THE ROLE OF Na^+/H^+ EXCHANGER-1 (NHE1) IN MAMMARY BRANCHING MORPHOGENESIS AND MAINTENANCE OF TISSUE ARCHITECTURE

By

Edmund C. Jenkins Jr.

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Edmund C. Jenkins Jr.

Advisor: Dr. Jimmie E. Fata

Abstract

Branching morphogenesis *in vivo* is a highly ordered process that necessitates spatially and temporally choreographed cues by growth factors and hormones, as well as mechanical and signal feedback from the extracellular matrix. Successful completion of this developmental process results in the architectural, and thereby the functional, basis for the lung, collecting ducts of the kidney, salivary, and mammary glands. The quest to understand the basic biological mechanisms underlying this developmental morphogenesis has lead to many seminal findings in the field of epithelial tube generation, as well as provided valuable insight into the pathogenesis of cancer. The primary focus of this thesis was elucidating the role of the Na+/H+ exchanger type 1 (NHE1) in branching morphogenesis of the mouse mammary gland. To accomplish this goal, we used three-dimensional (3D) primary tissue culture of mammary gland pieces (organoids) in a four day organotypic assay of growth factor induced branching morphogenesis. NHE1 is a ubiquitously expressed master regulator of intracellular pH (pHi). We found that blocking the function of this exchanger in the presence of growth factor stimulation led to altered kinase signaling, inhibition of growth factor induced alkalization, sustained proliferation after four days, ectopic expression of keratin 6 (K6), and a dramatic failure to undergo branching

morphogenesis.

These findings led us to question the role of NHE1 in the maintenance of branched mammary tissue architecture. We, therefore, inhibited NHE1 function on fully branched structures in our assay and found that NHE1 inhibition led to rapid loss (within 24 hours) of branched architecture by a process of branch fusion, with complete loss of the branched morphology after four days. This was not accompanied by cell death or altered proliferation, however, we did record altered intracellular pH (pHi) in the end buds of branched structures that had NHE1 inhibited. NHE1 localization, F-actin organization, and myoepthelial cell location were altered in structures that had undergone a loss of architecture, indicating a loss of tissue organization. Finally, we found that NHE1 inhibition resulted in a decrease in mammary E-cadherin.

Having found that NHE1 function is vital for both branching morphogenesis and the maintenance of branched architecture, we considered the role that NHE1 could be playing in the pathology of breast cancer. Both intracellular and extracellular pH is deregulated in cancer. This could be attributed to over activity of NHE1. Additionally, NHE1 is over expressed in many cancers. We used the ER+ breast cancer cell line MCF7 to investigate the therapeutic potential of chemotherapy augmentation by NHE1 inhibition. We found that Cycylophosphamide, a DNA alkylating chemotherapeutic agent known to be more effective in an acidic environment, was roughly 5 times more effective when used along with NHE1 inhibition. These findings indicate that NHE1 is a critical regulator of branching morphogenesis and tissue stability, as well as suggests a potential therapeutic target for the treatment of breast cancer.

Co-Authorship

This dissertation is composed from work that has been previously published or submitted for publication. Co-authors for this work include: Edmund C. Jenkins Jr., Jimmie E. Fata, Shawon Debnath, Stephen Gundry, Sajini Gundry, Sophia Verriano, Kaitlin Kelly, Nidhi Khanna, Diane Narouz, and Mino Abdelmissah. All experimental procedures were performed by Edmund C. Jenkins Jr. except for the following: Shawon Debnath performed the subcellular fractionation and immunoblotting for the signaling and protein expression referred to in chapters 1 and 2. Stephen Gundry developed imaging software for intracellular pH visualization. Sajini Gundry and Sophia Verriano assisted in tissue isolation for morphogenic experiments and immunocytochemistry. Nidhi Khanna, Diane Narouz, Kaitlin Kelly and Mino Abdelmissah performed many of the MTT assays referred to in chapter 4. Edmund C. Jenkins Jr. and Jimmie E. Fata wrote chapters 1 and 2 of this dissertation. Edmund C. Jenkins Jr. wrote chapters 3 and 4. The data in Chapter 3 represents a collaborative effort with Dr. Edward Gresik.

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Dedication

I would like to dedicate this work to:

My parents, Edmund and Valerie; whose continued support has provided me with the means to achieve my highest goals,

Lou-Fu and Kuey Fen Ni, who are my second parents,

William, whom I have the pleasure and honor to call "brother" and mean it in the very best sense of the word, *I Sam 20:23*

Dave and Carolyn, who have long watched and waited for me to reach this point,

and to my beloved Sara, my present and future. *Eph 5:25*

I can do all things through Christ which strengthenth me. *Phil 4:13*

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List of Abbreviations

2D – Two dimensional
3D – Three dimensional
μm – Micrometer
μM – Micromolar
mM – Millimolar
BCECF-AM – 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein
bFGF – Basic Fibroblast Growth Factor
BMP – Bone morphogenic protein
BSA – Bovine Serum Albumin
CPA – Cyclophosphamide
Dox – Doxorubicin
DMEM – Dulbecco's Modified Eagle Medium
ER – Estrogen Receptor
FCS – Fetal calf serum
GDNF – Glial cell line-derived neurotrophic factor
IC_{50} – Inhibitory concentration for 50% of a population
MAPK – Mitogen Activated Protein Kinase
MEC – Mammary epithelial cell
MEK – Mitogen-activated protein/extracellular signal-regulated kinase kinase
MIA – 5-(N-methyl-Nisobutyl)-amiloride
MM – Metanephric mesenchyme
NHE – Na+/H+ Exchanger
PBS – Phosphate buffered saline
PH3 – Phospho Histone H3
PI – Propidium iodide
PI3K – Phosphatidyl-inositol-3-kinase
PLCcl – phospholipase Cc1
SDS-PAGE – Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Shh – Sonic hedgehog
SMG – Submandibular gland
TGFα – Transforming Growth Factor Alpha
UB – Ureteric bud
K6 – Keratin 6
K8 – Keratin 8
K14 – Keratin 14

Introduction

The Swedish physician, botanist, and zoologist Carl Linnaeus first used the term *mamalia* in 1758 in the 10th edition of *Systema Naturae*. The word is derived from the Latin word *mamma*, meaning "pap" or "teat." This is the etiology of the modern scientific term *mammal*, which is a classification of animals that share, among other things, the ability to nurse their young by milk production through mammary glands. Indeed, the mammary gland is one of the defining characteristics of mammals. Early studies of this gland were conducted in the mid 19th century using swine, goats, and cattle. These works showed us that mammary glands are branched networks of epithelial tubes in fatty stroma that convey milk toward a common point, i.e. the nipple. In 1882, Anthony Bowlby gave the world a model of the developmental progression of the human mammary gland that is still correct today (Bowlby, 1882). Following these works, hormonal control of milk production and branching in the mammary gland began to be explored in the mid-late 20^{th} century (Voogt, 1978; Warner, 1976). Despite these classical and essential works, insights into the molecular mechanisms of hormonal control, as well as stromal cell and extracellular matrix contributions, that underly the development and dynamic regulation of the mammary gland *in vivo* could not be obtained until the late 20^{th} and early 21^{st} centuries (Daniel and Silberstein, 1987; Fata et al., 1999; Furumura et al., 1983; Imagawa et al., 1990; Kimata et al., 1985; Levay-Young et al., 1990; Parmar and Cunha, 2004; Silberstein and Daniel, 1987a; Silberstein and Daniel, 1987b; Sternlicht et al., 2006; Sternlicht et al., 2005; Wiseman and Werb, 2002).

The mouse mammary gland has become a powerful tool to study the developmental and morphogenic processes that lead to the mature gland. The gland starts as a placode of epithelial cells subjacent to the dermis, with the first rudimentary branches arising on embryonic day 16

(E16) (Cowin and Wysolmerski, 2010; Watson and Khaled, 2008). These remain dormant until ovarian hormones are released during the onset of puberty (3 to 4 weeks of age), at which time systemic ovarian hormone circulation drives branching morphogenesis of the mouse mammary gland by a combination of three processes: elongation of the primary ducts, bifurcation of end buds, and lateral side branching. Each of these processes is governed by a delicate reciprocating balance of hormonal, juxtacrine, and paracrine signaling, as well as mechanical and molecular signaling from surrounding extracellular matrix (ECM) (Sternlicht et al., 2006; Wiseman and Werb, 2002). Understanding the control and initiation of the molecular signaling mechanisms that generate the branched architecture of the mammary gland is intimately related to our understanding of the etiology of breast cancer, as many of the developmental and regulatory pathways in the mammary gland are deregulated in breast cancer (Booth et al., 2007; Booth and Smith, 2007; Bundy et al., 2005; Busser et al., 2011; Dimri et al., 2005; Incassati et al., 2010; Incassati et al., 2009).

Cellular pH regulation is a critical aspect of homeostatic regulation in mammalian cells and has been shown to be de-regulated early in transformation by the action of the Na+/H+ exchanger subtype 1 (NHE1) (Cardone et al., 2005; Reshkin et al., 2000). NHEs are a family of integral membrane proteins that regulate intracellular pH (pHi) by catalyzing the electro-neutral exchange of intracellular H+ for extracellular Na+ in a 1:1 stochiometry (Casey et al., 2010). In the chapters below, we show that NHE1, a master regulator of pHi, is critically necessary for both mammary branching morphogenesis and the maintenance of branched mammary architecture. We investigated its role in the development of other branched organs in the mouse, i.e. the lung, kidney, and salivary gland, and explored the therapeutic potential of NHE1 inhibition for augmentation of chemotherapeutic agents. Our results show a novel role for NHE1

and pH homeostasis in both the development and maintenance of a branched mammary structure, and suggest that NHE1 should be a potential target for the treatment of breast cancer.

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Chapter 1: Intracellular pH regulation by Na^+/H^+ exchanger-1 (NHE1) is required for growth factor-induced mammary branching morphogenesis.

Abstract

Regulation of intracellular pH (pHi) and protection against cytosolic acidification is primarily a function of the ubiquitous plasma membrane Na+/H+ exchanger-1 (NHE1), which uses a highly conserved process to transfer cytosolic hydrogen ions (H+) across plasma membranes in exchange for extracellular sodium ions (Na+). Growth factors, which are essential regulators of morphogenesis, have also been found to be key activators of NHE1 exchanger activity; however, the crosstalk between both has not been fully evaluated during organ development. Here we report that mammary branching morphogenesis induced by transforming growth factor-alpha (TGFα) requires PI3K-dependent NHE1-activation and subsequent pHi alkalization. Inhibiting NHE1 activity after TGFα stimulation with 10 μM of the NHE1-specific inhibitor N-Methyl-N-isobutyl Amiloride (MIA) dramatically disrupted branching morphogenesis and induced extensive proliferation, and ectopic expression of the epithelial hyper-proliferative marker Keratin-6. These findings indicate that mammary morphogenesis is dependent on a novel mechanism that requires a TGFα>PI3K>NHE1>pHi alkalization signaling.

Introduction:

Branching morphogenesis of an epithelial tube network is a highly conserved process observed in the development of the lung and kidney as well as the salivary and mammary glands. The latter uniquely undergoes most of its branching during postnatal development at the onset of puberty when the release of systemic ovarian hormones begins (Fata et al., 2004; Khokha and Werb, 2010; McNally and Martin, 2011; Sternlicht et al., 2006; Wiseman et al., 2003; Woodward et al., 1998). Three dimensional (3D) organotypic culture assays that model in vivo mammary branching morphogenesis have furthered our knowledge by defining important signaling cascades (Andersen et al., 2011; Fata et al., 2007; Pasic et al., 2011; Sternlicht et al., 2005) and morphogenetic-associated cellular movement (Ewald et al., 2008). These organotypic models, as well as studies investigating in vivo morphogenesis, have highlighted the essential role of how regulated growth factor signaling induces mammary ductal development and how deregulated growth factor signaling promotes tumor initiation and progression (Booth and Smith, 2007; Hynes and Watson, 2010; Smith et al., 1995; Stull and Wood, 2003).

Determining how mammary tissue integrates and regulates signals elicited by growth factors is critical to a better understanding of both morphogenesis and cancer. In cell cultures, an inherent downstream event of growth factor signaling is an associated intracellular pH (pHi) alkalization, which has been implicated to induce metabolism and proliferation (Grinstein et al., 1989; Moolenaar et al., 1983; Putney and Barber, 2003; Steffan et al., 2010; Strazzabosco et al., 1995). Yet at the tissue level, the occurrence, regulation, and subsequent importance of pHi alkalization during growth factor-induced morphogenesis has not been investigated. Several signaling cascades, such as the PI3K and MAPK signaling pathways, which lay downstream of growth factor signaling, have been implicated in regulating intracellular pH changes in cells

(Bianchini et al., 1997; Khaled et al., 2001; Kintner et al., 2005; Luo et al., 2007; Malo et al., 2007; Meima et al., 2009; Takahashi et al., 1999; Tominaga and Barber, 1998; Yan et al., 2001) and in regulating branching morphogenesis in several organs including the mammary gland (Fata et al., 2007; Hynes and Watson, 2010; Ren et al., 2010; Wickenden and Watson, 2010). However, whether intracellular pH regulation by these pathways is an essential mechanism for branching morphogenesis has not been investigated.

Sodium hydrogen exchangers (NHEs) are a family of integral membrane proteins that regulate intracellular pH (pHi) by mediating the electro-neutral exchange of intracellular hydrogen with extra-cellular Na+. To date, there have been 9 subtypes of NHE identified, exhibiting tissue and membrane specific expression patterns (Casey et al., 2010; Putney et al., 2002; Slepkov et al., 2007). NHE isotypes 1-5 are found on the plasma membrane while isotypes 6-9 are localized to intracellular organelle membranes. NHE1, which is ubiquitously expressed in epithelial cells, has been assigned three primary cellular functions: the main regulator of epithelial pHi (Jang et al., 2006; Steffan et al., 2009), a regulator of cellular acidosis induced apoptosis (Casey et al., 2010; Lagadic-Gossmann et al., 2004; Pedersen, 2006; Wang et al., 2008) and a regulator of cell migration (Koliakos et al., 2008; Martin et al., 2011; Stock et al., 2008; Stock et al., 2005; Stock and Schwab, 2006; Yang et al., 2010; Yang et al., 2011). Additionally, NHE1 has been shown to be responsible for intracellular alkalization following growth factor stimulation (Grinstein et al., 1989; Moolenaar et al., 1983; Strazzabosco et al., 1995; Takahashi et al., 1999) and NHE1 activity is often upregulated in oncogene-induced epithelial tumor cells leading to extracellular acidification (Cardone et al., 2005; Harguindey et al., 2005; McLean et al., 2000; Reshkin et al., 2000; Stock and Schwab, 2009). Moreover, a number of kinases, such as ERK, PKC, NIK and p90rsk are capable of activating NHE1

exchanger function by direct or indirect phosphorylation (Bianchini et al., 1997; Kintner et al., 2005; Luo et al., 2007; Malo et al., 2007; Meima et al., 2009; Moor and Fliegel, 1999; Takahashi et al., 1999; Tominaga et al., 1998; Yan et al., 2001). NHE1 has also been implicated in acting as an upstream regulator of signaling cascades (Pedersen et al., 2007). While a number of studies have confirmed the cross talk between growth factor signaling and NHE1 regulation of epithelial intracellular pH, the function of NHE1 exchanger activity as a regulator of growth factor-induced tissue development has not been fully defined.

To analyze whether controlled intracellular pH changes occur via growth factor-regulated NHE1 activation and to determine if this pathway is essential for mammary morphogenesis we used 3D organotypic mammary cultures, precise measurements of pHi and the NHE1 specific inhibitor N-Methyl N-isobutyl amiloride (MIA). Our findings define a novel developmental mechanism that requires growth factor activation of NHE1 regulated intracellular pH alkalization. We provide further evidence that inhibition of NHE1 exchanger function, in the presence of growth factor stimulation, leads to drastic reduction of mammary branching morphogenesis, sustained proliferation, and severe loss of tissue architecture.

Materials and methods

Mammary gland tissue isolation:

Mammary gland isolation was performed primarily as described previously (Fata et al., 2007). The fourth inguinal mammary glands were isolated from virgin Balb/C mice 14-30 weeks postnatal. The glands were diced using two standard razor blades held parallel to each other. Diced glands (2) were digested for 30 min under gentle shaking at 37°C in a 10 ml collagenase/trypsin mixture (0.2% trypsin/ 0.2% collaginase, 5% fetal calf serum, 1000 U/ml

Penicillin/Streptomycin). After digestion, the suspension was centrifuged at 201g for 10 minutes. The pellet was resuspended in 10 mLs of DMEM/F12 and spun again at 201g for 10 min. The supernatant was discarded and the pellet was suspended in 4 ml of DMEM/F12 plus DNase (2 U/µl) with gentle shaking at room temperature for 5 minutes. The suspension was again spun at 201g for 10 min and the DNase solution was discarded. Pieces of mammary epithelial ducts (mammary organoids) were separated from single cells by differential centrifugation. This included pulse spins to 67g a minimum of 3 times, discarding the supernatant every time. The final pellet was used for either 3D culturing in Matrigel or 2D cultures on plastic. For live video-microscopy, tissue was maintained in a fully enclosed microscope (Zeiss Axio Observer) with humidity, CO_2, and temperature control.

3D Branching Assay:

Mammary organoids were suspended in matrigel s and cultured in a 96 well format. Before the organoids were transferred to a 96 well plate, a supporting layer of 40 µl matrigel was laid down in each well and allowed to solidify for at least 30 min at 37 ^{o}C. Mammary organoids were cultured in 10% matrigel at a density of 70 organoids/well in basal media (DMEF/F12 with 1% insulin, transferrin, selenium, and 1% penicillin/streptomycin) for 24 hours. After 24 hours, organoids were stimulated with 18 nM TGFα in the presence or absence of 5-(N-methyl-N-isobutyl)-amiloride (MIA) (Sigma-Aldrich, Saint Louis, MO) a NHE1 specific inhibitor. Every other day all samples were replenished with basal media alone. To determine the morphogenic response we counted all organoids within each well having 3 or more branches and divided this number by the total number of organoids per well (Fata et al., 2007). The MEK inhibitor PD98059 (40 uM; Sigma-Aldrich, Saint Louis, MO) and the PI3K inhibitor LY294002 (25 uM;

Cell Signaling Technologies, Boston, MA) were pre-incubated for 90 minutes prior to the addition of growth factor. Cultures on plastic (2D) were seeded at 70 organoids/well plus 5% FCS in basal media, with media changes every other day.

2D cell culture:

Pieces of primary mammary epithelial tissue (organoids) were isolated as described above and cultured at 70 organoids/chamber in an 8-well chamber slide (BD Falcon). Organoids were suspended in MEC media plus 5% FCS and allowed to adhere to the slide and grow out for at least 24 hours prior to treatment conditions. Primary mammary epithelial cells (MECs) were allowed to grow off the organoids and form sheets. Tissue residues were not removed directly, but in many cases were rinsed off in subsequent media changes.

Immunostaining for Keratins 8, 14, and 6:

Mammary organoids cultured in 3D were fixed with methanol/acetone 1:1 for 20 min at -20˚C and rinsed with PBS twice for 5 min in the 96 well culture plate. After fixation and washing, the matrigel was removed from the well and partially air-dried onto an eight chamber slide. Blocking was performed with 10% horse serum, 2% bovine serum albumin, and 0.5% Triton X-100 in PBS for 1 hour gently shaking at room temperature. Primary antibodies against keratin-14 (K14) (clone #LL002, Novocastra, UK), keratin-8 (K8) (Troma-I-c, DSHB, Iowa City) or keratin-6 (K6) (PRB-169P, Covance Research Products, Berkeley) were incubated overnight shaking at 4˚C. Following a wash series (10 min PBS plus 0.5% triton X-100, 2x10min PBS), fluorescent conjugated secondary antibody (Invitrogen) incubation was performed at a concentration of

1:1000 for 1-3 hours. After another wash series, samples were stained with DAPI or PI for 5 min and imaged.

Immunostaining for phosph- histone H3 and NHE1, NHE2 and NHE3:

Cultures were fixed with 4% paraformadehyde for 5 minutes at 37˚C. The following primary antibodies were suspended in blocking buffer (see above) and were incubated overnight shaking at 4˚C: rabbit polyclonal anti-human phospho-histone H3 (Ser10, Millipore, Billerica, MA), rabbit polyclonal anti- human NHE1 (H-160:sc 28758; Santa Cruz Biotechnologies, CA.), goat polyclonal anti- rat NHE2 (V-20:sc-16099; Santa Cruz Biotechnologies, CA.), and goat polyclonal anti- human NHE3 (C-20: sc-16103; Santa Cruz Biotechnologies, CA.). Detection was performed as described above for keratin immunostaining.

