with HGF for indicated lengths of time before being washed with PBS and being fixed with 4% PFA for twelve minutes on ice. Cells were left in the refrigerator in immunofluorescence blocker with DAPI until ready for use. Cells were stained with 100uL primary antibody solution for 45 minutes. (CaMKII gamma antibody NBP2-15685 from Novus, CaMKII delta antibody sc-5392 from Santa Cruz Biotech, Non-Muscle Myosin II antibody sc-47199 from Santa Cruz Biotech). Coverslips were washed 3 times for 5 minutes

each and stained with 100uL of secondary antibody solution. Coverslips were then washed 4 times for 5 minutes each and stained with alexa fluor-coupled phalloidin (Invitrogen) for 20 minutes. Coverslips were then washes 3 times for 5 minutes and mounted on slides with Vectashield and imaged under an Olympus BX41 microscope with a 60X oil lens.

FRET Analysis

FRET movies were obtained by imaging Camui transfected cells with both CFP and CFP/YFP filter cubes. These were done with both 10X and 40X air objective lens and a 1.6X optical zoom. FRET movies were analyzed using Slidebook to obtain the fluorescent intensity of the colony through masks, and background fluorescent intensity through an ROI, for each time point. This data was exported to Excel where the background intensity was multiplied by the mask area at each time point, which was subtracted from the mask total fluorescent intensity for each time point, to give a corrected colony fluorescent intensity that accommodated cell size changes during cellular spreading. This corrected intensity of FRET fluorescence was divided by the intensity of CFP fluorescence for each time point, and then all colonies from all similar experiments were averaged for the same time points to give data shown in Figure 2.5.

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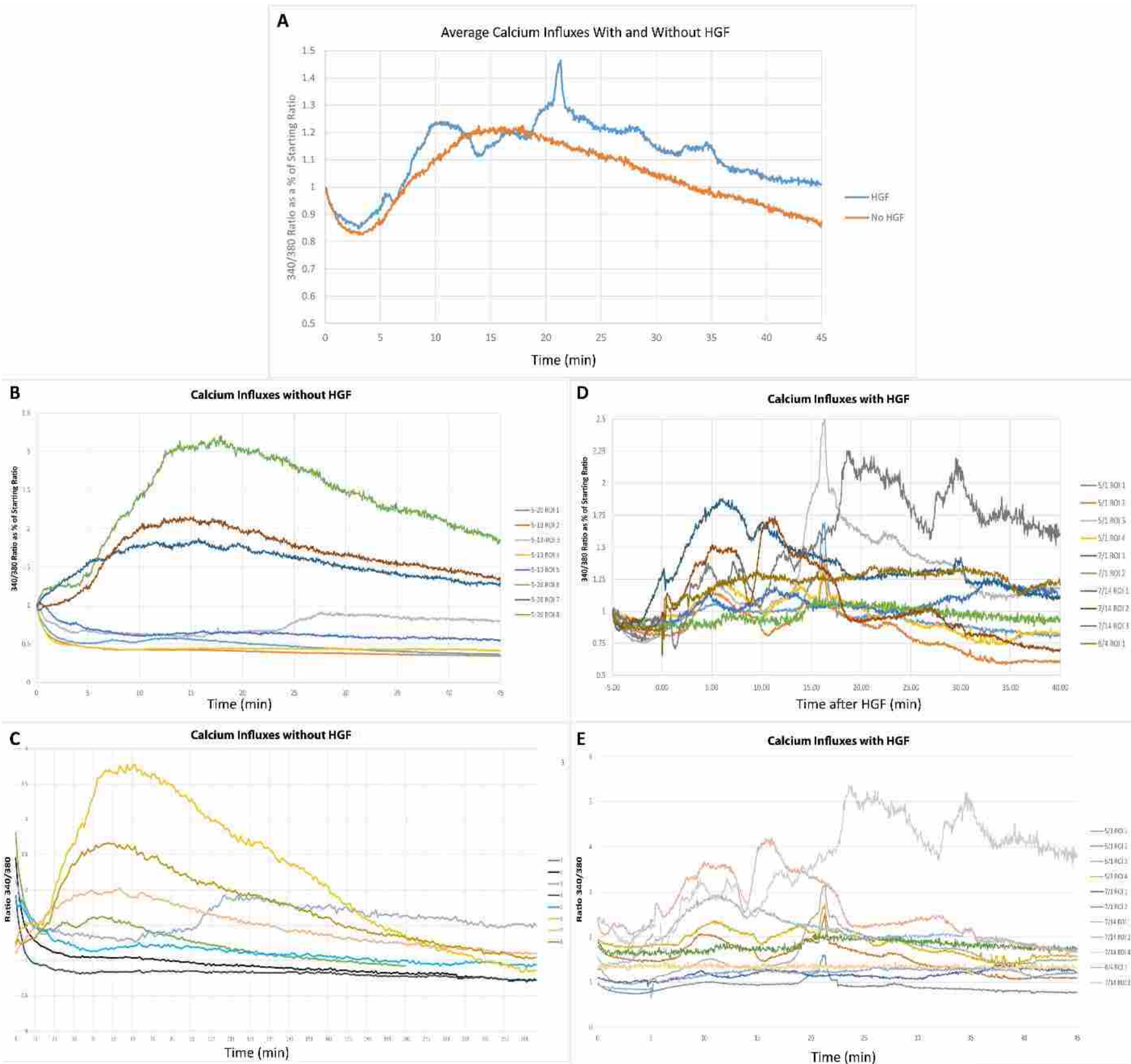