Cell death assays

Propidium iodide was used to detect cells with compromised plasma membranes in tissue cultured in 3D. 60 µg/mL of propidium iodide was added to live tissue cultures and allowed to incubate at 37˚C for 5 minutes. Tissue was then imaged using bright-field and fluorescent microscopy. The bright-field image was then overlaid with the fluorescent image using ImageJ. Quantification was performed by counting the number of cells positive for propidium iodide staining and dividing that number by the total area of the tissue. Area was measured in ImageJ.

Measurement of intracellular pH using BCECF-AM:

For the generation of a standard intracellular pH curve, primary MECs were isolated and cultured in 2D before incubation with known pH adjusted buffers as described previously

(James-Kracke, 1992; Thomas et al., 1979). The buffers consisted of 135 mM KCl, 10 mM NaCl, 10 mM glucose, 10 mM Tris Base, 10 mM MES, and were adjusted to pH 6.6, 6.8, 7.0, 7.2, 7.4, 7.6, or 7.8 with HCl or NaOH. The standard curve was generated by incubating primary MECs (cultured in 2D) with nigericin (10 μM; Invitrogen) in pH adjusted buffers for 40 min at 37˚C. After incubation, a fresh volume of buffer at each pH was added containing the pH-sensitive fluorescent dye BCECF-AM (2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein; 5 μM; Acros Organics). Tissue was incubated with BCECF-AM for 10 min at 37˚C before being rinsed off with 10 μM nigericin in pH adjusted buffers.

Fluorescent images were captured using a filter set designed for ratio imaging of BCECF-AM, including a dichroic filter for detecting emission at 535nm, and two filters for alternative excitation of 490nm (the pH sensitive wavelength) and 440nm (the isobestic point) (Carl Zeiss, Germany). The light source was a mercury lamp. An image was created showing the ratio value (i.e., pixel by pixel division) of the 440 nm and 490 nm fluorescent images using the image calculator function of ImageJ (Abramoff et al., 2004). This ratio image was then imported into the MATLAB (by MathWorks) computational environment. In this setting, we implemented the Henderson-Hasselbalch equation on each pixel within the 1040 x 1388 tif ratio and calculated the corresponding pH value using the following formula: $pH_i = pK_a - \log [(R_{max} - R)/(R - R_{min})]$. The constant values used in the formula were $pK_a = 6.97$, $R_{min} = 0.3233$, and $R_{max} = 1.7167$. The values for R_{min} and R_{max} were obtained from our standard curve and are the ratio values measured for our pH 6 and pH 8 standards respectively. These pH values were chosen as our upper and lower bounds because the 440nm/490nm ratio values of BCECF are linear in this range. We chose to spread the color spectrum over a range of pH values that would encompass the pH range

observed in a given experiment and would visually depict the fine pH changes (~0.1 pH units) that we observed upon the addition of growth factor. These values were then mapped to a 16-bit discrete color representation from blue to red.

Generation of an intracellular acid load with NH_4 prepulsing

Primary MECs cultured in 2D were used for acid loading and NHE1 mediated recovery by NH_4 prepulsing using a similar method, which has been previously described (Jang et al., 2006). An intracellular acid load was generated by incubating MECs with a modified Ringer's buffer containing 20 mM NH_4Cl with 20 mM HEPES for 5 minutes. The ringer's buffer was then washed away in buffer containing neither NH_4Cl nor Na+, and incubated for 1-2 minutes. Recovery was allowed by the addition of Na+ to the extracellular environment in the form of 140 mM NaCl in 20mM HEPES buffer or base media containing 81.9 mM NaCl and 44.05 mM sodium bicarbonate. In our assay, intracellular pH was recorded using the BCECF method outlined above 5 minutes after addition of Na+ containing media.

Results

TGFα induces rapid and transient intracellular alkalization in mammary tissue.

In response to growth factors and receptor tyrosine signaling, cell lines exhibit a well known increase in intracellular pH (alkalization). To investigate if mammary tissue also exhibits this growth factor induced change in intracellular pH, we analyzed the pHi response to TGFα using BCECF-AM (see materials and methods). In the absence of TGFα mammary tissue had a basal pHi of 7.00 ± 0.01 SE (Fig. 1A). We observed a rapid (within 5 minutes) and significant ($p < 0.05$) increase in intracellular pH to 7.09 ± 0.01 in mammary epithelial tissue stimulated with the

growth factor TGFα (18 nM) that was sustained for at least 24 hours and absent by 48 hours (data not shown). This growth factor-induced alkalization of pHi was significantly inhibited by 10 μM of the NHE1 inhibitor MIA (pHi = 7.01 ± 0.02; $p<0.05$). In a matching experiment, where primary mammary epithelial cells were cultured in 2D instead of 3D, similar findings were found (Supplemental 1). In order to determine intracellular pH concentrations we generated a standard intracellular pH curve using primary MECs isolated and cultured in 2D (Supplemental 2 and see Materials and Methods).

Inhibition of the MAPK pathway, with the MEK inhibitor PD98059, did not block the TGFα-induced alkalization (pHi = 7.08 ± 0.01); however, inhibition of the PI3K pathway, with LY294002, significantly blocked TGFα-induced alkalization (pHi = 7.01 ± 0.02; $p<0.05$). This result is consistent with the finding that PI3K acts downstream of RTKs and that PI3K has been previously shown to activate NHE1 (Ma et al., 1994; Meima et al., 2009). Intracellular pH changes in response to various conditions were visualized by color coding BCECF-determined intracellular pH gradients (Fig 1B).

NHE1 is present and functional in mouse mammary cells

To detect whether MECs express any of the three common epithelial isotypes of NHE, we performed immunostaining for NHE1, NHE2, and NHE3 on freshly isolated mammary tissue. NHE1, but not NHE2 or NHE3 was found to be present in all mouse mammary epithelial cells including myoepithelial cells (Fig. 2A).

Recovery from an intracellular acid load is the primary method in determining NHE exchanger function (see materials and methods; (Kintner et al., 2004)). Combining this method with MECs cultured in 2D we determined the capacity of MEC-expressed NHE1 exchanger

function during acid load recovery in the presence or absence of increasing concentrations of MIA (Fig. 2B). Without Na+ in the media, the NHE1 expressed on MECs is incapable of functioning to alleviate the acid load caused by the addition of NH_4+ and, as a result, the intracellular acid load (pH 6.69 ± 0.08) remained. Recovery back to basal pHi occurred within 5 minutes when Na+ was added back to the system by the addition of the MEC media or with modified Ringer's buffer (Fig. 2B and data not shown). Increasing the concentration of MIA suppressed the acid-load recovery in a dose dependant manner, with maximal suppression occurring at 100 μM MIA (Fig. 2B). These findings confirm that the regulator of intracellular pH in MECs is NHE1, which can be specifically inhibited by MIA. Furthermore, inhibition of recovery by MIA occurred in media containing HCO_3^- , which rules out a role for activation of a Na-coupled HCO_3- transporter (NBC) in allowing for acid load recovery. These results were also observed with recovery media containing sodium chloride with no sodium bicarbonate (data not shown). Intracellular pH changes in response to acid load, recovery and inhibition of recovery in the presence or absence of MIA were visualized by color coding BCECF-determined pH gradients (Fig. 2B).

Inhibition of NHE1 exchanger function suppresses mammary morphogenesis.

To determine whether NHE1 exchanger function is needed for mammary branching morphogenesis we added increasing concentrations of the NHE1 specific inhibitor MIA (0.1 – 10 μM) to TGFα-stimulated mammary tissue. Our results along with previous studies indicate that TGFα induces branching morphogenesis within 4 days when added to mouse virgin mammary tissue cultured in 3D matrigel (Andersen et al., 2011; Ewald et al., 2008; Fata et al., 2007;

Sternlicht et al., 2005) (Fig 3A). Increasing concentrations of MIA inhibited TGFα-induced branching morphogenesis in a dose dependent manner (Fig. 3A). At the lowest concentration of MIA (0.1 μM), a 30 percent decrease in branching was observed (Fig. 3A), while a complete absence of any branched structure was evident in tissue exposed to 10 μM MIA (Fig. 3A). Still images and video microscopy revealed that significant morphological differences occurred as early as 48 hours (Day 2) when comparing TGFα-induced versus TGFα plus MIA (10 μM; Fig. 3B; Supplemental Movie 1 and Supplemental Movie 2). This difference becomes discernable approximately 48 hours after stimulation and becomes increasingly obvious between 48 hours and 96 hours (Fig 3B; Supplemental Movie 1 and Supplemental Movie 2). Inhibition of NHE1 by MIA leads to the growth of an "amorphous" tissue mass with no discernable branches and occurred when the tissue was stimulated to branch with TGFα or with bFGF (Figure 3A, B and Supplemental 3). We further determined that the development of this abnormal tissue mass requires both PI3K and MAPK signaling, since inhibitors to these pathways abolished this phenotype (Fig 3C). In the absence of TGFα signaling, 10 μM of MIA alone had no obvious effect on mammary tissue and is indistinguishable from un-stimulated tissue (data not shown).

Independent of its exchanger function, NHE1 is known to mediate migration in a number of cell types via its cytoplasmic tail binding to the actin cytoskeleton (Cardone et al., 2005; Denker and Barber, 2002; Meima et al., 2009; Schneider et al., 2009; Stock and Schwab, 2006; Stuwe et al., 2007)and MIA has been shown to only affect NHE1 exchanger function and not actin binding by NHE (Counillon et al., 1993; Slepkov et al., 2007). To support this statement, we noted no decrease in branching morphogenesis when we added MIA to mammary tissue at 48 hours. This represents a time when pHi had equilibrated and when a considerable amount of cell movement is necessary to produce a branched phenotype in this assay (Ewald et al., 2008).

Therefore, NHE1 exchanger function is necessary for branching morphogenesis and its function is only required sometime within the first 48 hours after TGFα stimulation (Fig. 3B).

Inhibition of NHE1 leads to lumen filling and expression of keratin-6 during branching morphogenesis.

We noted a number of unique tissue and cellular phenotypes when TGFα-induced NHE1 exchanger activity was inhibited during branching morphogenesis. An examination of the overall architecture on day 4 of the branching assay, using confocal images of DAPI-stained tissue, revealed that NHE1 inhibition leads to the development of large lumens partially filled with cells (Fig 4A). This is distinctly abnormal when compared to the bi-layer of cells (myoepithelial and epithelial) found in *in vivo* mammary ducts (Gudjonsson et al., 2005) and with branching assays where NHE1 is not inhibited (Fig 4A; (Fata et al., 2007). Analysis of K14 (myoepithelial marker) and K8 (epithelial marker) on day 4 revealed that mammary tissue inhibited in NHE1 exchanger function retains normal polarity in that K14-positive external myoepithelial cells surround K8-positive epithelial cells (compare Fig 4Bb [normal] with 4Be [NHE1-inhibited]). Those cells that filled the lumen, when NHE1 was inhibited were K8-positive epithelial cells (4Be).

We found that K6-positive cells were scattered as single cells throughout tissue stimulated to branch with TGFα (Fig4Bc), which confirms a previous finding (Fata et al., 2007) and is similar to that found *in vivo* (Grimm et al., 2006). However, inhibition of NHE1 exchanger function during branching morphogenesis resulted in ectopic expression of K6 in the majority of cells including those filling the lumen (Fig. 4Bf). Inhibiting TGFα-induced NHE1

activity with MIA also induced ectopic expression of K6-positive cells when mammary tissue was cultured on 2D plastic (Supplemental 4).

Inhibition of TGFα-induced NHE1 activity leads to sustained proliferation.

Whenever NHE1 exchanger function was inhibited during morphogenesis we noted an increase in tissue mass and an increase in cell number when compared to normal tissue undergoing branching. This suggested that NHE1 exchanger function may be a key regulator of controlling proliferation and/or apoptosis in mammary tissue. To investigate this observation we analyzed at day 2 and day 4 the presence of phospho-histone H3, a well-defined marker of cellular proliferation (McManus and Hendzel, 2006; Tsuta et al., 2011). These time points were chosen because they were either the time at which proliferation is known to be ongoing leading to a branched structure (day 2) or when proliferation is minimal after completion of branching (day 4) (Fata et al., 2007). On day 2, there was no significant difference in the number of phospho-histone H3 positive cells between the organoids exposed to TGFα (9.45±.69) or exposed to TGFα and 10 μM MIA (8.18±2.25; Fig 5A). However, on day 4, in tissue with inhibited NHE1 for the first two days of development, there was a significant 13-fold increase in proliferating cells when compared to controls (Fig 5; 5.89±0.1 vs. 0.44±0.2; $p<0.0001$).

To determine if cell death regulation was altered in NHE1-inhibited tissue we assayed for apoptosis associated caspase-3 activation and for cell death possibly caused by necrosis using cell impermeable propidium iodide. In both of these assays we found no differences between normal branching mammary tissue and mammary tissue inhibited in NHE1 exchanger function (Supplemental 5A and B).

Disscussion/Conlcusion

We have uncovered a novel regulatory checkpoint essential for growth factor-induced branching morphogenesis of mammary tissue. Specifically, we demonstrated that TGFα stimulation of mammary tissue led to rapid and sustained NHE1-dependent induction of intracellular pH (alkalization) that required PI3K activation and not MAPK activation. Finally, we observed that inhibition of NHE1 activity, in the presence of growth factor signaling led to abnormally sustained proliferation, induced ectopic expression of keratin-6, and drastically suppressed branching morphogenesis. Together these findings point to intracellular pH regulation by NHE1 as an important determinant of how mammary tissue responds to growth factor stimulation.

A number of key morphogens, such as growth factors and their cognate signaling cascades are essential to the induction, regulation, and development of the branched epithelial ducts evident in the mammary gland (Hynes and Watson, 2010; Smith et al., 1995; Stull and Wood, 2003). A tight regulation of growth factor signaling cascades is absolutely necessary since deregulation has been attributed to loss of tissue architecture and breast cancer (Booth and Smith, 2007; Bundy et al., 2005; Busser et al., 2011; Dimri et al., 2005; Schwertfeger, 2009; Smith et al., 1995; Sternlicht and Sunnarborg, 2008; von Lintig et al., 2000). Epidermal growth factor (EGF) and other similar ligands like TGFα, through their binding with EGF-receptors (EGFR), are well known elements for inducing mammary branching morphogenesis (Booth and Smith, 2007; Fata et al., 2007; Fata et al., 2004; Sternlicht et al., 2005). EGF has also been shown to stimulate NHE activity in the gastrointestinal track and in other organs and cell lines (Chiang et al., 2008; Furukawa et al., 1999; Ghishan et al., 1992; Haimovici et al., 1994; Johnstone et al., 2007; Khurana et al., 1996), and our data is the first to connect EGFR signaling

and NHE1 activity as co-regulators of organogenesis, specifically mammary branching morphogenesis.

A number of classical studies have detailed the importance of pHi during a variety of developmental events. These include, intracellular alkalization occurring during Xenopus oocyte maturation (Chambard and Pouyssegur, 1986) and fertilization of Xenopus and sea urchin eggs (Grandin and Charbonneau, 1990; Shen and Steinhardt, 1979; Webb and Nuccitelli, 1981), as well as prior to pre-spore development in Dictyostelium (Van Lookeren Campagne et al., 1989). Our observations add mammary gland development to the list of developmental events that associate with and require changes in intracellular pH.

Intracellular pH has been shown to be important in determining cell cycle progression. Studies using smooth muscle cells have found that an elevation of pHi is permissive for proliferation and precedes cell division (Putney and Barber, 2003; Quinn et al., 1996; Wakabayashi et al., 2006). On the other hand intracellular acidosis has been observed as a strong inhibitor of proliferation (Bussolino et al., 1989; Delvaux et al., 1990; Horvat et al., 1992; Svegliati-Baroni et al., 1999; Turturro et al., 2004; Wakabayashi et al., 2006). These findings have led to a number of investigations into NHE inhibitors as effective cancer therapies (Harguindey et al., 2009; Matthews et al., 2011). We had anticipated that NHE1 inhibition would indeed lead to intracellular acidosis and subsequent inhibition of proliferation but we did not observe this association. Instead, at 10 μM of MIA, we observed only the inhibition of growth factor-induced alkalization and no associated acidosis. Moreover, mammary tissue inhibited in an ability to alkalinize in response to TGFα stimulation exhibited abnormal development, amorphic growth, and sustained proliferation with associated expression of proliferative markers. This suggests that during the development of a tissue or disease, the cellular response (normal or

abnormal) to growth factor signaling may be determined by an ability to regulate the pHi environment through NHE1 exchanger activity.

We observed that inhibition of NHE1 exchanger function during growth factor stimulation leads to sustained proliferation and ectopic expression of K6 in the majority of cells. We have previously reported that fibroblast growth factor-7 (FGF7, KGF) promotes ectopic K6 expression in an identical MEC branching assay and associates with sustained proliferation (Fata et al., 2007). Keratin-6 has been reported as a marker of hyper-proliferative cells in the mammary gland (Smith et al., 1990) and is abundant in Wnt1-induced mammary tumors (Li et al., 2003). Our observation of ectopic expression of K6 could be explained by the ability of NHE1 to alter gene transcription depending on its activity (Putney and Barber, 2004). We speculate that continued proliferation and ectopic expression of K6 are in part responsible for the inability of the tissue to undergo branching morphogenesis. This suggests that abnormal or increased proliferation and ectopic expression of keratins, such as that seen in mammary hyperplasias and breast cancers may be caused or sustained by NHE1 deregulation in the presence of growth factor signaling. An alternative hypothesis of the unusual growth seen in our system may be attributed to inhibiting localized and discrete removal of ECM required for directional movement of branches. This mechanism is seen in breast cancer cells that exhibit activated NHE1 on invadapodia to create an acidic extracellular environment conducive to ECM degradation and subsequent metastasis (Busco et al., 2010).

Our findings imply that the concentration of hydrogen ions in which signal transduction occurs is a critical determinant of tissue architecture and development in the mammary gland. Such a phenomenon has broader implication in diseases like cancer where pHi is known to be deregulated while the tissue is constantly being exposed to growth factors from a variety of

sources. Further work into the role of pHi dynamics as it relates to tissue morphogenesis will likely lead to novel functions ascribed to sodium hydrogen exchangers (NHEs) and could help identify new therapies for diseases like cancer.

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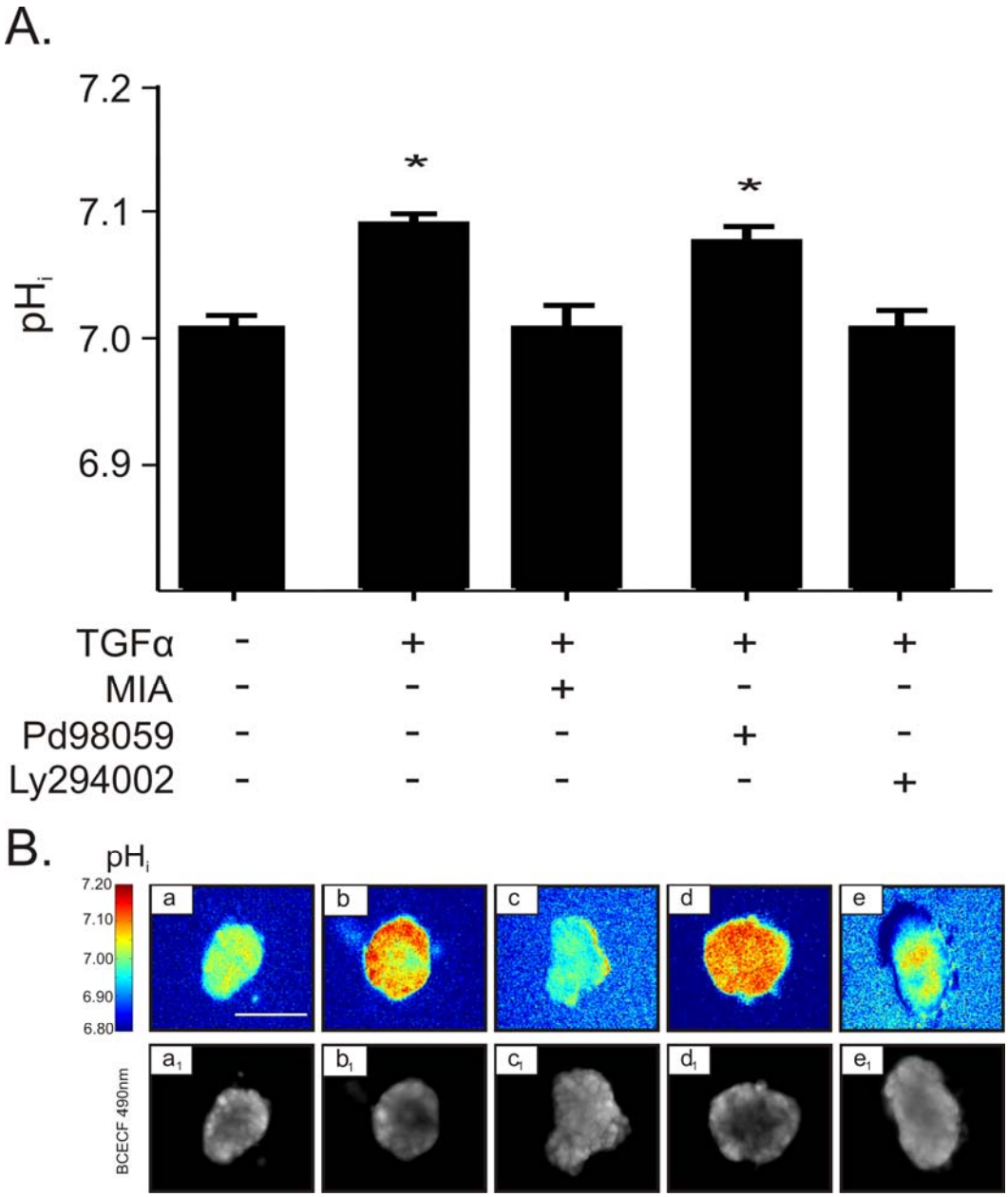


Figure 1. TGFα induces intracellular pH alkalization in mammary epithelial cells (MECs). (A) Measurement of pHi in primary mammary epithelial tissue cultured in 3D using BCECF-AM. Tissue was cultured for 24 hours prior to loading with BCECF-AM and stimulation with TGFα or stimulation/exposure with TGFα and MIA. Both PD98059 (40 μM) and LY294002 (25 μM) were pre-incubated for 90 minutes prior to growth factor stimulation. Data was collected five minutes following stimulation. MECs stimulated with TGFα alone showed a significant increase in pHi ($p < 0.005$), while this increase was abrogated by the addition of 10 μM MIA or inhibition of PI3K with LY294002. (B) Intracellular pH visualized by pH maps of 3D cultured MECs (a = untreated, b = 18 nM TGFα, c = 18nM TGFα and 10 μM MIA, d = 18 nM TGFα and 40 μM PD98059, and e = 18 nM TGFα and 25 μM LY294002). Lower panels (a1-e1) represent fluorescent intensity of BCECF-AM at 490 nm excitation. Scale bar = 50 μm.

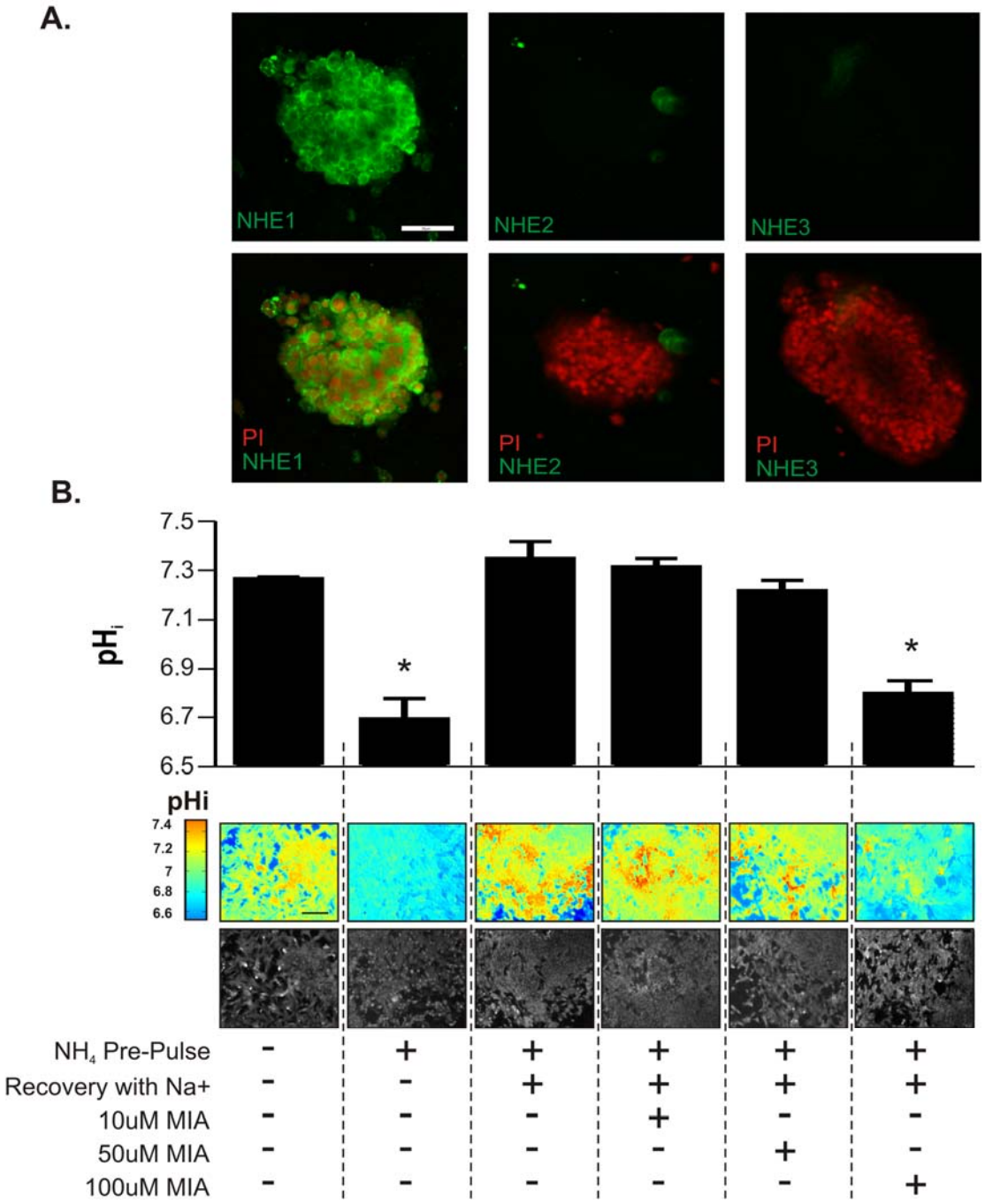


Figure 2. Functional NHE1 localizes to MECs and is inhibited by MIA (A) NHE 1 is present in all mouse mammary epithelial cells as determined by immuno-localization. NHE2 and NHE3 were not detected. The red channel represents nuclei stained with propidium iodide. (B) Measurement of pHi in primary mammary epithelial cells culture in 2D following NH4pre-pulsing. Recovery from acid load was assayed in the presence of increasing amounts of the NHE1 inhibitor MIA (* $p < 0.002$ when compared to column 1). Results of panel B were visualized by pH maps (immediately below the graph). Scale bar in A = 35 μm and B = 50 μm.

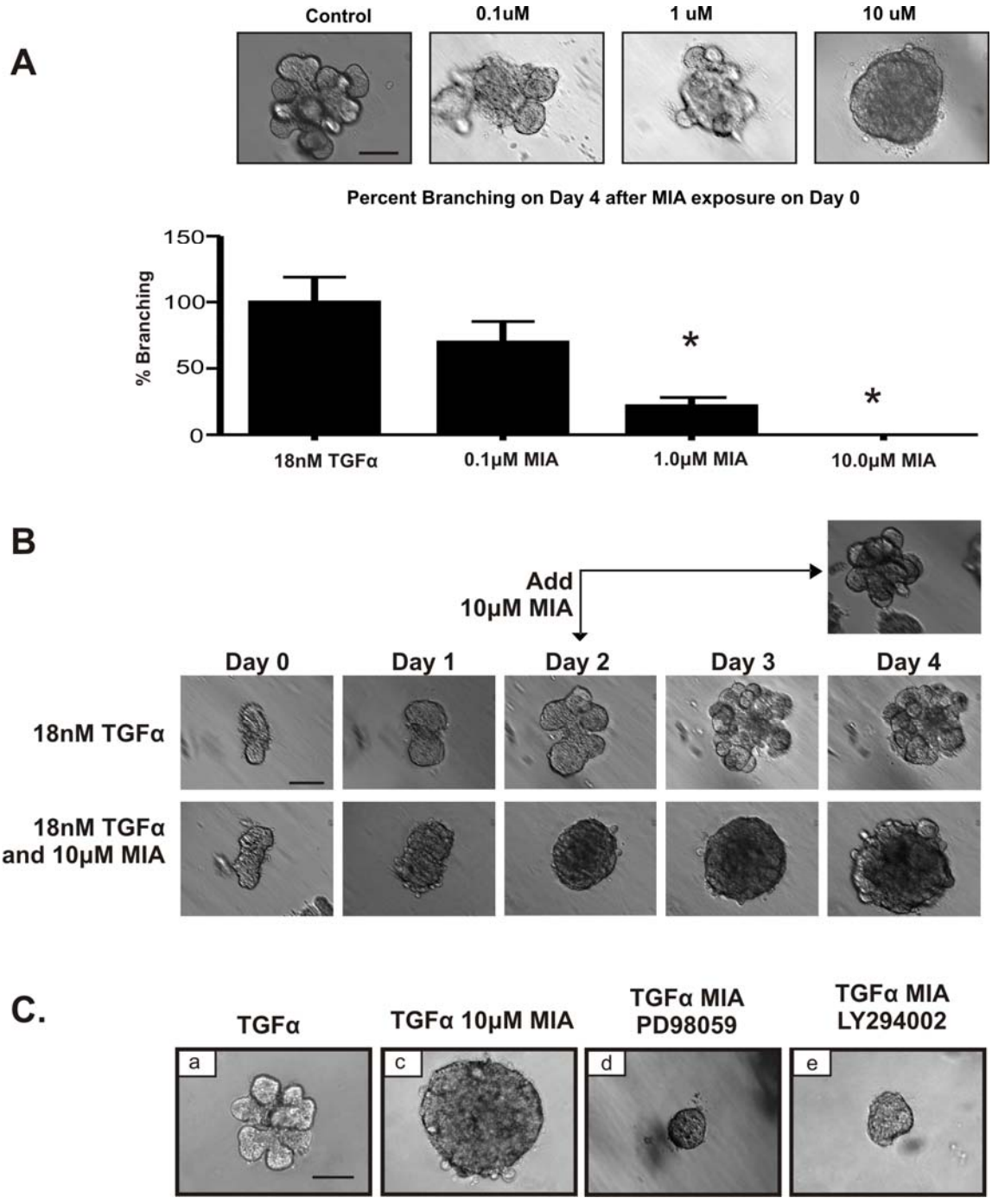


Figure 3. Inhibition of NHE1 function suppresses mammary branching morphogenesis. (A) Dose-dependent inhibition of branching morphogenesis by the NHE1 inhibitor MIA. Stimulation/exposure with TGFα and 10 μM MIA leads to the development of an amorphic structure. Percent branching was normalized to the control. (B) Time course of tissue stimulated with TGFα or stimulated/exposed with TGFα and MIA. Addition of MIA on day two of the assay does not inhibit branching. Scale bars = 100 μm.

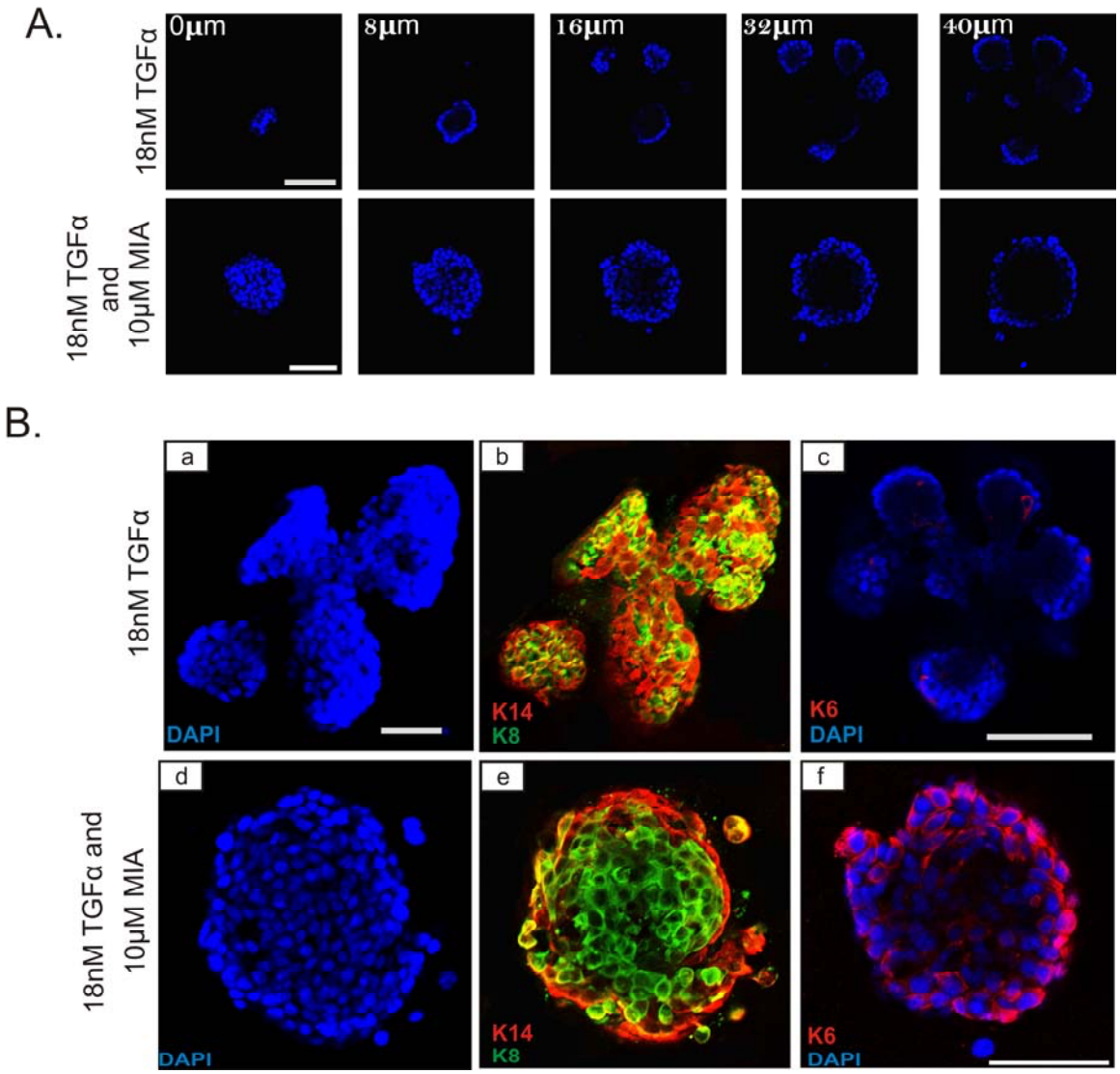


Figure 4. NHE1 inhibition during mammary branching morphogenesis leads to lumen filling and ectopic expression of keratin-6. (A) Optical sectioning by confocal microscopy of day 4 mammary organoids cultured in 3D. Top row stimulated with TGFα alone and bottom row stimulated with TGFα and inhibited in NHE1function with MIA. Cells filling the lumen in NHE1-inhibited tissue were evident at 8 and 16 µm sections, while control tissue retains a bi-layered polarity. Scale bar top = 75 µm and bottom = 50 µm. (B) Immuno-localization of keratin-8, -14, and − 6 on day 4 mammary organoids. K14/K8 polarity was not disrupted in the NHE1 inhibited tissue; however, ectopic expression of K6 expressing cells was observed (compare panels "c" and "f"). Scale bar for panels a,b,d, and e = 45 µm. Scale bars for panels c and f = 75 µm and 50 µm respectively. K8 = keratin-8, K14 = keratin-14, Nuclei labeled by DAPI.

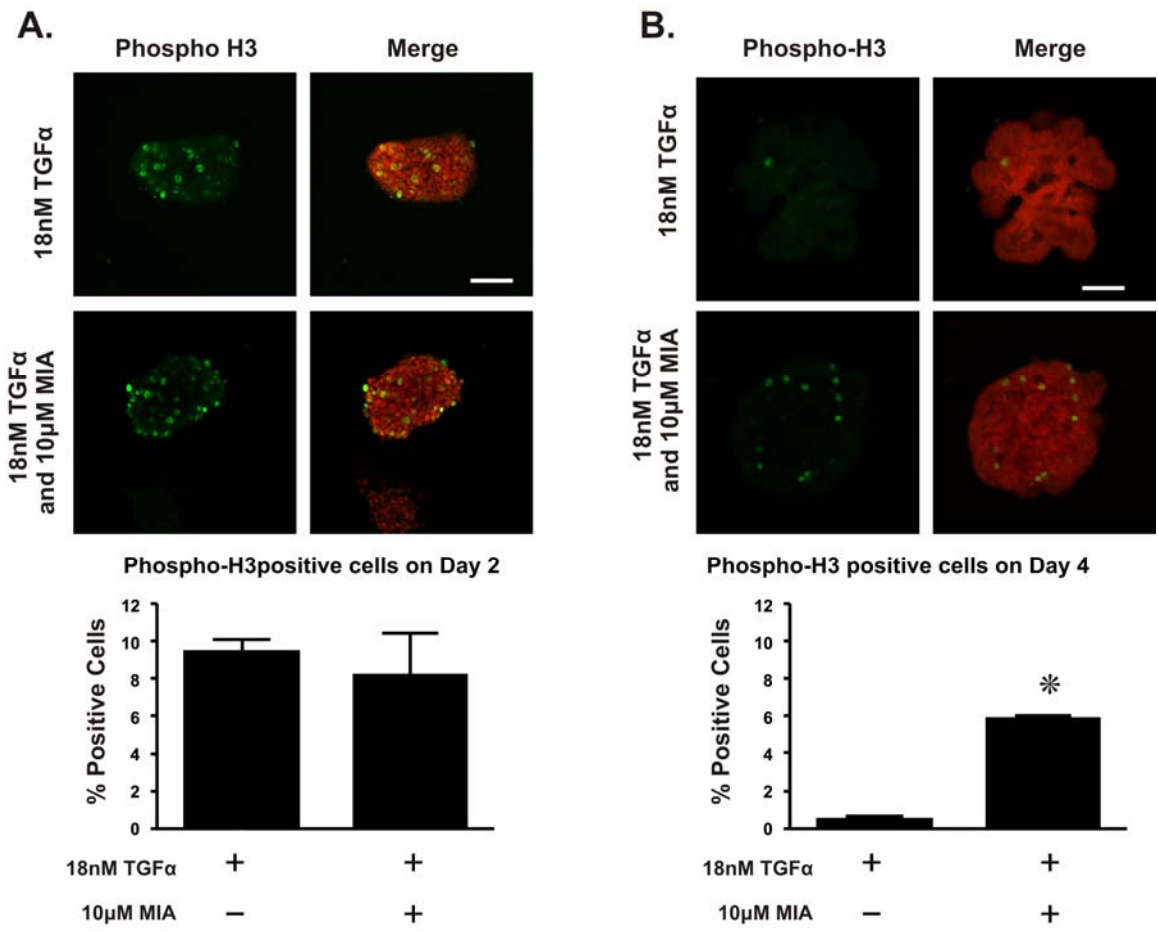


Figure 5. Inhibition of NHE1 in TGFα-stimulated mammary organoids leads to sustained proliferation. Phospho-histone H3 immunostaining and quantification on day 2 (A) or day 4 (B) mammary organoids stimulated with TGFα ± NHE1 inhibition by MIA. On day 4 a significantly greater percentage of proliferative cells are evident in TGFα stimulated mammary organoids inhibited in NHE1 function compared to control tissue ($p < 0.005$) - this difference was not evident on day 2. Scale bars = 80 μm.