Figure 2.1: Calcium Influxes After HGF Stimulation.

Calcium imaging with Fura2-AM measuring total calcium concentration with and without HGF addition. Both treatments were imaged for 1000ms at both 340 and 380. (A) Averages of B and D overlaid, with values represented as percentages of the starting 340 to 380 fluorescent intensity ratios. (B-E) several experiments overlaid, with (B-C) unstimulated cells and (D-E) cells treated with HGF at time point 0 in (D) and 5 minutes in (E). (B, D) 340/380 ratios represented as percentages of the starting ratio. (C, E) 340/380 ratios left as raw data. Cells left untreated only show a single, smooth, large peak while cells stimulated with HGF show random calcium peaks in addition to the same peak seen in unstimulated cells.

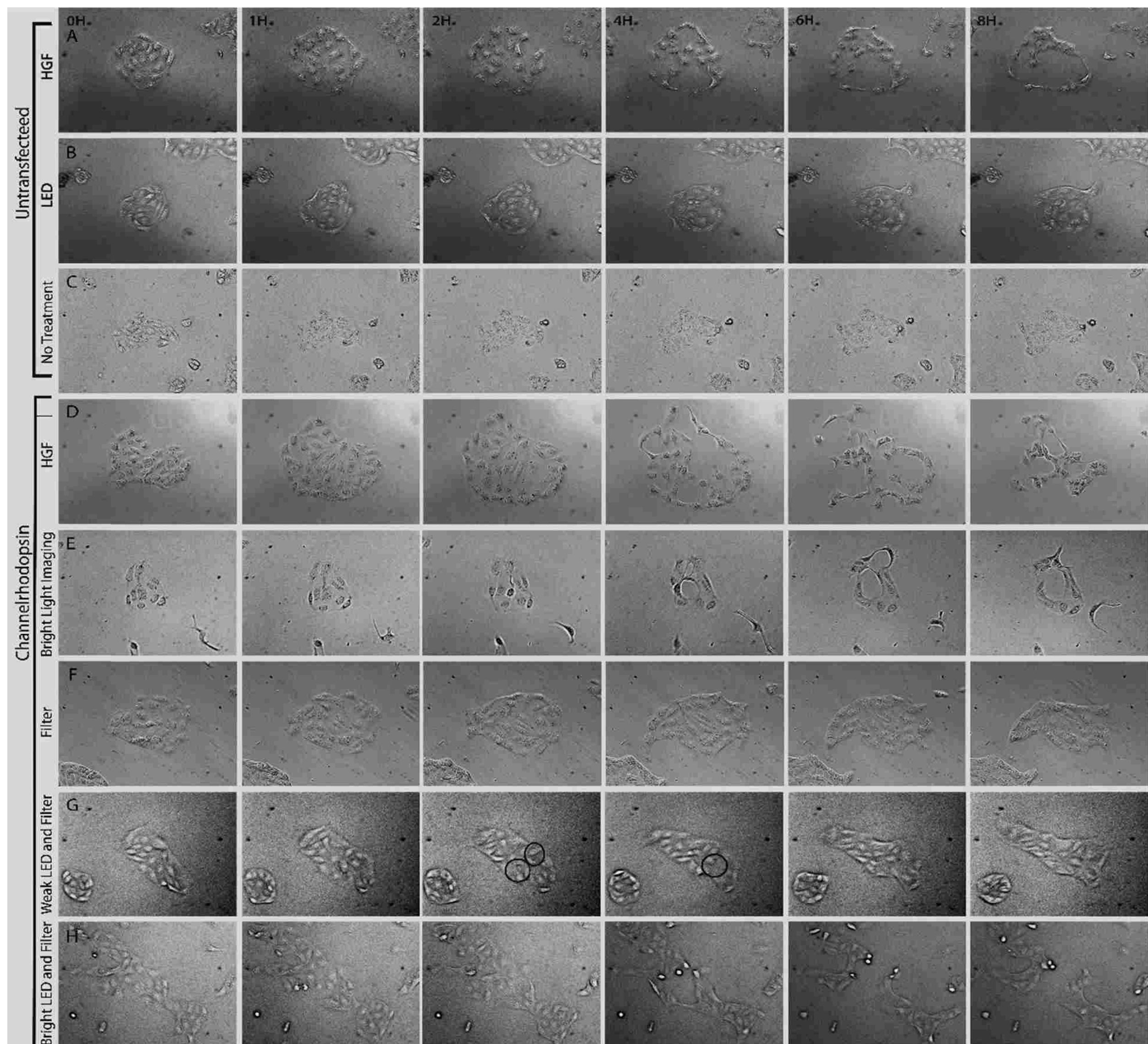

Figure 2.2: Optigenetic Stimulation Results in Disassembly of Cell-Cell Junctions.