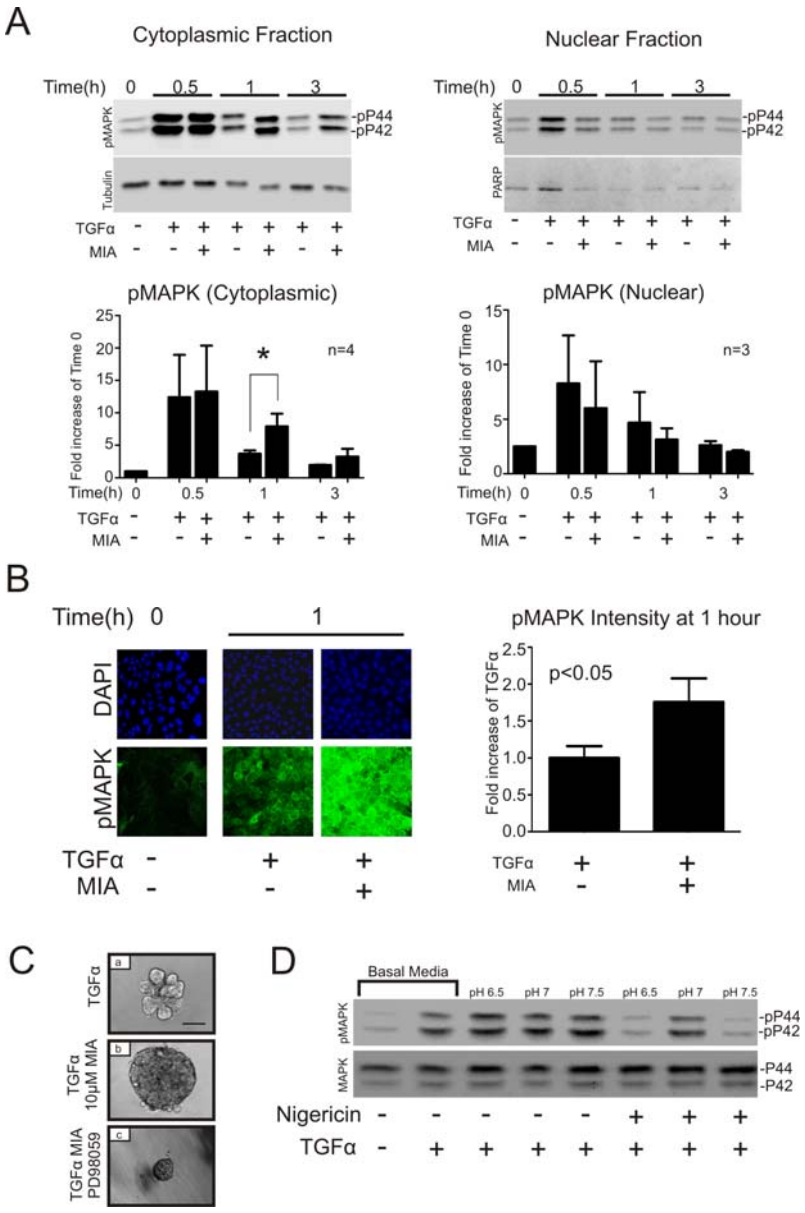
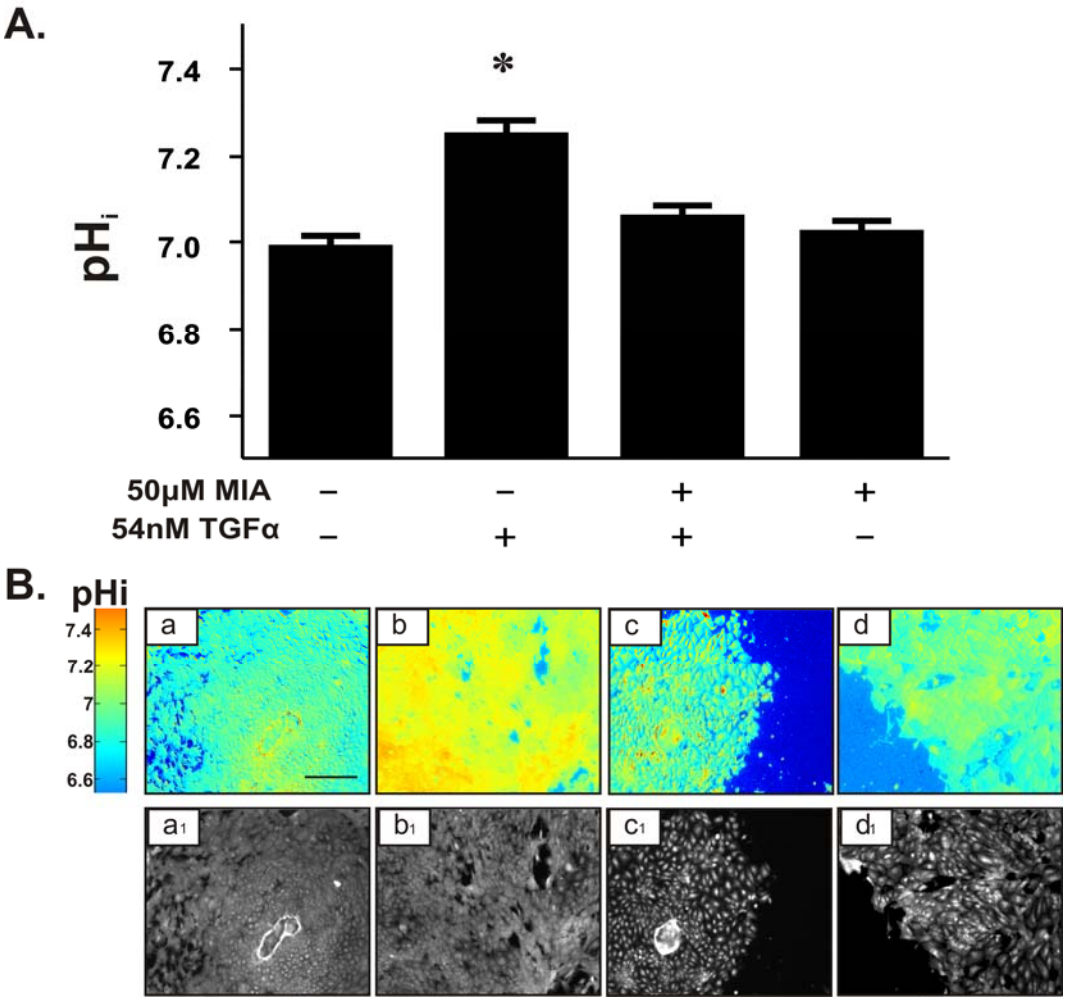
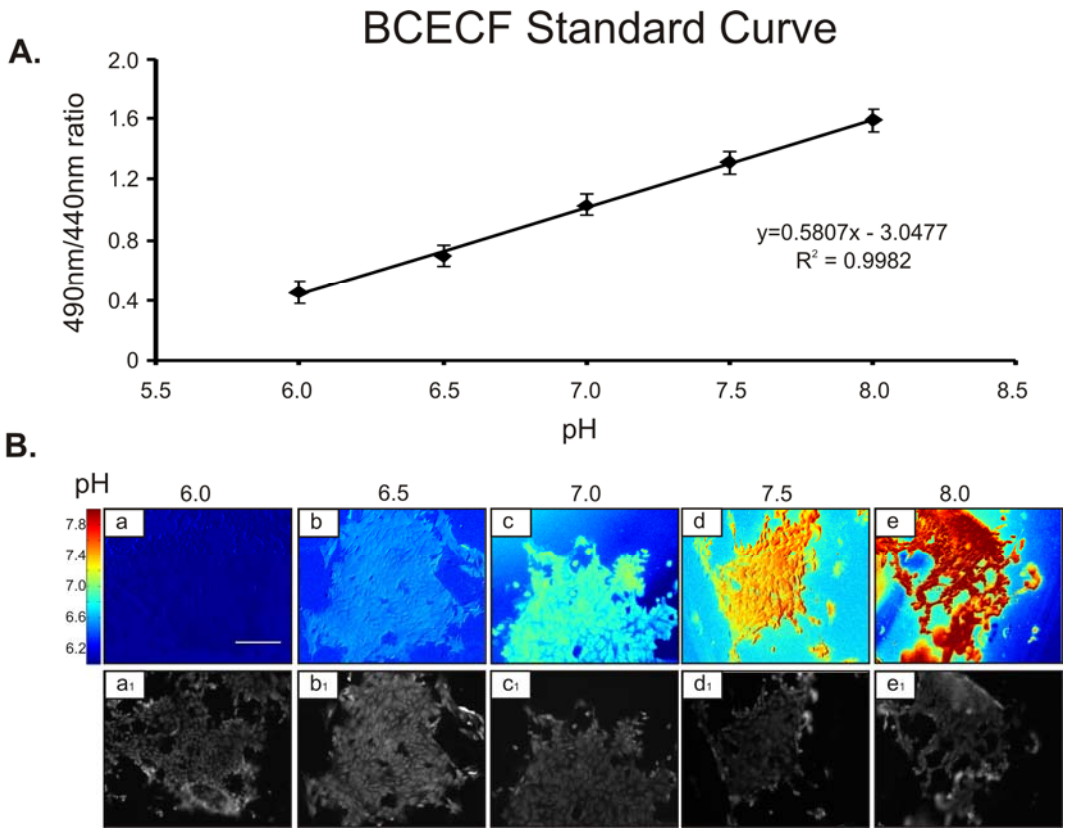


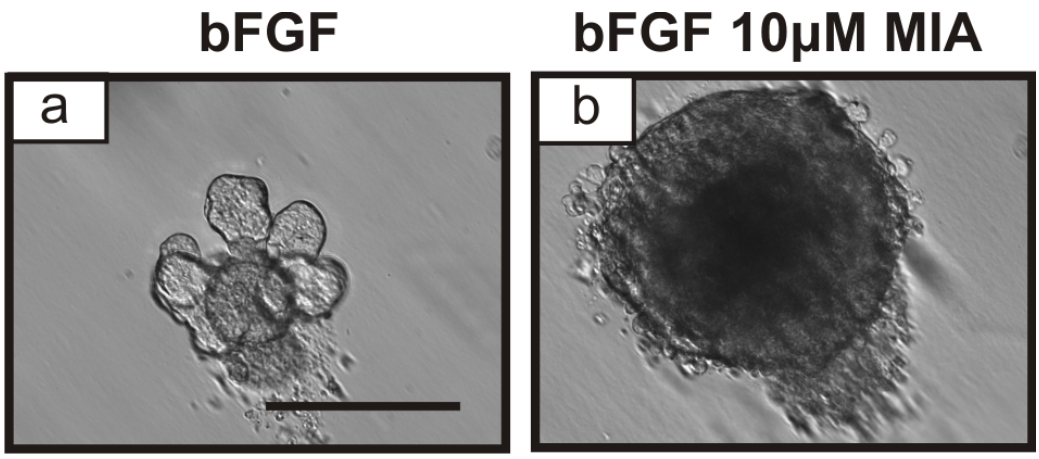
Figure 6. (A) Immunoblot analysis of TGFα-induced activated MAPK (pMAPK) found in the cytoplasmic and nuclear fractions of primary mammary epithelial cells. Increased activation of MAPK was evident in the cytoplasmic fraction at 1 hr and 3 hrs post-TGFα stimulation when NHE1 was inhibited with MIA (50 μM) compared to when NHE1 was not inhibited (*$p < 0.05$). No differences in MAPK activation was found in the nuclear fraction. (B) Immunocytochemistry of activated MAPK (pMAPK), 1 hr post-TGFα stimulation, indicates a significant increase in pMAPK when NHE1 is inhibited by MIA (50 μM), compared to when NHE1 is not inhibited ($p < 0.05$). (C) Inhibition of the MAPK pathway with PD98059 (40 μM) completely suppresses the TGFα-induced amorphic growth evident in mammary tissue inhibited in NHE1 function (compare panel c with panel b). (D) Immunoblot analysis of TGFα-induced activation of MAPK under varying pHi conditions. Nigericin is added to equilibrate external pH with internal pH. In the absence of Nigericin external pH does not affect internal pH. Scale bars = 100 μm.



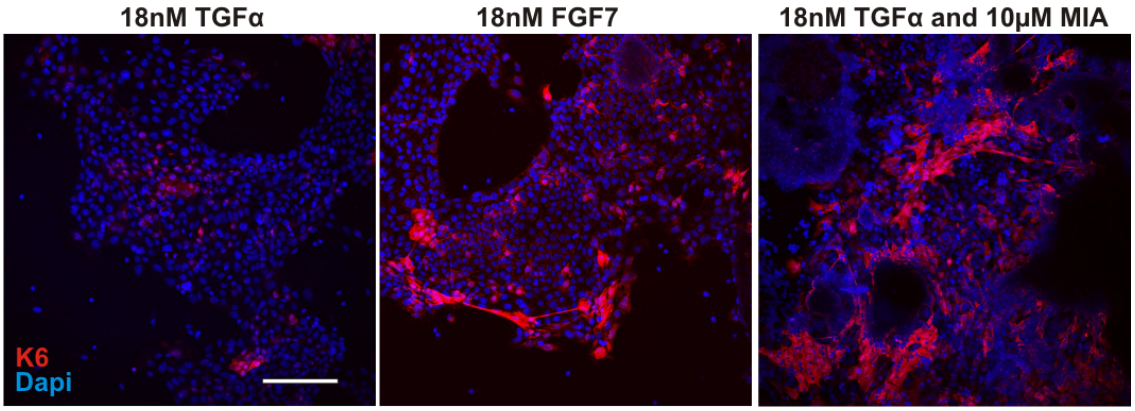
Supplemental 1. TGFα induces intracellular alkalization in mammary epithelial cells (A) Measurement of pHi in primary mammary epithelial cells cultured in 2D show TGFα induced a significant increase in pHi (alkalization; * $p < 0.05$) . This increase was inhibited by the NHE1 inhibitor MIA. Data was collected 24 hours after TGFα stimulation. (B) TGFα induced alkalization of pHi visualized by pH maps (a = untreated, b = 54 nM TGFα, c = 54 nM TGFα and 50 μM MIA, and d = 50 μM MIA). Data was collected 24 hours following stimulation. Panels a1-d1 correspond to a-d and represent BCECF-AM loaded MECs excited at 490 nm. Scale bar = 50 μm.



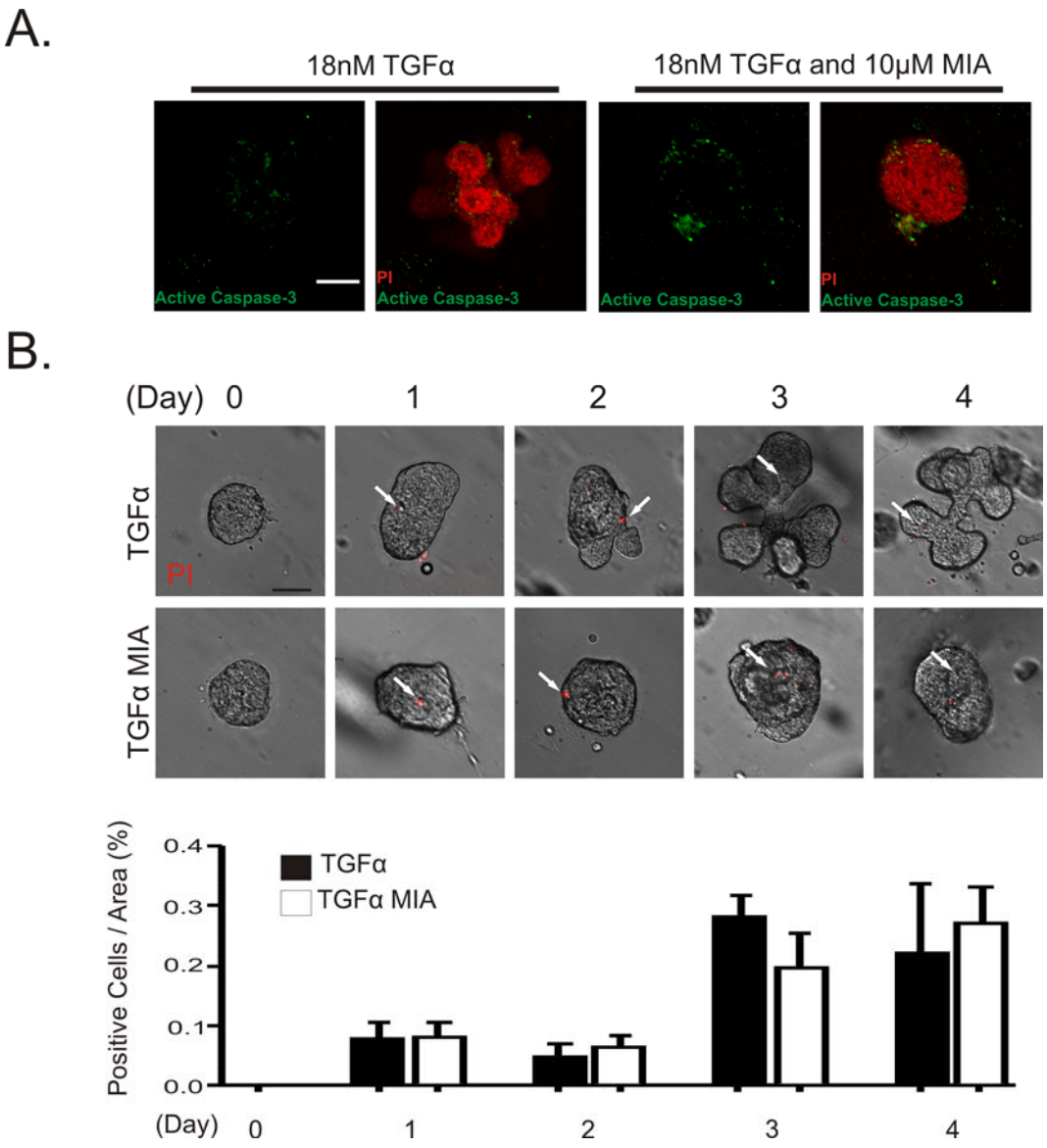
Supplemental 2. Standard curve for pHi measured with BCECF-AM. (A) Modified Ringer's buffers of known pH were incubated with nigericin on primary mammary epithelial cells to equilibrate internal pH with external pH. Ratio numbers were used to generate a standard curve relating fluorescent ratio intensity to intracellular pH (see material and methods). (B) The top row (panels a-e) represent pH maps for increasing pHi standards. The bottom row (panels a1-e1) correspond to a-e and represent BCECF-AM loaded MECs excited at 490 nm. Scale bar = 50 μm.



Supplemental 3. Other growth factors require NHE1 function for inducing mammary branching morphogenesis. bFGF induced a similar branched pattern in mammary organoids when compared to TGFα induction. Inhibition of NHE1 with MIA during bFGF stimulation blocked branching morphogenesis and resulted in a phenotype similar to that induced by TGFα plus MIA. Scale bar = 100 µm.



Supplemental 4. Inhibition of NHE1 during TGFα stimulation leads to upregulation of keratin-6 expression (red) in MECs cultured in 2D. Cells were stimulated with either 18nM TGFα, 18nM FGF7 (positive control), or 18nM TGFα in the presence of MIA. NHE1 inhibition by MIA led to an increase in the number of cells expressing keratin-6. Nuclei stained with DAPI (blue). Scale bare = 150 μm.



Supplemental 5. The NHE1 inhibitor MIA does not induce mammary cell death at 10 μM (A) Immuno-localization of cleaved caspase-3 (green) in mammary epithelial organoids on day 4 following stimulation with 18nM TGFα or stimulation/exposure with 18 nM TGFα and 10 μM MIA showed no significant differences. Propidium iodide (PI; arrows pointing to red cells) was used to detect cells with compromised plasma membranes, i.e. cells that have undergone apoptosis or necrosis. The presence of MIA did not significantly impact the number of PI positive cells when organoids were examined from day 0 to day 4. Scale bar in A = 80 μm and in B = 100 μm.

Chapter 2: Na^+/H^+ Exchanger 1 (NHE1) Function is Necessary for Maintaining Mammary Tissue Architecture

Abstract

The mammary gland is an ideal model to study the link between form and function in normal tissue. Perhaps as interesting as the cues necessary to generate this structure are the signals required to maintain its architecture over the lifetime of the organism, since likely these pathways are de-regulated in malignancies. Previously, we have shown that the Na+/H+ exchanger 1 (NHE1), a critical regulator of intracellular pH, was necessary for mammary branching morphogenesis. Here we provide strong evidence that NHE1 function is also necessary for maintaining mammary branched architecture. Inhibition of NHE1 with 5-N-Methy-N-isobutyl amiloride (MIA) on branched structures resulted in a rapid (within 24 hours) and reversible loss of branched architecture that was not accompanied by any overt changes in cell proliferation or cell death. NHE1 inhibition led to a significant acidification of intracellular pH in the branched end buds that preceded a number of events, including altered tissue polarity of myoepithelial cells, reversal of NHE1 cellular polarity, F-actin rearrangements and decreased E-cadherin expression. Our results implicate NHE1 function and intracellular pH homeostasis as key factors that maintain mammary tissue architecture thus, indirectly allowing for mammary function as a milk providing (form) and producing (function) gland.

Key Words: mammary tissue architecture; mammary epithelial ducts; sodium hydrogen exchanger; NHE1; intracellular pH; tissue polarity; cellular polarity

Introduction:

A branched epithelial ductal network is the architectural basis for the lung and collecting ducts of the kidney, as well as the salivary and mammary glands in mammals. Like other branched systems, the initial mammary branched pattern arises in embryonic development by crosstalk between the epithelium and the supporting mesenchyme (Wiseman and Werb, 2002; Parmar and Cunha, 2004). Unlike other branched systems, however, the mammary gland undergoes most of its branching at the onset of puberty, when circulating hormones, like progesterone and estrogen, begin systemic circulation (Woodward et al., 1998; Wiseman et al., 2003; Sternlicht et al., 2006; Fata et al., 2007; Khokha and Werb, 2011; McNally and Martin, 2011). In the mammary gland, epitheial ducts are the structural components that accomplish the function of conducting milk from the glandular component, called lobuloalveolar units, to the nipple where it is consumed by a suckling newborn. The ductal portions of the mammary gland are composed of two layers of epithelium, an internal layer of luminal epithelium, and an external layer of contractile myoepithelium sheathed in a thin layer of basement membrane. The maintenance of this architectural form allows for apical basal cellular polarity, which is essential for proper luminal secretions and the primary function of the mammary gland (Adriance et al., 2005). These epithelial components are embedded in a complex stroma, which plays a considerable role in the specification and maintenance of this highly ordered branched structure (Kratochwil, 1969).