(A) MDCK cells treated with HGF spread then pull apart and rupture cell-cell junctions. (B) LED treatment of untransfected MDCK cells results in the same behavior as untreated cells. (C) Untreated MDCK cells do not develop tears in the middle of colonies and no not move much. (D) ChR transfected cells treated with HGF scatter like untransfected cells. (E) Imaging with bright light results in cells contracting and disassembling cell-cell junctions due to the blue within the white light spectra. (F) Adding a filter that only allows red light through eliminates scattering behavior from channelrhodopsin cells. (G) Weak blue LED exposure results in small tears between cells in colonies. (H) Exposure to bright blue LED results in cells having a large increase in motility and ripping larger holes in the colony.

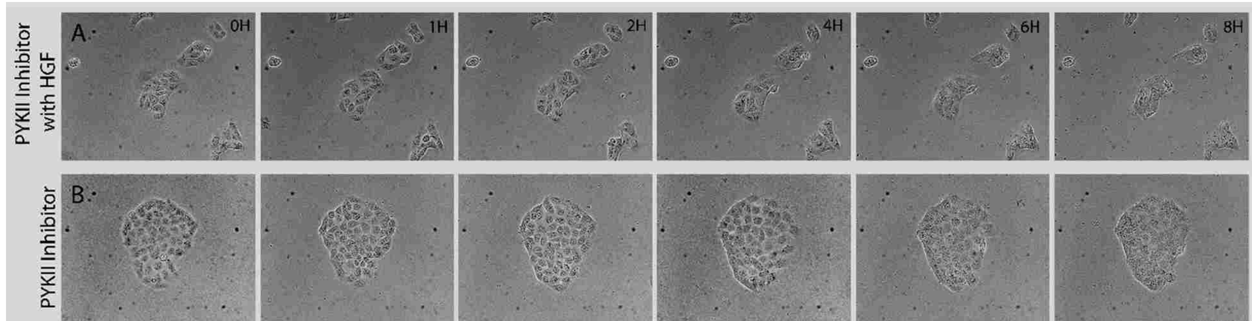

Figure 2.3: Inhibition of PYKII During EMT.

(A) Live cell imaging with the PYKII inhibitor PF-431396 results in increased cell shuffling within the colony. (B) With PF-431396 and HGF, the cells shrivel up and die.