While the generation of this pattern occurs in a matter of weeks in the mouse, it is maintained over the lifetime of the organism. Therefore, uncovering the factors that contribute to the maintenance of branched architecture is equally important to understanding the developmental cues that result in the form and function of the mammary gland. Three

dimensional (3D) cultures of normal mammary epithelial cells using a laminin rich ECM (lrECM) have been extremely valuable in determining the functional architecture and polarity of mammary epithelium (Bissell et al., 2003; Nelson and Bissell, 2005; Nelson and Bissell, 2006; Tanner et al., 2012). Continued research into the intricacies of branching morphogenesis in the mammary gland has highlighted the importance of extracellular factors such as hormones, extracellular matrix, mesenchyme, and growth factors, as well as numerous essential intracellular pathways, in the development of a branched structure (Simian et al., 2001; Sternlicht et al., 2005; Fata et al., 2007; Ewald et al., 2008; Andersen et al., 2011; Pasic et al., 2011; Basham et al., 2013). This heavy focus on better understanding branching morphogenesis in the mammary gland has provided seminal findings in the field of ductal development, as the mammary gland continues to provide researchers with a malleable model to investigate many aspects of biology.

Sodium Hydrogen Exchangers (NHEs) are a family of integral membrane proteins that catalyze the electroneutral exchange of extracellular Na+ for intracellular H+. The sodium hydrogen exchanger type 1 (NHE1) is a ubiquitously expressed member of this family that functions as a master regulator of pHi (Jang et al., 2006; Steffan et al., 2009). Currently, there are 9 subtypes of NHEs identified, with expression profiles following tissue and membrane specific patterns (Putney et al., 2002; Slepkov et al., 2007; Casey et al., 2010). NHE1 has been found to have roles in cellular proliferation (Denker et al., 2000), migration (Denker and Barber, 2002; Stock et al., 2005; Stock and Schwab, 2006; Koliakos et al., 2008; Stock et al., 2008; Yang et al., 2010; Martin et al., 2011; Yang et al., 2011), intracellular acidosis induced apoptosis (Lagadic-Gossmann et al., 2004; Pedersen, 2006; Wang et al., 2008; Casey et al., 2010) and critical developmental processes (Patel and Barber, 2005; Zhou and Baltz, 2013). Activation of this exchanger occurs by phosphorylation (Bianchini et al., 1997; Tominaga and Barber, 1998; Moor

and Fliegel, 1999; Takahashi et al., 1999; Yan et al., 2001; Kintner et al., 2005; Luo et al., 2007; Malo et al., 2007; Meima et al., 2009), intracellular acidosis (Lagadic-Gossmann et al., 2004; Casey et al., 2010), or osmotic stress (Holt et al., 2006). Additionally, NHE1 has been shown to become activated as a result of growth factor stimulation (Moolenaar et al., 1983; Grinstein et al., 1988; Strazzabosco et al., 1995) and integrin activation (Tominaga and Barber, 1998). Previously, we have shown that its function is also necessary for mouse mammary branching morphogenesis, and that its inhibition alters growth factor induced signaling (Jenkins et al., 2012).

In an effort to elucidate NHE1 function as it relates to the maintenance of organized tissue architecture, we first generated an established mammary branched structure using a previously described organotypic 3D-culture model. We then asked whether NHE1 was necessary to maintain tissue architecture in this model by exposing the tissue to the NHE1 specific inhibitor N-Methyl-N-isobutyl Amiloride (MIA). In doing so, we found that NHE1 inhibition resulted in a rapid loss of tissue organization and cellular polarity that was not accompanied by cell death or altered proliferation in our system. Additionally, NHE1 inhibition acidified intracellular pH (pHi) in end bud portions of branched tissue and altered actin localization in that region. Finally, we provide evidence that functional NHE1 is necessary for regulating E-cadherin in mammary epithelium. Our findings suggest NHE1 has a novel role in the maintenance of tissue architecture.

Results

NHE1 function is required for the maintenance of mammary tissue branches:

A fully established branched mammary tissue was first developed over the course of 4 days in a 3D tissue culture in order to ask whether NHE1 function was required to maintain this architecture (Jenkins et al., 2012) (Figure 1A). After branches were established (Day 4) (Figure 1A), NHE1 function was inhibited with 5(N-Methyl)-N-Isobutyl Amiloride (MIA) (50 μM) in the presence of growth factor TGFα (18 nM) (Figure 1B) and tissue architecture was followed by time-lapse photography for the next 4 days (Day 4 to Day 8). In the absence of NHE1 inhibition, branches were maintained in both structure and number over the course of 4 days (Figure 1B top panels). In contrast, inhibition of NHE1, with MIA (50 μM) led to a rapid and drastic disruption of the branched architecture (Figure 1B, bottom panels). Disruption was noted as early as 24 hours and involved the "fusing" of existing branches such that after 4 days only a mass of tissue existed often containing few if any branches (Figure 1B, compare bottom panels with top panels) When compared to control tissue at Day 8 (4 days after MIA addition), inhibition of NHE1 function led to a greater than 65% reduction (100 ± 5.16 vs. 33.5 ± 10.59, control (adjusted to 100%) vs. MIA treated; $p < 0.01$) in the number of structures that exhibited at least 3 or more branches (Figure 1C). Importantly, in tissue where NHE1 inhibition induced loss of architecture, TGFα-induced branching could be reinitiated after MIA was washed out of the culture system (Figure 1D and supplemental movie 1). These findings indicate that maintenance of a branched mammary tissue requires NHE1 function and loss of NHE1 function induces a rapid non-permanent loss of tissue architecture.

NHE1 inhibition acidifies intracellular pH in mammary tissue endbuds:

NHE1 is the key regulator of intracellular pH (pHi) in epithelial cells (Lagadic-Gossmann et al., 2004; Cardone et al., 2005; Casey et al., 2010). The exchanger performs this function by

exchanging intracellular H+ for extracellular Na+ upon activation. Previously, we have shown that NHE1 inhibition disrupts branching morphogenesis and blocks growth factor induced alkalization (Jenkins et al., 2012). Here, we investigated the effect of NHE1 inhibition on pHi of branched structures 30 minutes after NHE1 inhibition by MIA (50 μM; Figure 2). Using the ratiometric pH indicator BCECF-AM and our previous methods for detecting intracellular pH in mammary epithelial cells (Jenkins et al., 2012), we found that pHi in endbuds of branched structures was significantly lower when NHE1 was inhibited (6.85 ± 0.02, N = 34 endbuds counted) compared to when NHE1 remained functional (6.97 ± 0.01; N = 19; $p < 0.0001$) (Figure 2A and B). Therefore, in 3D branched mammary tissue, the addition of MIA, which inhibits NHE1 function, rapidly induces intracellular pH acidification that can be effectively measured with the pH indicator BCECF-AM and ratiometric imaging.

Loss of tissue architecture is not due to increased proliferation or cell death:

NHE1 function has been shown to have a permissive role in both cellular proliferation (Pouyssegur et al., 1984) and acidosis induced apoptosis (Li and Eastman, 1995; Lang et al., 2000). To characterize whether the loss of MIA-induced tissue architecture was a consequence of altered proliferation or cell death, we analyzed cell proliferation by phospho-histone H3 staining and cell death by Propidium Iodide (PI) exclusion, respectively (Figure 3). An analysis of proliferation at Day 6 (data not shown) and Day 8 (Figure 3A) indicated that the loss of tissue architecture was not associated with any significant changes in proliferation (0.57 ± 0.11 vs. 0.72 ± 0.13, control vs. MIA treated; $p = 0.7$). To investigate cell death, we stained live organoids at Day 8 with propidium iodide (PI) to fluorescently label dead cells in which the plasma membrane had become compromised. Cell death analysis revealed no significant differences

between control tissue (0.05 ± .006) and tissue treated with 50 μM MIA (0.04 + 0.005; Figure 3B). We treated live tissue with 100 μM of MIA as a positive control for cell death, which induced approximately 100% of the cells to die after 4 days. These results show that inhibition of NHE1 function by MIA results in loss of mammary tissue architecture that is not associated with any changes in proliferation or cell death.

Disruption of mammary tissue architecture within 24 hours of NHE1 inhibition

We noticed that a rapid loss of mammary tissue architecture occurred in the first 24 hours following NHE1 inhibition by MIA (50 μM). To further explore this time window, we performed live-video microscopy of branched structures for the first 24 hours after NHE1 inhibition by MIA. An analysis of the resultant movies revealed that, when mammary tissue was inhibited in NHE1 function, it lost its architecture by a process of branch fusion, in which the endbuds of the structure merged first, followed by the stalks (Figure 4A and Supplemental movies 2 and 3). This process of branch fusion was rarely evident in mammary tissue not inhibited in NHE1 function (Figure 1A; compare top panels with bottom panels). Moreover, propidium iodide staining of live tissue on day 5 indicated that this process was not accompanied by any significant pronounced cell death (Figure 4B). It should be noted that branch fusion could occur with the addition of MIA (50 μM) alone or when it was co-administered with TGFα (18 nM).

Tissue polarity of myoepithelial cells is disrupted when NHE1 function is inhibited:

The mouse mammary gland has a distinct cellular organization pattern, with keratin 14 (K14) expressing myoepithelial cells surrounding keratin 8 (K8) expressing epithelial cells on

the stalks of the branch, while K8 positive and double positive (K8 and K14) cells are found at the tips of the endbuds (Figure 5A, left panel). Using immunocytochemistry for these keratins we determined if this organizational pattern was distorted when the loss of architecture occurred after NHE1 inhibition. Within 24 hours of NHE1 inhibition (Day 5), the continuous sheet of K14-postive myoepithelial cells seen in the stalk areas did not exist anymore (Figure 5A). Instead, K14-positive cells became discontinuous throughout the entire surface of the tissue (Figure 5A). We determined that the changes in K14-positive cells were likely not a consequence of ectopic expression since the amount of K14 protein was equal between control tissue and MIA-treated tissue (Figure 5B). To further analyze the K14-phenoytpe we performed optical sectioning of K14-immunostained tissue by confocal microscopy (Figure 5C). This analysis revealed that in mammary tissue inhibited in NHE1 function, myoepithelial cells could be found on the surface as well as deep within the altered tissue as compared to control tissue where myoepithelial cells are only found on the external surface (Figure 5C). These changes indicated that altered tissue organization was accompanied by abnormal tissue polarity of myoepithelial cells within 24 hours after NHE1 inhibition.

Inhibition of NHE1 function leads to a reversal of NHE1 cellular polarity.

In epithelial cells, NHE1 can be found to exhibit prominent basal-lateral polarity with a marked absence from the apical surface (Damkier et al., 2009). As expected, in mammary tissue freshly isolated from the mouse, immunostaining revealed both myoepithelial and epithelial cells expressed NHE1 and that in areas containing lumens, a basal-lateral polarity was evident with lower amounts on the apical side (Figure 6A). In mammary tissue that had undergone branching in our assay, normal NHE1 expression and polarity were still maintained throughout the

branched structure (Figure 6B, top panels). However, in mammary tissue inhibited in NHE1 function, the polarity of NHE1 became disrupted. Under these conditions where NHE1 function was inhibited the basal polarity of NHE1 appeared to reverse, such that NHE1 became absent on the basal side and evident on the apical side (Figure 6B, bottom panels). When we quantified this reversal of polarity, we found that NHE1 inhibition led to an 8.5 fold increase of cells with apical lateral polarity on the periphery of disrupted structures compared with branched structures where NHE1 function was not perturbed (Figure 6B, $p < 0.0001$). Therefore, the rapid loss of tissue architecture caused by NHE1 inhibition is accompanied by a reversal of NHE1 cellular polarity.

F-actin and E-cadherin are altered by NHE1 Inhibition:

Since we observed rapid fusing of branches during the loss of architecture, we hypothesized that NHE1 inhibition could alter the cytoskeleton and/or cell-cell adhesion molecules necessary for maintaining epithelial structures. Filamentous actin (F-actin) is a structural cytoskeleton protein known to have an important role in maintaining cellular and tissue structure (Yamazaki et al., 2007; Tang and Brieher, 2012) and is known to be affected by changes in pHi (Lagarrigue et al., 2003). In an effort to determine the pattern of F-actin organization in both the day 5 branched structure (-MIA) and the day 5 disrupted structure (+MIA), we stained both conditions with phalloidin. We found that branched tissue had thick rings of circumferential actin on the apical side of the end bud lumens (Figure 7A, top panels), consistent with what has been observed in other polarized epithelial systems (Yonemura et al., 1995; Smutny et al., 2010). In contrast, in tissue where the architecture was disrupted by NHE1 inhibition, these thick highly organized bands were not observed at any z-plane (Figure 7A, bottom panels). No other differences were apparent when examining F-actin by phalloidin labeling. To investigate cell

adhesion molecules, we cultured mammary primary epithelial cells in 2D, exposed them to MIA for 24hrs and then immunostained for E-cadherin. When confocal fluorescent images were captured under identical conditions and identical z-planes we found that NHE1 inhibition associated with a decrease in E-cadherin expression (Figure 7B). Lower levels of E-cadherin expression were also noted in tissue cultured in 3D that had become disrupted due to NHE1 inhibition (Figure 7C). In 3D cultures, this loss of E-cadherin expression was most prominent deep within the tissue mass and, in both 2D and 3D mammary epithelial cultures, E-cadherin loss could be found within 24 hours of inhibiting NHE1 with MIA. We had noted that organoids were able to reorganize themselves back into branched structures once NHE1 inhibition was removed from 3D cultures. We therefore hypothesized that E-cadherin expression would need to be restored to normal levels for this to occur. To test this hypothesis, we isolated protein from organoids that were cultured in 2D and probed to detect E-cadherin expression by immunoblot. We found that NHE1 inhibition for 24 hours lowered E-cadherin expression and that this effect could be rescued once the NHE1 inhibitor was removed from culture for 72 hours (Figure 7D; compare lanes 2, 3 and 4). These results provide strong evidence that, in mammary tissue, NHE1 function is necessary to maintain both normal F-actin cytoskeleton arrangement and E-cadherin expression.

Discussion:

Branching morphogenesis is a highly ordered and efficient process that generates a ductal network critical to the function of a number of biological structures, including the mammary gland. Perhaps as interesting as how a branched structure is generated is how these structures are maintained in form and function over the lifetime of the organism. Previously, we found that

NHE1 disruption during growth factor induced branching morphogenesis dramatically disrupted branching morphogenesis, indicating this hydrogen exchanger is integral to this developmental process. Here we demonstrated that NHE1 is also necessary for the maintenance of branched tissue architecture and that its inhibition resulted in a rapid but reversible loss of tissue organization.

Establishing a functional branched structure requires dynamically regulated interaction between the stroma and epithelium, as well as between the epithelium and the basement membrane (Bissell et al., 2002; Bissell and Bilder, 2003; Bissell et al., 2003; Parmar and Cunha, 2004; Adriance et al., 2005). These interactions provide vital contextual cues that regulate processes such as cellular polarity, which is critical for vectoral milk production in mammary epithelium (Barcellos-Hoff et al., 1989), and proliferation, which is controlled within specific windows during branching morphogenesis (Fata et al., 2007; Ewald et al., 2008). Additionally, cell migration, a process that requires both regulated cell adhesion and modification of the ECM, has been shown to play a pivitol role in driving mammary branching morphogenesis (Ewald et al., 2008) and in generating epithelial tubes (Mori et al., 2009). It stands to reason that the regulated pathways necessary to establish mammary tissue architecture are also necessary to maintain it. The importance of these pathways is no better illustrated then when one considers cancer, where functional tissue disregards normal polarity and growth regulating pathways and begins to proliferate in a disorganized and dysfunctional manner.

Na+/H+ exchanger function has been shown to be involved in establishing cellular polarity (Patel and Barber, 2005) and in creating favorable micro-environments for ECM interaction and actin stabilization (Bernstein et al., 2000), as well as actin filament organization downstream of growth factor stimulation (Meima et al., 2009). Importantly, actin regulation

plays a critical role in maintaining epithelial cell interaction by E-cadherin (Yamazaki et al., 2007; Tang and Brieher, 2012), and has been shown to be dynamically regulated at cell-cell contacts (Kovacs et al., 2011; Yamada et al., 2012). Here, we have shown the perturbing NHE1 function altered both actin organization and E-cadherin expression resulting in a reversible loss of branched tissue architecture. This decrease in E-cadherin is similar to what has been reported during mammary branching morphogenesis, specifically when the epithelium is rearranging during ductal elongation (Ewald et al., 2008). Taken together, a possible mechanism for our observations arises. NHE1, by its ability to maintain pH homeostasis, contributes to the stabilization and localization of actin to the zona adherins of polarized epithelium. When NHE1 function is altered, dynamic actin localization in the apical perijunctional region is disrupted and E-cadherin protein is decreased, thus making tissue architecture unstable. Our findings suggest a novel homeostatic regulatory pathway involving NHE1 > E-cadherin expression > tissue stability.

The loss of mammary ductal architecture is clearly evident in the formation of tumors, which develop from established ducts by disregarding the influence of normal polarity pathways. Therefore, understanding the factors that maintain mammary tissue architecture not only feeds our understanding of mammary gland development and function, but also provides information that impacts how polar tissue becomes apolar during tumor development. Both intracellular and extracellular pH are known to be deregulated in cancer and are thought to play a major role in the progression of the disease (Reshkin et al., 2000; Cardone et al., 2005; Harguindey et al., 2005; Stock and Schwab, 2009). Here, we have found that deregulation of intracellular pH leads to loss of tissue architecture analogous to cancer progression. A number of studies have shown that cellular context and tissue architecture greatly influence cancer phenotypes despite considerable

genetic aberration (Mintz and Illmensee, 1975; Bissell et al., 2002; Wang et al., 2002; Kirshner et al., 2003). Our results suggest a novel role for NHE1 and pH homeostasis in maintaining normal tissue structure and function independent of direct genetic manipulation. This role should not be underestimated when considering that the maintenance of tissue architecture is absolutely necessary for mammary function and that loss of this architecture is a hallmark of breast cancer.

Experimental Procedures

Mammary Tissue (Organoid) Isolation:

Mouse mammary tissue was isolated and cultured as previously described (Fata et al., 2007; Jenkins et al., 2012). Briefly, the fourth inguinal mammary glands [2] were isolated from virgin Balb/C mice 14-30 weeks of age, diced with two standard razor blades, and placed in 10mL of a trypsin/collagenase mix (0.2% trypsin/0.2% collagenase, 5% fetal calf serum (FCS), 1000 U/ml Penicillin/Streptomycin) for 25 minutes gently shaking at 37º C. Following digestion, the suspension was centrifuged for 5min at 70 xg. The pellet was washed twice in 7mLs of DMEM/F12 from CellGro and was then resuspended in 4mLs of DMEM/F12 with DNase (2U/μL) gently shaking at room temperature for 5 minutes. The larger pieces of mammary gland (mammary organoids) were then separated by differential centrifugation, i.e. pulse spins to 70 xg at least three times with the supernatant being discarded every time. The final pellet was used for three dimensional (3D) mammary tissue cultures.

Three Dimensional (3D) Branching and Loss of Architecture Assay:

Three dimensional culturing of mammary tissue in matrigel was performed as previously described (Fata et al., 2007; Jenkins et al., 2012). A 40 μL supporting layer of matrigel (BD

Sciences) was first added to each well in a 96 well format and allowed to solidify in the incubator (37º C and 5% CO_2) for 30 min. Isolated mammary organoids were suspended in 100% matrigel plated at 40 µl/well and at a density of 50 organoids/well. The organoid containing layer was allowed to solidify for 30 min before the addition of 100 µl of basal media, also called mammary epithelial cell (MEC) media (DMEF/F12 with 1% insulin, transferrin, selenium, and 1% penicillin/streptomycin). After 24 hours, organoids were stimulated with TGFα (18 nM). This was considered day 0. Media was changed to basal media after 48 hrs (day 2). Branching was considered complete on day 4 at which time organoids were either re-stimulated with TGFα (18 nM) with or without 5(N-Methyl)-N-Isobutyl Amiloride (MIA) (50 µM). Media was again changed to basal (MEC) media alone on day 6. The assay was considered complete two days later on day 8 when all remnants of organoid branches were no longer apparent following NHE1 inhibition. A structure was scored as branched if it had three or more distinct branches (endbuds and stalks) protruding in separate directions (Fata et al., 2007). To capture the loss of architecture, live video microscopy was conducted as previously described (Jenkins et al., 2012). Briefly, cultures were kept in a fully enclosed inverted Microscope (Zeiss Axio Observer) and imaged by brightfield microscopy every 1 or 24 hours.

Two Dimensional (2D) Culture of Organoids.