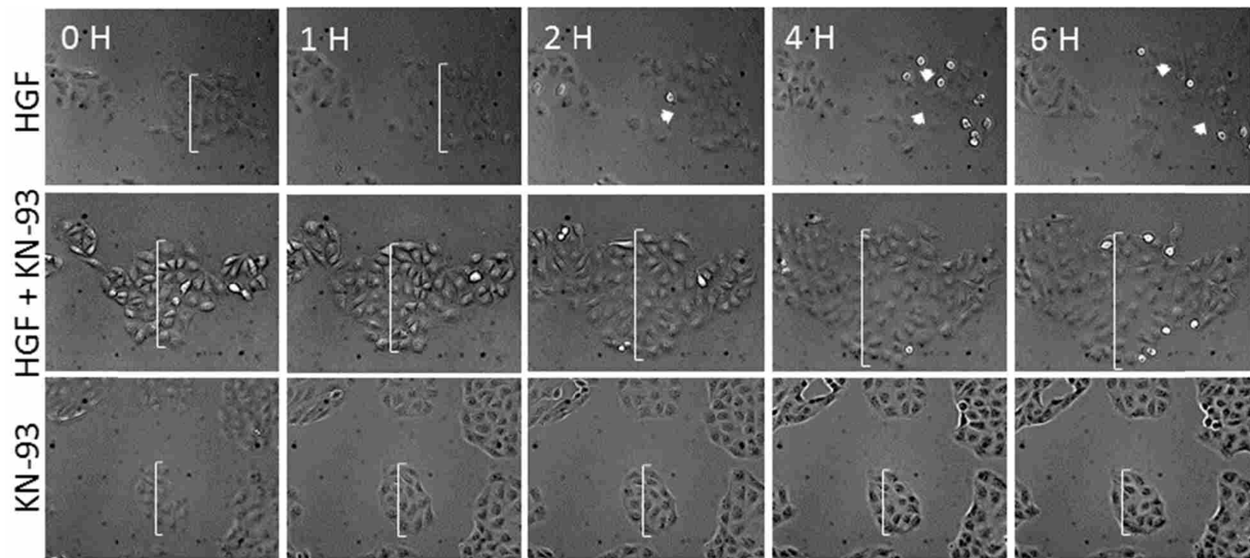

Figure 2.4: Inhibition of CaMKII During EMT.

Live cell imaging of MDCK cells treated with HGF results in an increase in colony surface area as cells spread, and cellular contraction resulting in cells pulling away from their neighbors. CaMKII inhibitor KN-93 stops all cell movements when added alone, and causes cell spreading without subsequent cellular contraction and pulling apart when HGF is also added.

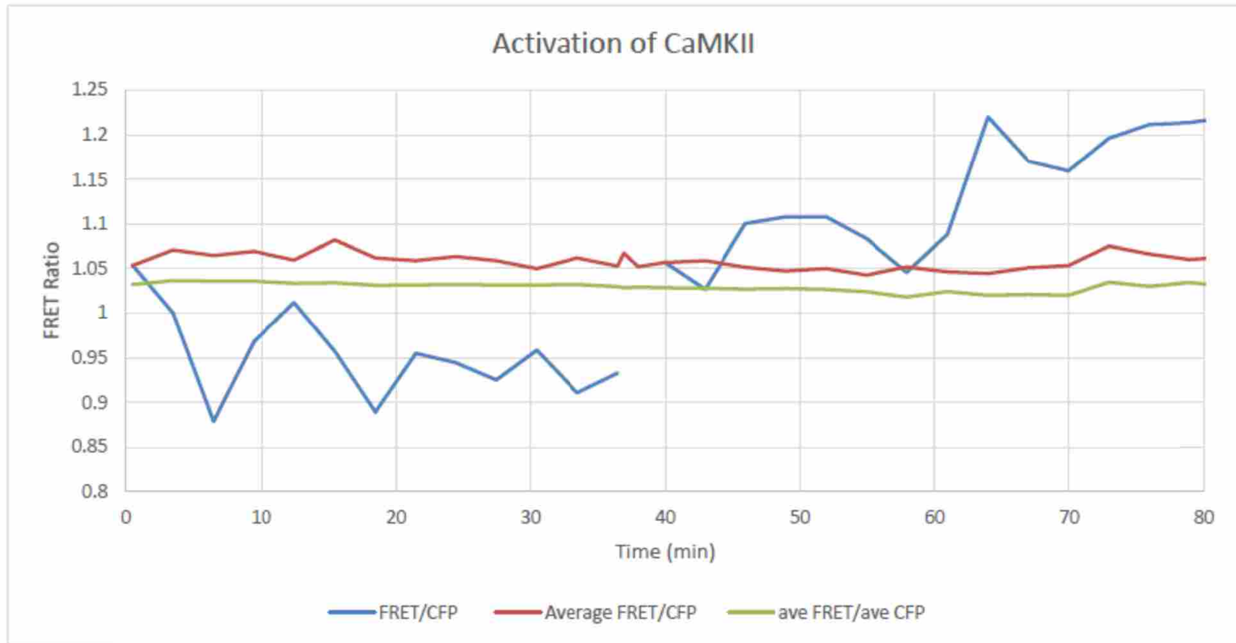

Figure 2.5: Activation of CaMKII after HGF Addition.