Mammary organoids were isolated as described above and cultured in MEC media supplemented with 2% fetal calf serum (FCS) at a density of 100 organoids/well in a 48 well plate. Organoids were allowed to adhere and spread out for 48hrs before being serum starved for 24hrs in MEC media supplemented with 0.5% FCS. The tissue residues were not removed directly, however many washed away in subsequent media washes. All cultures were serum

starved before experimentation. Subsequent experimental materials (growth factors and/or inhibitors) were suspended in MEC media supplemented with 0.5% FCS. For recovery from NHE1 inhibition, organoids were cultured in MEC media containing 2% FCS once the NHE1 inhibitor was removed.

Cell Death Assay:

Cell death in organoids was assessed as described previously (Jenkins 2012). At day 5 and at day 8, live tissue was stained with propidium iodide (PI) (100 μM) to detect cells in which the plasma membranes had become compromised by necrosis or apoptosis. Tissue was then imaged by brightfield and fluorescent microscopy. Cells positive for PI staining were scored as dead. Quantification was performed by dividing the number of dead cells by the area of the tissue imaged. Area was measured using ImageJ.

Immunostaining for NHE1, phospho-histone H3, and E-cadherin:

Mammary organoids cultured in 3D were fixed with 4% paraformaldehyde at 37º C for 5 minutes. The cultures were then washed two times (5 minutes each) with phosphate buffered saline (PBS) before being permeabilized with PBST (PBS+ 0.5% Triton X-100) for 10 minutes. Blocking was performed with blocking buffer (10% horse serum, 2% bovine serum albumin, and 0.5% Triton X-100 in PBS) for 1 hour. Primary antibodies against phospho-histone H3 (1:100)(Ser10, Millipore, Billerica,MA), NHE1 (1:50) (clone 2F5: WH0006548M1; Sigma-Aldrich) (H-160:sc 28758; Santa Cruz Biotechnologies, CA.), or E-cadherin (clone EP700Y; Millipore) were suspended in blocking buffer and incubated overnight at 4ºC. After incubation with primary antibody, samples were washed (2 times with PBS for 5 minutes each then 1 time

with PBST for 5minutes) and incubated with a species appropriate Alexa Fluor conjugated secondary antibody (Invitrogen; 1:1000) in blocking buffer for 1-3 hours shaking at room temperature. After another wash series, samples were stained with 4',6-diamidino-2-phenylindole (DAPI) or propidium iodide (PI) for 5 minutes and imaged. Quantification of phospho-histone H3 staining was performed by capturing optical sections of organoids and dividing the number of positive phospho-histone H3 cells by the total number of cells found in the structure by PI staining. Quantification of NHE1 polarity was accomplished by capturing optical sections of organoids. Cells on the periphery from the center slice of each structure were scored for polarity.

Immunostaining for keratin 8 (K8) and keratin 14 (K14):

3D cultures were fixed with methanol/acetone 1:1 for 20 min at − 20 °C. They were then rinsed twice with PBS for 5 minutes in the 96 well culture plates. The matrigel was then removed from the well and partially air-dried onto an eight chamber slide (BDsciences). Samples were then blocked as described above. Primary antibodies against keratin-14 (K14) (clone #LL002, Novocastra, UK) (1:100) or keratin-8 (K8) (Troma-I-c,DSHB, Iowa City) (1:100) were suspended in blocking buffer and incubated overnight at 4ºC. Secondary antibody staining was performed as described above for NHE1 and phospho-histone H3 staining.

BCECF-AM staining for detection of intracellular pH (pHi)

BCECF-AM was used exactly as described previously (Jenkins et al., 2012). Briefly, day 4 branched organoids were stimulated with TGFα (18 nM) in the presence or absence of NHE1 inhibition by MIA (50 μM) for 30 minutes before pHi was measured. The structures were then incubated with BCECF for 5 minutes and imaged by fluorescent microscopy using a filter set

designed specifically for BCECF-AM (Carl Zeiss, Germany). BECEF-AM loaded organoids were then alternatively excited with 440nm and 490nm light, recording emission at 535nm. The light source was a mercury bulb. An image (1390x1390) was captured at each excitation wavelength. Ratiometric analysis, i.e. pixel by pixel division of the 440nm and 490nm images and a standard curve of pHi were then used to determine the pHi as described previously (Jenkins et al., 2012). The ratio image was then mapped to a discrete 16 bit color map so that pH values could be visualized.

Actin Staining with Phalloidin

Actin organization was visualized by staining with fluorescent conjugated phalloidin (Alexa Fluor® 594 phalloidin from Invitrogen). Organoids were cultured in a 96 well format and stimulated to branch (day 0), then re-stimulated (day 4) with TGFα in the presence or absence of NHE1 inhibition by MIA (50 μM). Organoids were fixed with the Actin Visualization Biochem Kit from Cytoskeleton (Cat. BK005) on day 5. Fixation and staining were performed in the well according to the manufacturer's specifications. After staining was complete, the samples were removed from the well and lightly air-dried to a glass slide to be imaged by confocal microscopy.

Immunoblotting

Immunoblotting was performed as previously described (Jenkins et al., 2012). All primary antibodies were suspended in Tris buffered Saline (TBS) containing 5% BSA and 0.05% Tween-20. Rabbit anti-E-cadherin (Cell Signaling # 3195) (1:1000) was used to detect E-cadherin. Mouse monoclonal anti-keratin-14 (K14) (clone #LL002, Novocastra, UK) (1:100) was used to

detect keratin 14. β-Actin was detected with rabbit polyclonal anti-β-Actin (Cell Signaling #4967) (1:2000). All samples were briefly sonicated on ice prior to loading.

Statistics

Statistical significance was determined using the Student's t-test function in GraphPad Prism 5 for Windows (GraphPad Software, San Diego California USA).

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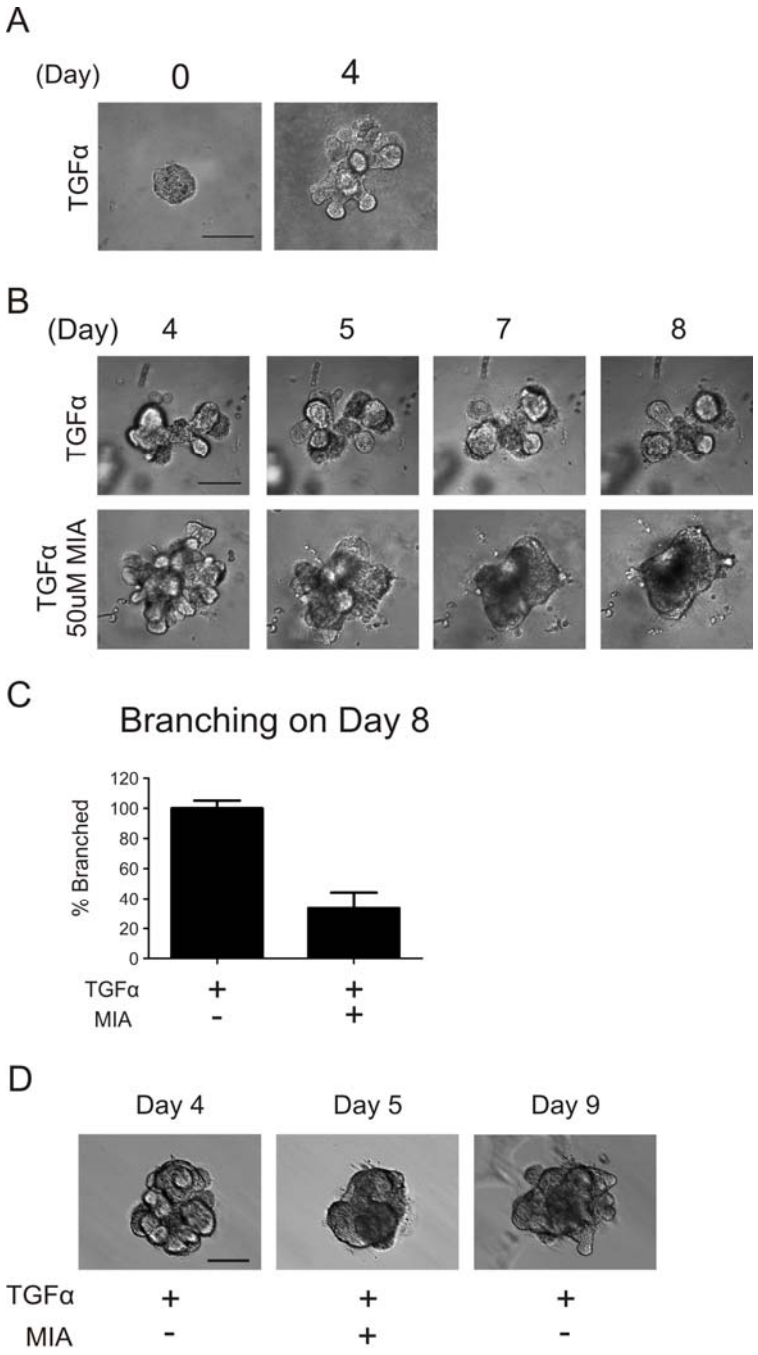


Figure 1: NHE1 inhibition causes a loss of branched architecture. A) TGFα (18nM) induces branching of organoids in 4 days. B) Inhibition of NHE1 with 50μM MIA caused a loss of branched architecture that was evident after 24hours (day 5) and was complete after 96hrs (day 8). C) Quantification of branching on day 8 (-MIA =99.98%±7.85% vs. +MIA =45.28%±12.93%) ($p<0.01$). Quantification was normalized to the control (- MIA) condition. D) Structures that had undergone a loss of architecture by NHE1 inhibition with MIA were able to re-establish a branched structure after the NHE1 inhibitor (MIA) was removed from the culture. Scale bars equal 100μm.

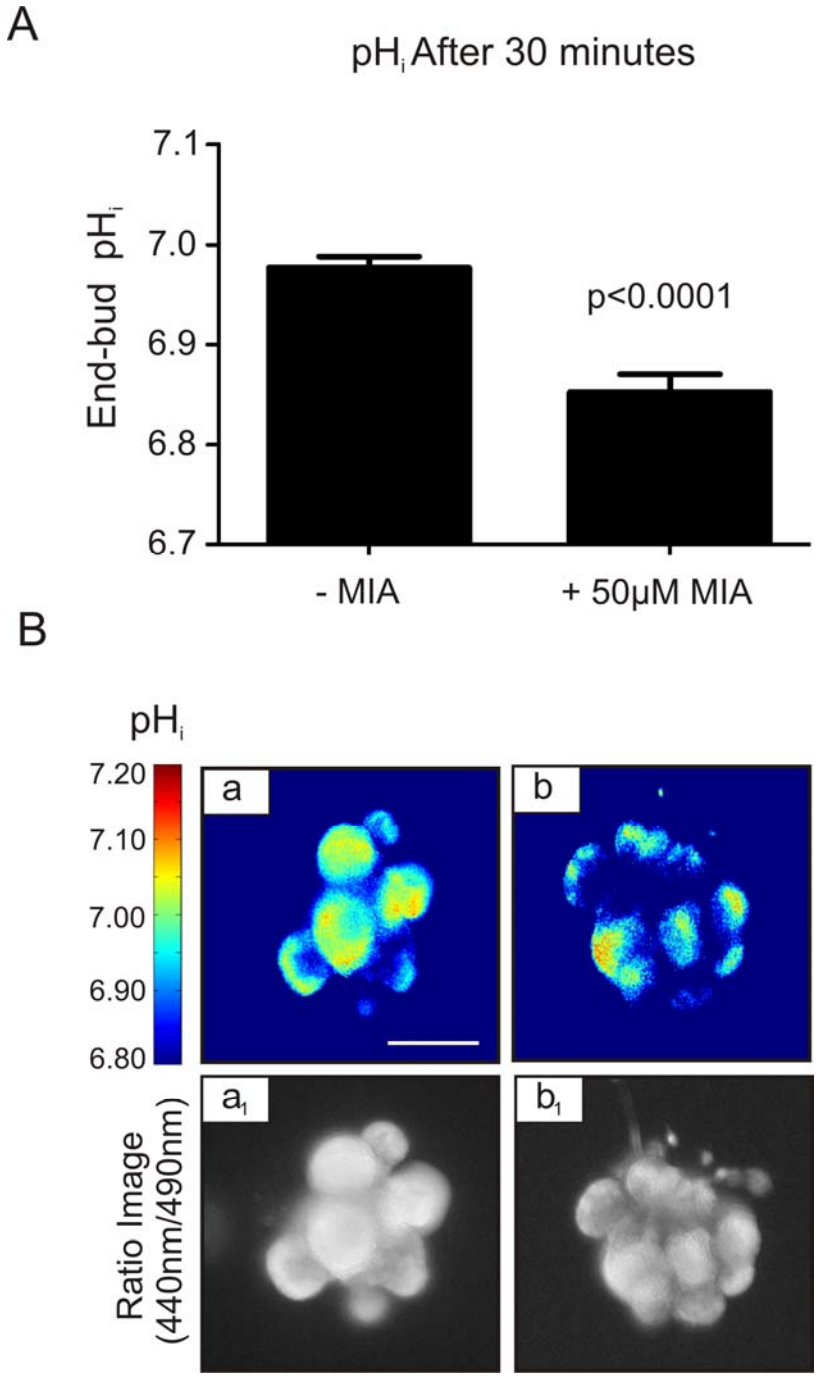


Figure 2: NHE1 inhibition with MIA caused decreased intracellular pH (pHi) in end buds of branched organoids. A) Quantification of pHi using BCECF-AM. Inhibition with MIA (50μM) lead to significantly lower pHi in the end buds of branched structures (-MIA =6.99±0.02; n=19 vs +50μM MIA=6.88±0.03; n=34 vs. 6.99±0.02) ($p<0.0001$). Values were measured from individual end buds regions. B) Intracellular pH visualized by pH maps of branched organoids (a= -MIA, b= +50μM MIA). Lower panels (a_1 and b_1) represent the image division of the 440nm and 490nm image with no pseudo-coloring. Scale bar equals 100μm.

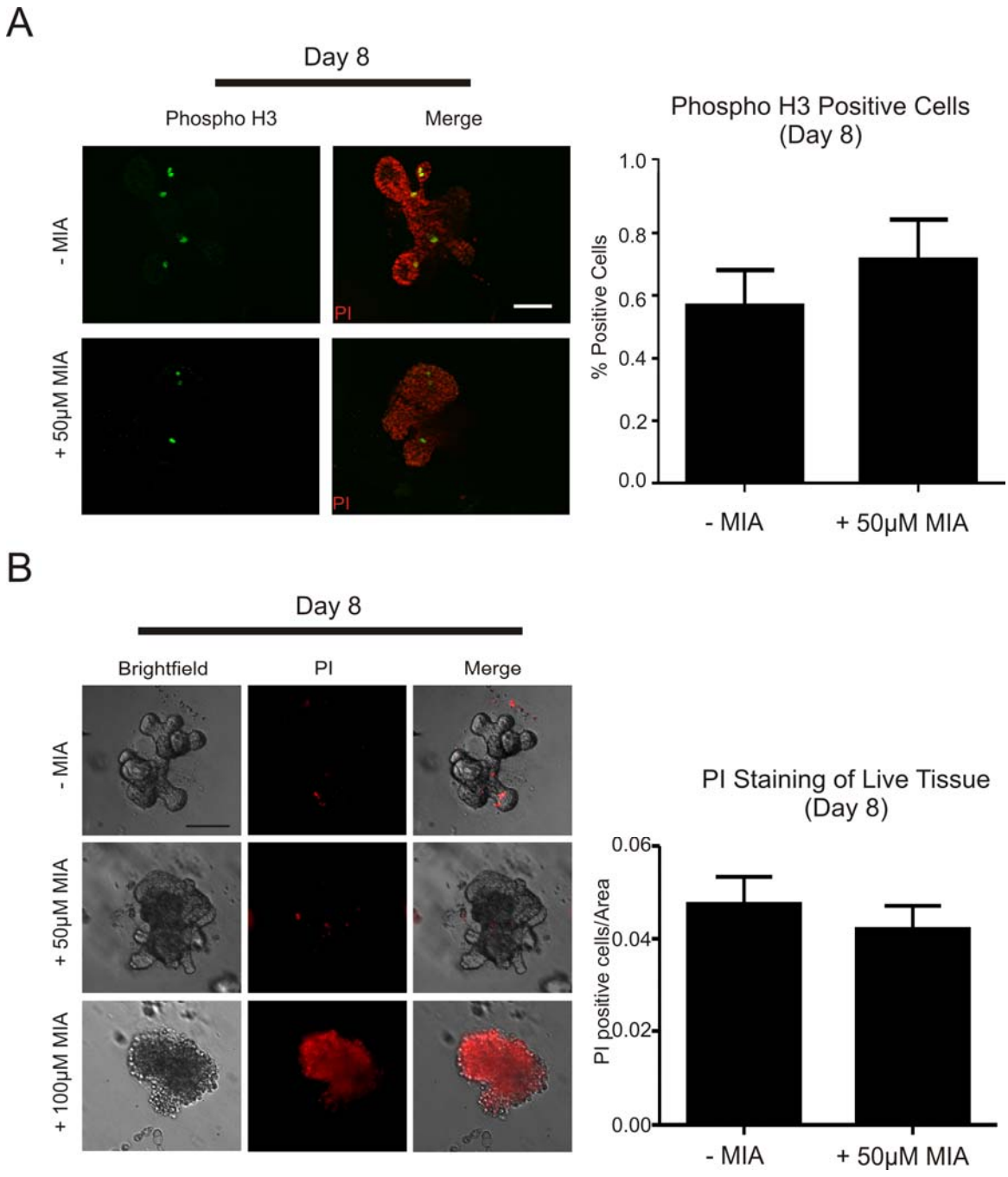


Figure 3: NHE1 inhibition did not alter proliferation or cell death in organoids. A) phospho-histone H3 immunostaining and quantification of organoids ± 50µM MIA on day 8 (-MIA= 0.57%±0.11%; n=10, +50µM MIA=0.72%±0.11% n=10). The numbers reflect counts taken across z-stacks. The images in panel A were selected from near the center of the two respective structures. B) Propidium iodide (PI) staining and quantification of dead cells in organoids ± MIA on day 8. (-MIA=0.048±0.005; n=4, +50µM MIA= 0.042±0.005; n=8). NHE1 inhibition with 100µM was used as a positive control for PI staining. Scale bars equals 100µm.

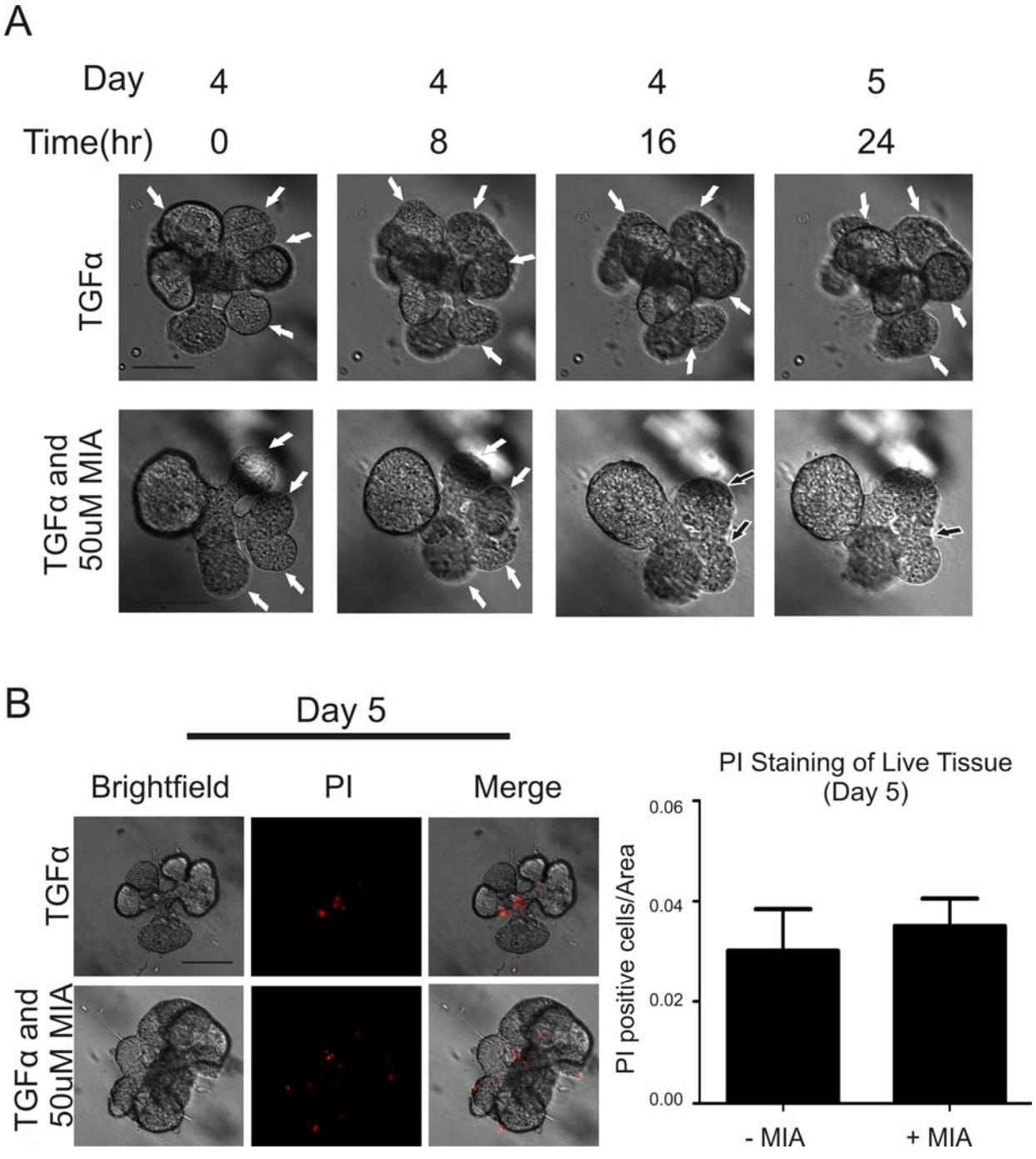


Figure 4: NHE1 inhibition causes a loss of branched architecture by branch fusion in 24hrs. A) Brightfield images of organoids ± NHE1 inhibition with 50μM MIA. White arrows indicate the borders of discrete endbuds. Black arrows indicate areas of fusion. B) Propidium Iodide (PI) staining and quantification of dead cells in organoids on day 5 (-MIA=0.03±0.008; n=9, +50μM MIA=0.04±0.006; n=10). Scale bars equal 100μm.

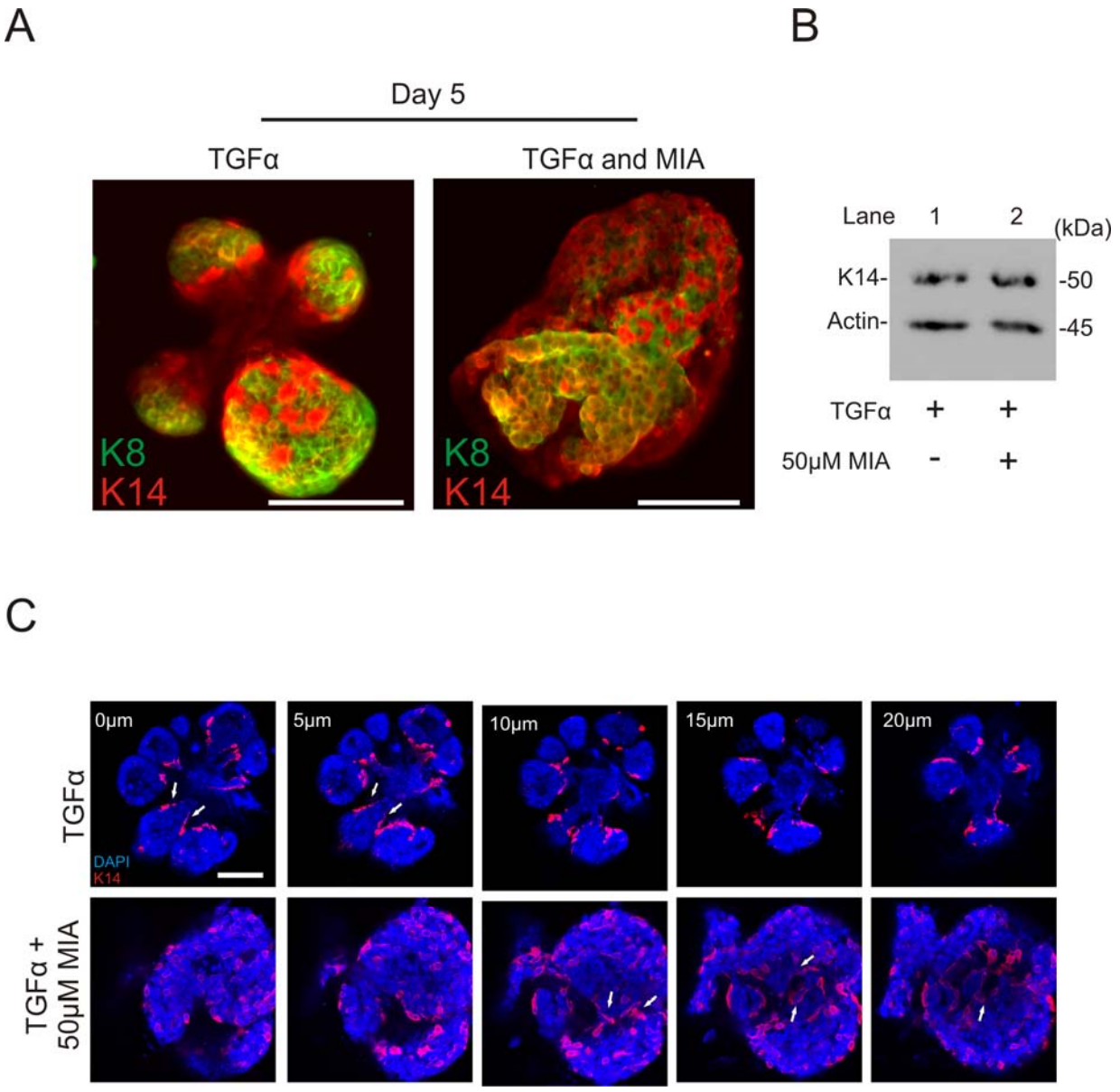


Figure 5: Tissue polarity of myoepithelial cells is altered in disrupted structures. A) Immunostaining of keratin-14 positive myoepithelial (K14; red) and keratin-8 positive epithelial cells (K8;green) in branched (TGFα) and disrupted (TGFα + 50μM MIA) organoids on day 5. B) Immunoblotting of protein isolated from organoids on day 5 cultured in 3D that either had (+MIA) or had not (-MIA) undergone a loss architecture. Actin was used as a loading control. C) Immunostaining and optical sectioning of branched (TGFα) and disrupted (TGFα + 50μM MIA) organoids on day 5. K14 positive cells (red) are indicated with white arrows. Scale bars equal 100μm.

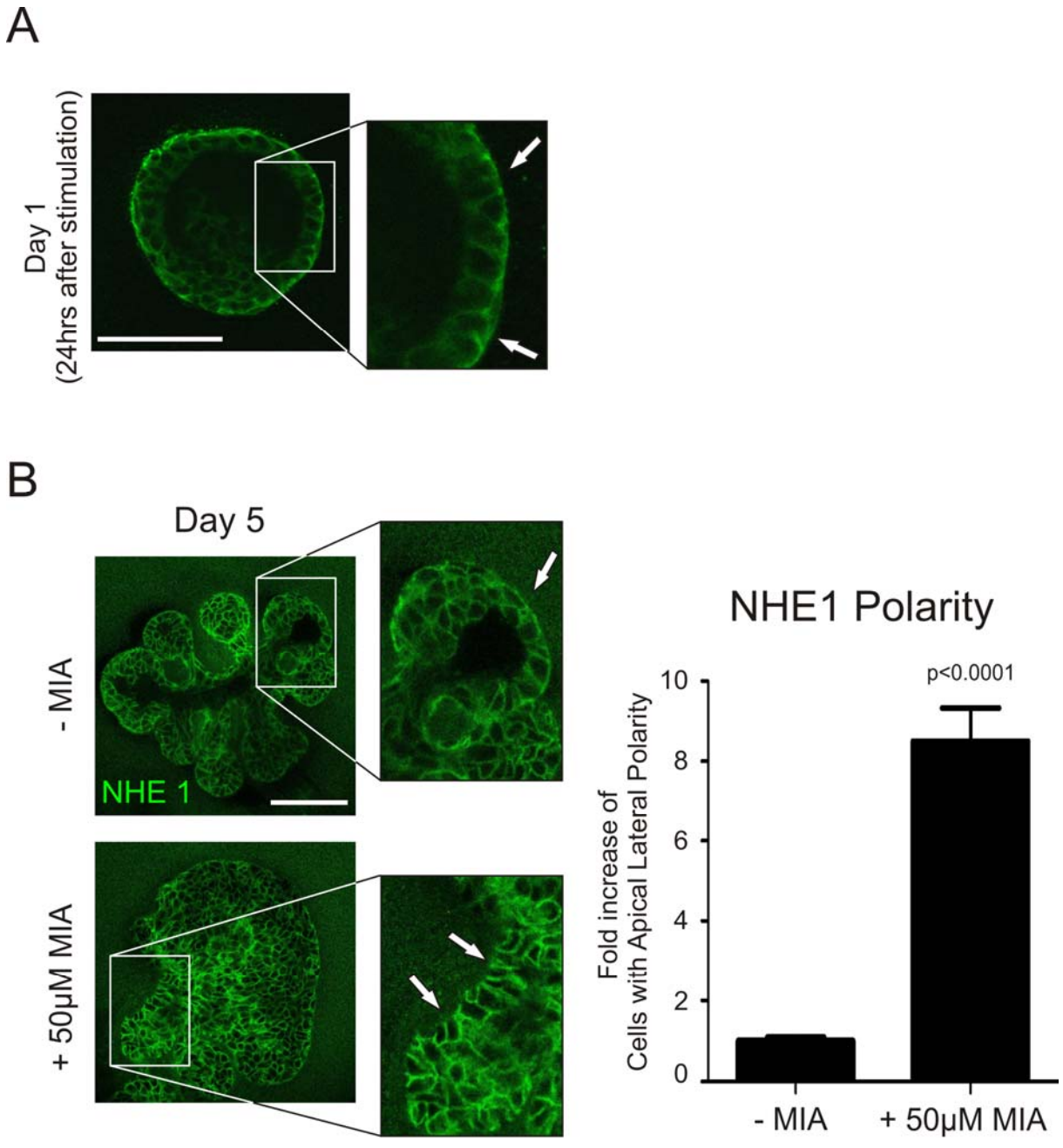


Figure 6: NHE1 polarity is disrupted in structures that have undergone a loss of architecture. A) Immunostaining for NHE1 (green) on day 1. B) Immunostaining for NHE1 (green) of organoids that either had (+50μM MIA) or had not (-MIA) undergone a loss of architecture on day 5. The number of cells with apical lateral polarity along the periphery of the center slice of each structure was quantified and expressed as fold increase (-MIA=1±0.09; n=5, +50μM MIA 8.5±0.83; n=10) ($p<0.0001$). Scale bar in A= 50 μm and B=100 μm.

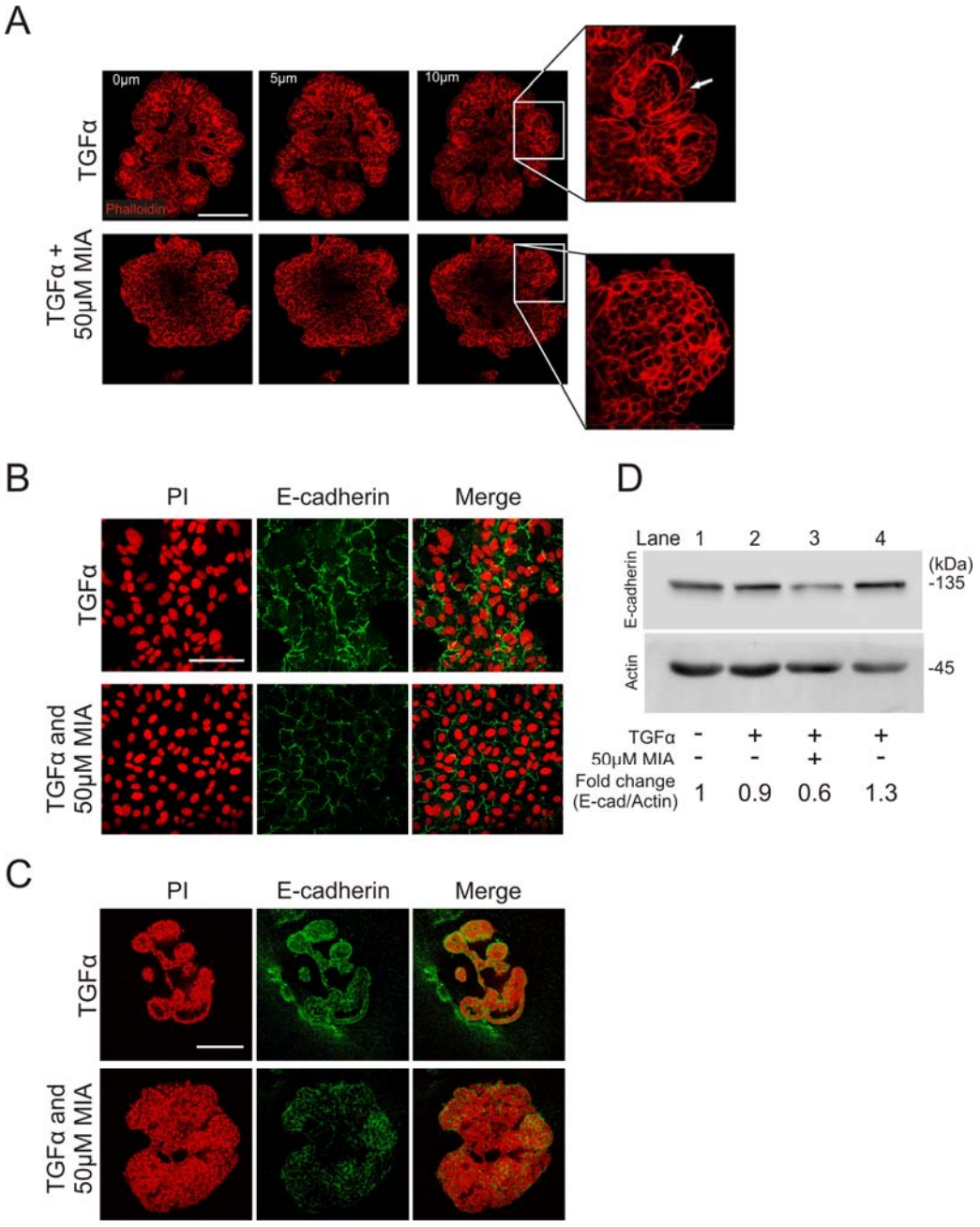
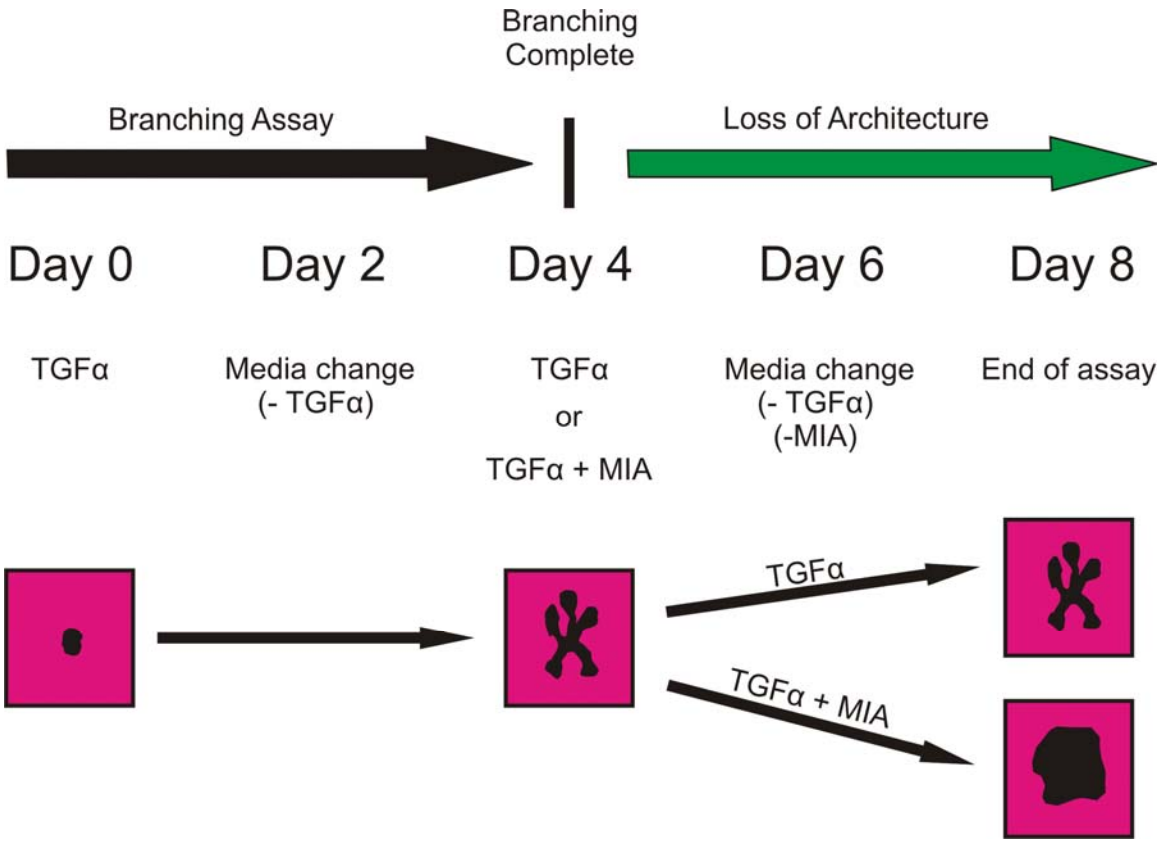
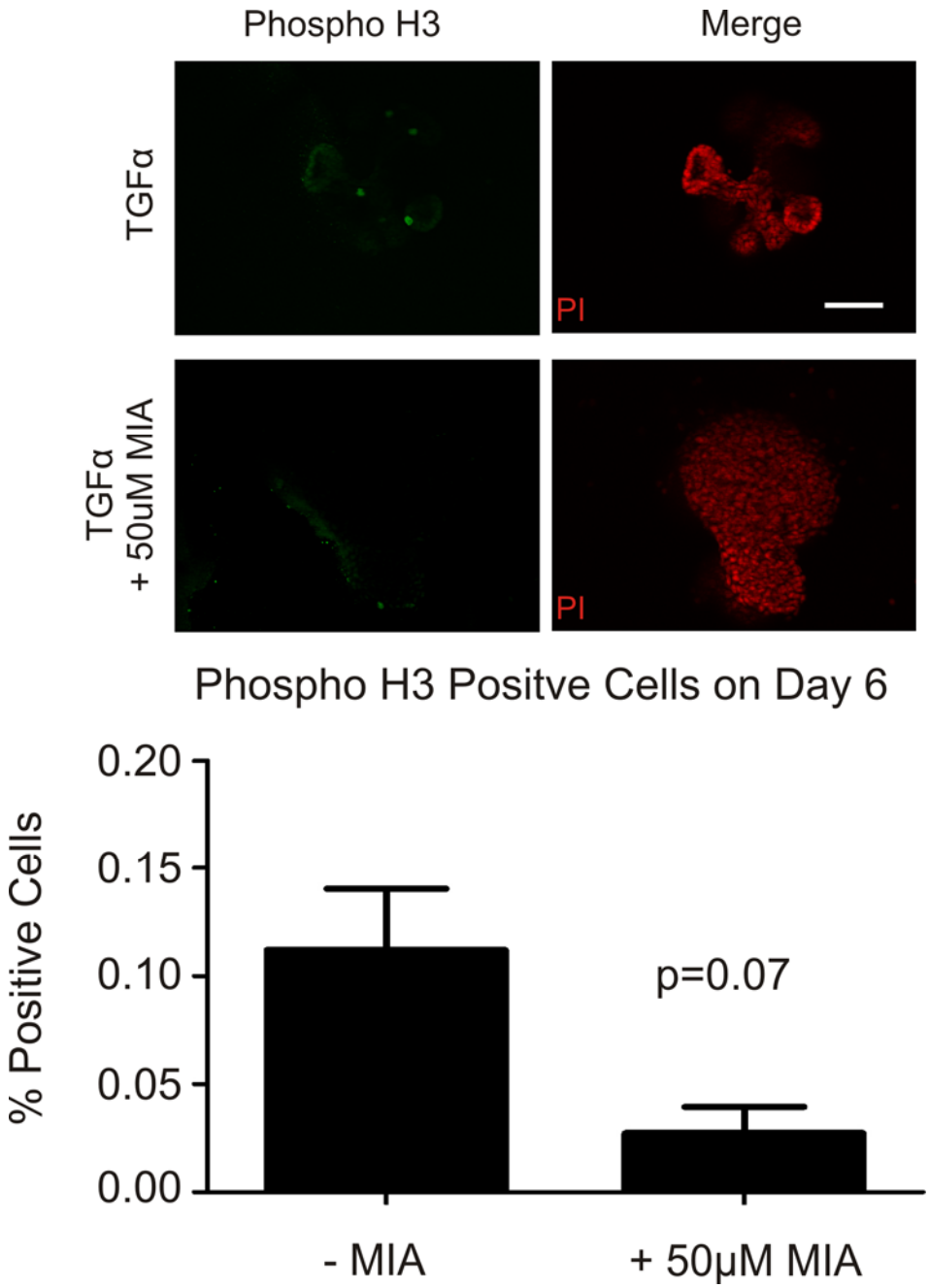


Figure 7: Actin organization and E-cadherin expression are altered in disrupted structures. A) Phalloidin staining of branched (TGFα) and disrupted (TGFα + 50μM MIA) organoids on day 5. Images (left to right) are optical sections of organoids. Thick bands of circumferential actin rings (white arrows) can be seen on the apical side of endbuds in the branched structures (TGFα). B) Immunostaining for E-cadherin (green) in organoids cultured in 2D ± NHE1 inhibition with MIA. Images were captured by confocal microscopy under identical conditions. C) Immunostaining for E-cadherin (green) of branched (TGFα) and disrupted (TGFα + 50μM MIA) organoids on day 5. Images are optical sections from the center of each structure. D) Immunoblot for E-cadherin of protein isolated from organoids that were cultured in 2D. Lane 1: no stimulation, Lane 2: stimulation with TGFα, Lane 3: stimulation with TGFα and NHE1 inhibition with MIA, and Lane 4: stimulation with TGFα and NHE1 inhibition with MIA for 24 hours followed by recovery in the absence of NHE1 inhibition in basal media. Protein in lanes 1-3 was collected 24 hours after stimulation. Protein in lane 4 was collected 72 hours after removal the NHE1 inhibitor. Scale bar in A=50 μm, B and C=100 μm.