The CaMKII biosensor Camui has an increase in FRET/CFP when the enzyme is inactive, and a decrease when active and the active site is opened. When a CaMKII inhibitor is added, the FRET/CFP ratio increases. With no drugs or treatment, the average level of FRET/CFP does not change. With HGF treatment, the FRET/CFP ratio stays flat, indicating that the enzyme is not being activated within an hour of HGF stimulation.

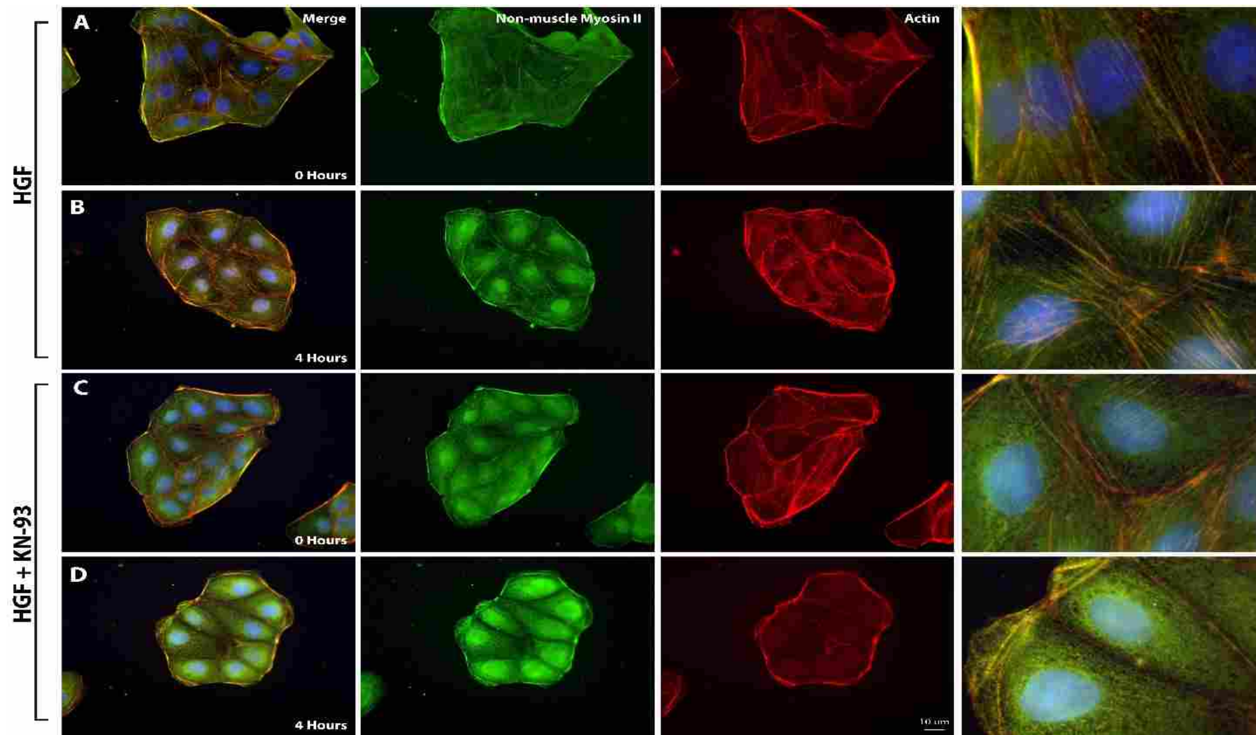

Figure 2.6: HGF Stimulated Actin Rearrangements are Prevented When CaMKII is Inhibited.

Immunofluorescence staining for actin and NMII. (A) Untreated cells exhibit some stress fibers. (B) HGF treatment causes reorganization of the actin cytoskeleton, specifically an increase in contractile stress fibers and trans-cellular actin networks. (C) Pre-treatment with KN-93 results in fewer stress fibers than untreated cells. (D) Pre-treatment with KN-93 and subsequent treatment with HGF results in no stress fibers or transcellular actin networks.

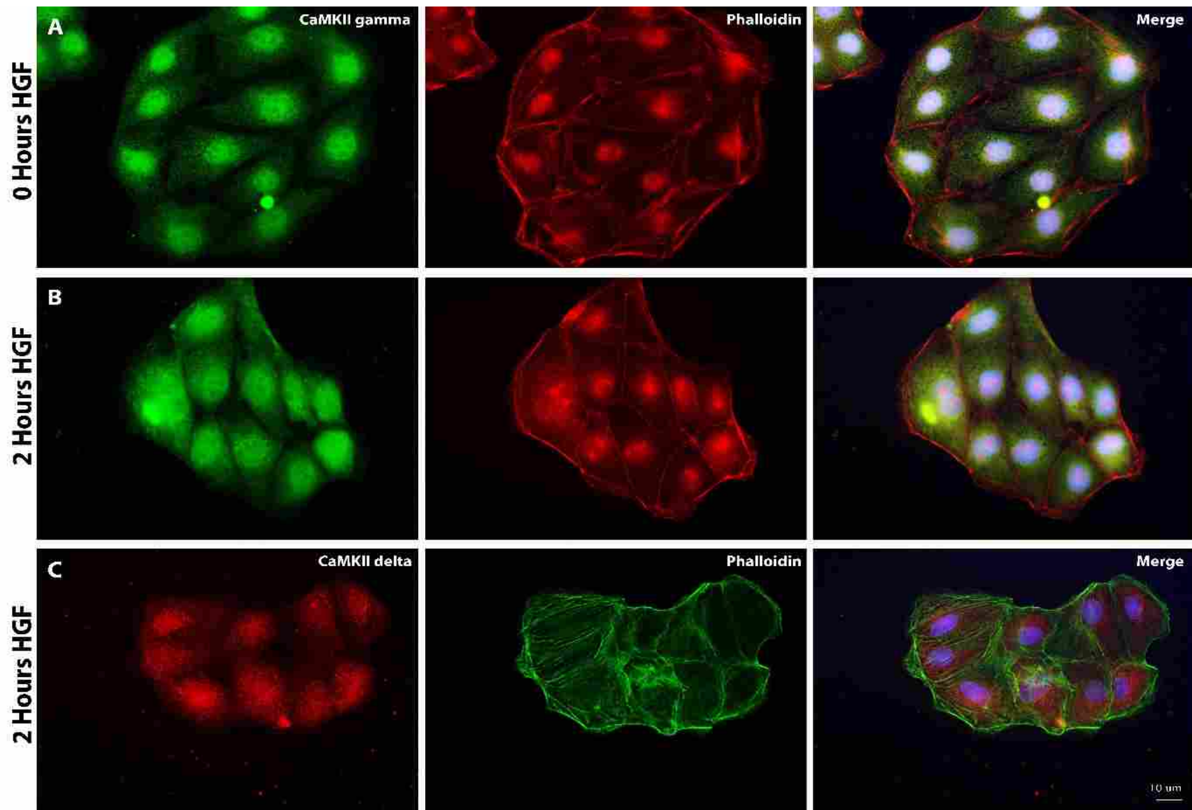

Figure 2.7: CaMKII Does Not Colocalize With Actin.

Immunofluorescence with (A-B) CaMKII gamma, (C) CaMKII delta, and phalloidin to stain for actin. CaMKII in neurons colocalizes with and bundles actin as a structural protein. In MDCK cells, actin and CaMKII have little to no colocalization so CaMKII is likely not acting as a structural actin bundling protein.

S100 protein	S100 protein potential functions in EMT
S100A1	– Inhibits microtubule assembly in a Ca^{2+} dependent manner in vitro. – Knockout in some cell lines results in reduced migration in wound healing assays. – Purified S100A1 and F-actin co-sediment, and in cultured vascular smooth muscle cells they colocalize (Gross, Sin, Barraclough, & Rudland, 2014).
S100A10	– Knockout decreases invasion, but not migration, of colorectal cancer cells and fibrosarcoma cells. – In epithelial squamous carcinoma cell lines and aggressive lung cancer cells, knockdown reduces migration in wound healing assays. – A heterotrimeric complex of S100A10 and Annexin 2 bundles actin filaments at the plasma membrane in a calcium dependent manner. – The RhoGAP DLC1 interacts with S100A10 to prevent it from interacting with Annexin 2 and to tag it for degradation (Gross, Sin, Barraclough, & Rudland, 2014).
S100A11	– Involved in cytoskeletal reorganization and cellular motility, as well as tumorigenesis. – Associates with actin, β-tubulin, intermediate filaments, and Annexin 1. – Overexpression results in an increase in pseudopodia (Ji, Huang, Jiang, Ni, & Xiao, 2014; Gross, Sin, Barraclough, & Rudland, 2014).
S100A14	– Decreases the stability of wild type p53, which may increase expression of MMP proteins, allowing increased invasiveness. – The opposite effect was seen with mutant p53 (Donato et al., 2013).

Figure 4.1: Less Studied S100 Proteins and Potential Roles in EMT.

CURRICULUM VITAE

Melissa McNeil

melissamcneil6@gmail.com   509 North 800 East Provo, UT 84606   Cell (949) 290-5196

Education

Masters of Science, Physiology and Developmental Biology,
Brigham Young University, Provo, UT, Dec 2015, 4.0 GPA
Thesis: Calcium signaling in Epithelial to Mesenchymal Transition
Advisor: Marc Hansen

Bachelors of Science, Physiology and Developmental Biology,
Brigham Young University, Provo, UT, Dec 2012, 3.59 GPA

Research Experience

Dr. Marc Hansen's Lab   Apr 2014 – Present
Brigham Young University, Provo, UT

- Applied molecular biology and bacteria culture to alter, clone, and purify plasmids
- Mastered tissue culture techniques for MDCK cells
- Stably transfected MDCK cells to grow three different monoclonal cell lines
- Oversaw the creation of an electronic LED amplifier controlled by a computer to do optigenetics with channelrhodopsin transfected cells
- Performed calcium and live cell imaging microscopy techniques to monitor cell behavior after initiation of epithelial to mesenchymal transition
- Conducted immunofluorescence to detect colocalization and reorganization of proteins and the actin cytoskeleton
- Utilized FRET analysis and western blots to detect activation of CaMKII
- Trained other lab members on tissue culture, live cell imaging, immunofluorescence, transfection, and FRET.

Dr. Arminda Suli's Lab   Rotation Jan 2014 – Mar 2014
Brigham Young University, Provo, UT

- Developed PCR primers, extracted DNA from zebrafish, performed PCR on extracted DNA, and cloned PRC product into bacteria to develop a plasmid containing an enhancer region that targets lateral line neurons in zebrafish.

Dr. Dixon Woodbury's Lab   Rotation Feb 2014 – May 2014
Brigham Young University, Provo, UT

- Studied the biophysics of exocytosis and SNARE proteins using protein fluorescence, lipid bilayer assay techniques, quantification of oxidation state of cysteines in SNARE proteins, and a protein binding assay using a quartz crystal microbalance.

Dr. Jeffery Barrow's Lab   May 2010 – Jul 2011
Brigham Young University, Provo, UT

- Performed PCR genotyping on thousands of DNA samples from lab mice
- Trained new lab members on PCR protocol and equipment

- Dissected mice and chick embryos at various stages of development

Grants and Awards

Simmons Center for Cancer Research Summer Fellow 2015

- Project title "Calcium signaling in cancer metastasis"
- Competitive grant that awards stipend support for research

Teaching Assistantships (5)

Research Assistantships (2)

Publications

- Calcium signaling in EMT. M. A. McNeil, H. Haws, M. D. Hansen (Manuscript)
- Rab8 mediated vesicle trafficking leads to calcium influx, CaMKII activation, and actin reorganization in EMT. M. A. McNeil, H. Haws, B. Chandler, C. M. Freitas, S. Kris, M. D. Hansen

Posters

Poster accepted for 2015 American Society for Cell Biology Annual Meeting

- Poster title "An optigenetic approach to show that calcium in epithelial to mesenchymal transition activates Ca2+/calmodulin–dependent protein kinase II, increases cellular contractility and breaks cell–cell junctions"

Teaching Experience

Physiology Lab Instructor Jan – Dec 2014, Aug – Dec 2015

BYU Department of Physiology and Developmental Biology, Provo, UT

- Taught daily lectures on physiology concepts to labs of up to thirty students
- Supervised physiology experiments ranging from creating an osmometer out of dialysis tubing to performing EKG's and blood typing

Cell Biology Teaching Assistant Jan – Apr 2015

BYU Department of Physiology and Developmental Biology, Provo, UT

- Facilitated the understanding of complex scientific papers and cell bio concepts to undergraduates through guiding group discussions during class, holding office hours, and critiquing assignments.