Supplemental 1: Schematic representation of the branching and loss of architecture assay. Organoids were isolated from virgin Balb/C mice and cultured in matrigel. Organoids were stimulated to branch with TGFα (18nM). Branching was considered complete on day 4 at which time organoids re-stimulated with TGFα ± NHE1 inhibition by MIA (50 μM).



Supplemental 2: Loss of branched architecture by NHE1 inhibition does not alter proliferation on day 6. Phospho-histone H3 immunostaining and quantification of orgnanoids ± 50μM MIA on day 6 (-MIA= 0.11%±0.03%; n=4, +50μM MIA=0.03%±0.04%; n=3). The numbers reflect counts taken from z-stacks. The images were selected from near the center of the two respective structures.

Chapter 3: Na+/H+ exchanger-1 (NHE1) inhibition in other branched organs

Abstract

Branching morphogenesis is a developmental process that gives rise to the lung and kidney, as well as the salivary and mammary glands. Our previous work has shown the Na+/H+ exchanger subtype 1 (NHE1) is a critical regulator of mammary branching morphogenesis and is necessary for the maintenance of branched mammary tissue architecture. To explore the role of pH regulation by NHE1 in the development of other branched systems, we isolated and cultured embryonic lung, kidney and submandibular salivary glands (SMGs) in the presence or absence of the NHE1 inhibitor 5-N-Methyl N-isobutyl amiloride (MIA). We found that NHE1 inhibition did not drastically alter branching morphogenesis of any of these organs, however, the formation of the primary branches of the lung appeared to be altered by NHE1 inhibition.

Introduction

A branched epithelial duct network is an efficient approach to maximize surface area for fluid production or gas exchange. These networks form by branching morphogenesis, which is the developmental process that gives rise to the functional architecture of the mammary and salivary glands, as well as the lung and kidney. Though the formation of these branched systems utilize different signaling pathways, the basic processes of invasion, elongation and duct formation remain a common theme in the development of all of these structures.

The highly ordered branches observed in the lung function to exchange gases between the external environment and the circulatory system of an organism. The formation of this structure begins in embryonic development when lung epithelium from the ventral foregut endoderm invades into its surrounding mesenchyme. As with other branched systems, signaling crosstalk between the epithelium and its supporting mesenchyme specifies the epithelium for lung branching and dictates the extent and direction of subsequent branches. This process utilizes cell extrinsic factors such as fibroblast growth factors (FGFs), bone morphogenic protein (BMP), Sonic hedgehog (Shh), and retinoic acid, as well as cell intrinsic pathways such as Wnt and Sprouty (Kim and Nelson, 2012; Morrisey and Hogan, 2010 ; Warburton et al., 2000).

Kidney branching morphogenesis in the mouse begins at embryonic day 10.5-11 when a small portion of the epithelial nephric duct forms a bud (ureteric bud or UB) that invades through the metanephric mesenchyme (MM) (Little and McMahon, 2012). The formation and subsequent directed branching of the UB is dictated by the MM and utilizes a number of stimulatory (i.e. glial cell line-derived neurotrophic factor (GDNF and FGFs) as well as inhibitory (i.e BMPs and TGFβ) signaling molecules (Costantini and Shakya, 2006; Kim and Nelson, 2012; Nigam and Shah, 2009). As with the mammary gland, branch propagation in this system results primarily

from bi- and, some cases, tri-furcation of the invading epithelial bud.

The salivary glands utilize a highly ordered branched architecture to produce and deliver saliva into the mouth of an organism to aid in the digestion of food and protect against infectious pathogens. The adult has three major pairs of salivary glands: the sublingual, the submandibular, and the parotid. Though histologically unique, the patterns of branching morphogenesis that give rise to these structures have many common elements. Branching morphogenesis of the salivary glands occurs during embryonic development. Like the lung and kidney, the nascent salivary epithelium is surrounded by its supporting mesenchyme. Unlike the lung and kidney, however, branching is initiated by cleft formation rather than invasion and bifurcation of the epithelium. As in the development of the branched systems described above, crosstalk between the salivary epithelium and mesenchyme occurs by cell extrinsic factors such as epidermal growth factor (EGF) and FGFs which call upon intrinsic signaling pathways involving mitogen-activated protein kinase (MAPK), phospholipase Cc1 (PLCc1) and phosphatidyl-inositol-3-kinase (PI3K) among others (Kim and Nelson, 2012; Patel et al., 2006).

As described earlier in chapters 1 and 2, the mammary gland uniquely undergoes only rudimentary branching during embryonic development and then pauses until ovarian hormones such as estrogen and progesterone begin systemic circulation. Previously, we have shown that the Na+/H+ exchanger-1 (NHE1) function is critical for mammary branching in a three-dimensional (3D) organotypic model of branching morphogenesis (Jenkins et al., 2012). To investigate the role of NHE1 in branching morphogenesis of other organs, we dissected and cultured lung, kidney, and submandibular salivary gland from embryonic mice in the presence or absence of NHE1 inhibition.

Materials and Methods

Salivary (Submandibular) gland extraction and culture

The uterus of pregnant Balb/C mice was removed 13 days after mating and partially submerged in basal media (DMEF/F12 with 1% insulin, transferrin, selenium, and 1% penicillin/streptomycin) in a 10 cm plate (BDsciences). The uterus was cut above and below each embryo to allow the uterine muscles to contract and push out the embryos. Embryos were sectioned transversely across the thorax, then transversely across the neck, exposing the mandible. The mandible was cut along the midsagittal plane and pulled away exposing the contralateral submandibular glands. Most of the hindbrain was cut away with a coronal section, and then the submanidular glands were teased away from the mandible. The salivary mesenchyme was left intact. The glands were cultured at the fluid-air interface floating on non-abrasive, non-linting Ross Optical Lens Paper (Ladd Research Industries inc. part # 12700) filter paper in a 6 well plate (BDsciences) containing 400μL of basal media for 48hrs prior to stimulation with 18μM TGFα in the presence or absence of MIA (50μM).

Lung extraction and culture

Embryos were collected as described above. After extracting the submanidular glands, the thorax of embyonic day 13 Babl/C mice was incised sagittally, exposing the nascent lung epithelium and mesenchyme. The lungs were then removed, leaving the lung mesenchyme intact. Culturing and exposure to MIA were as described above for the salivary gland. Lung tissue was stimulated to branch with 5% serum in the presence or absence of 5-N-Methyl N-isobutyl amiloride (MIA) (20μM).

Kidney Extraction and culture

Embryos were collected as described above. After extracting the submanidular glands, the abdominopelvic cavities of embyonic day 13 Babl/C mice were incised sagittally, exposing the nascent kidney epithelium and mesenchyme. The kidneys were then removed, leaving the kidney mesenchyme intact. Culturing and exposure to MIA were as described above for the salivary gland. Kidney tissue was stimulated to branch with 5% serum in the presence or absence of MIA (20μM).

Imaging

Images were captured every 12 or 24 hours with a Zeiss dissection microscope (Discovery.V8 Stereoscope; Carl Zeiss, Germany). The extent of branching was determined by visible inspection of the tissue.

Results

MIA did not inhibit lung branching morphogenesis

Embryonic day 13 lung tissue was cultured as described above in the presence or absence of the NHE1 inhibitor MIA (20μM) and followed for 72 hours at 24 hour increments (Fig. 1). Though a considerable amount of branching occurred *in* vivo, we were able to stimulate continued branching *ex vivo* with 5% FCS (Fig. 1A). The addition of MIA did not dramatically reduce the amount of branching morphogenesis, however, the structure of the primary bronchi seemed to be altered by NHE1 inhibition (compare Fig. 1 panel A with panel B at 48 and 72 hours).

MIA did not inhibit kidney branching morphogenesis

Embryonic day 13 kidney tissue was cultured as described above in the presence or absence of the NHE1 inhibitor MIA (20μM) and followed for 24 hours at 12 hour increments (Fig. 2). Though it was evident that a considerable amount of branching morphogenesis had occurred *in vivo* before stimulation with FCS (5%), we were able to observe continued branching *ex vivo*. We did not, however, observe any obvious reduction in branching morphogenesis as a result of NHE1 inhibition.

MIA did not inhibit Salivary (Submandibular) gland branching morphogenesis

As alluded to above, the salivary gland responds to many of the same morphogens as the mammary gland. TGFα, an epidermal growth factor receptor (EGFR) ligand, was used in our mammary organoid branching assay (see chapters 1 and 2) to induce branching morphogenesis (Fata et al., 2007; Jenkins et al., 2012). We used this same growth factor to induce branching

morphogenesis in SMG tissue isolated from embryonic day 13 mice. Branching was followed for 72 hours at 12 hour intervals (Fig 3). We did observe SMG branching morphogenesis in response to TGFα (18nM), however, the NHE1 inhibitor MIA (50μM) did not have an obvious effect on this response.

Discussion

NHE1 is a ubiquitously expressed critical regulator of intracellular pH (pHi). Previously, we were able to show that NHE1 was a critical regulator of mammary branching morphogenesis in a 4 day organotypic model of mammary branching. We were not, however, able to demonstrate this same result in the lung, kidney, or salivary gland. This result does not prove that NHE1 is not essential for branching morphogenesis in these organs. Rather, it is likely that we did not observe an inhibition in branching due to the very different culture methods used in our 3D model of mammary branching morphogenesis versus the tissue culture methods described above. Most notably, the method of isolation and culture for the lung, kidney, and salivary gland used in this project left the respective mesenchyme of these tissues intact. Our 3D model of mammary branching utilized only the epithelial portions of the gland suspended in a laminin rich extracellular matrix (ECM). Additionally, we noted a dose dependent inhibition of branching in this system, suggesting that the amount of NHE1 inhibition on the epithelium directly affects the morphogenic response of the tissue. The intact mesenchyme of the lung, kidney, and salivary gland in the above cultures likely insulated the epithelium from NHE1 inhibition by MIA, thus, we were not able to inhibit branching morphogenesis in these organs. Alternatively, the intact mesenchyme of the three branching systems described above acted as a kind of living matrix, complete with fibroblasts, endothelial cells, and immune system cells that collectively may have

provided a more robust stimulus for branching than we observed in our model of mammary gland morphogenesis, which look place in non-living purified basement membrane (matrigel). In either case, further work should be done with higher concentrations of the NHE1 inhibitor to determine the role of NHE1 in the development of these systems.

Intracellular pH change has been associated with a number a developmental processes such as fertilization of *Xenopus* and sea urchin eggs (Charbonneau and Grandin, 1992; Grainger et al., 1979; Shen and Steinhardt, 1979) and during *Xenopus* oocyte maturation (Webb and Nuccitelli, 1981). Uncovering the role of NHE1 and intracellular pH regulation in branching morphogenesis of the lung, kidney, and salivary gland will require further work and potentially alternative culture methods.

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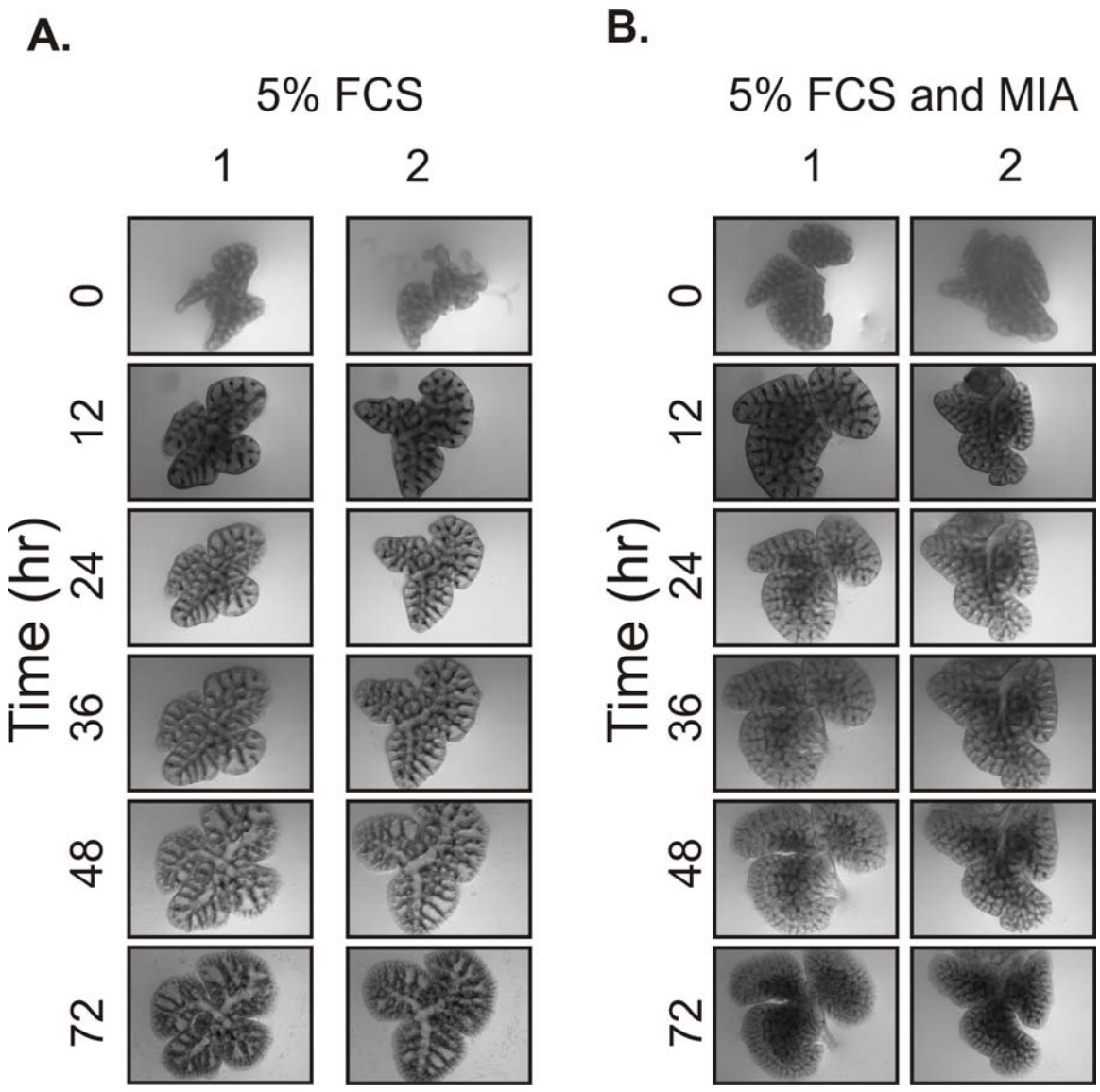

Figure 1: Lung branching morphogenesis in the presence of NHE1 inhibition by 5-N-Methyl N-isobutyl amiloride (MIA) (20 μM). A) Lungs were isolated from embryonic day 13 Balb/C mice (5) (time 0). Left lungs from each animal were stimulated to branch with 5% FCS and were cultured in the absence of NHE1 inhibition. Panel A represents two lungs from two animals. B) Lungs were isolated from embryonic day 13 Balb/C mice (5) (time 0). Right lungs from each animal were stimulated to branch with 5% FCS and were cultured in the presence of NHE1 inhibition by MIA (20 μM). Panel B represents two lungs from two animals.

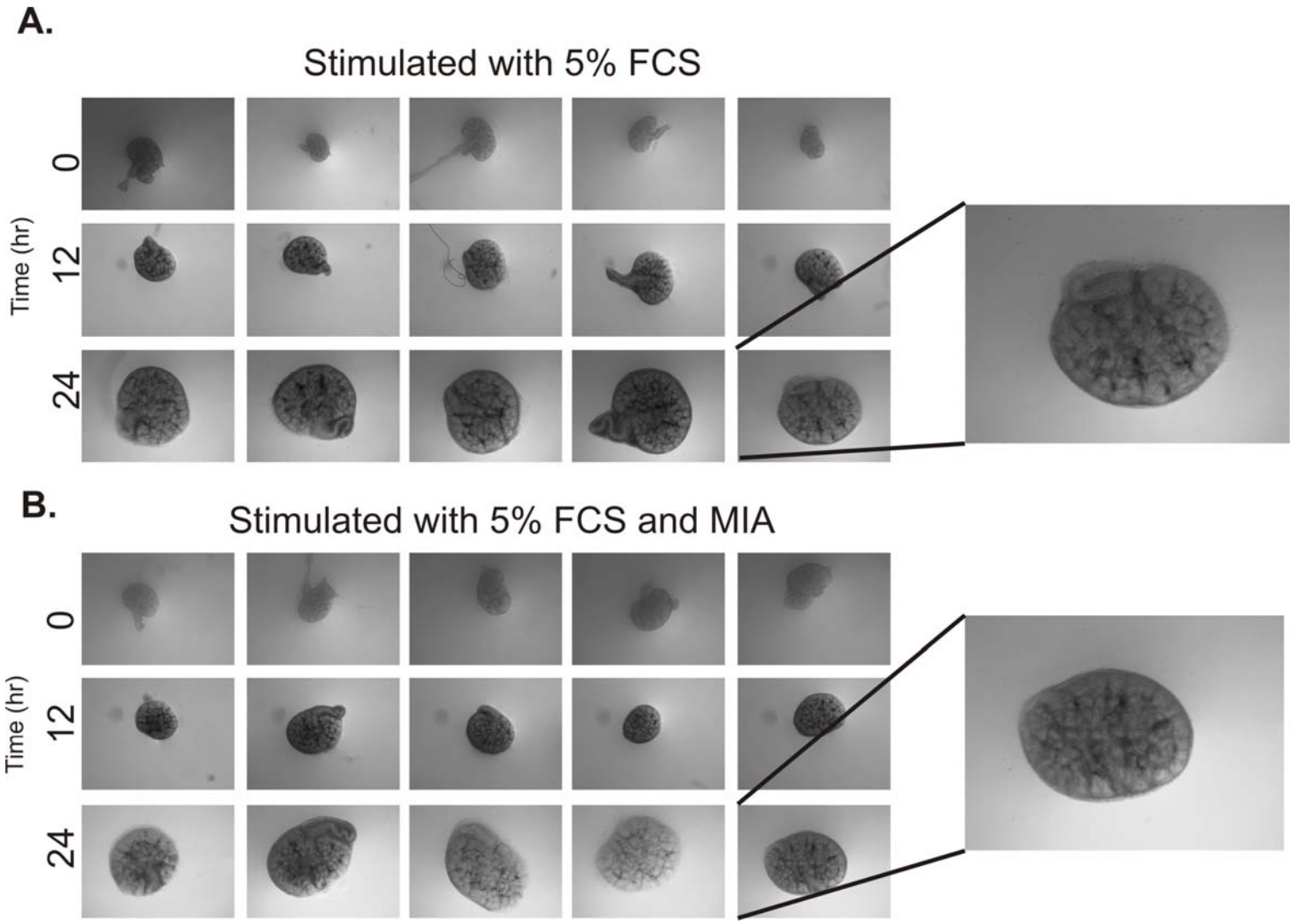

Figure 2: Kidney branching morphogenesis in the presence of NHE1 inhibition by 5-N-Methyl N-isobutyl amiloride (MIA) (20 μM). A) Kidneys were isolated from embryonic day 13 Balb/C mice (5) (time 0). Left kidneys from each animal were stimulated to branch with 5% FCS and were cultured in the absence of NHE1 inhibition. B) Kidneys were isolated from embryonic day 13 Balb/C mice (5) (time 0). Right kidneys from each animal were stimulated to branch with 5% FCS and were cultured in the presence of NHE1 inhibition by MIA (20 μM).

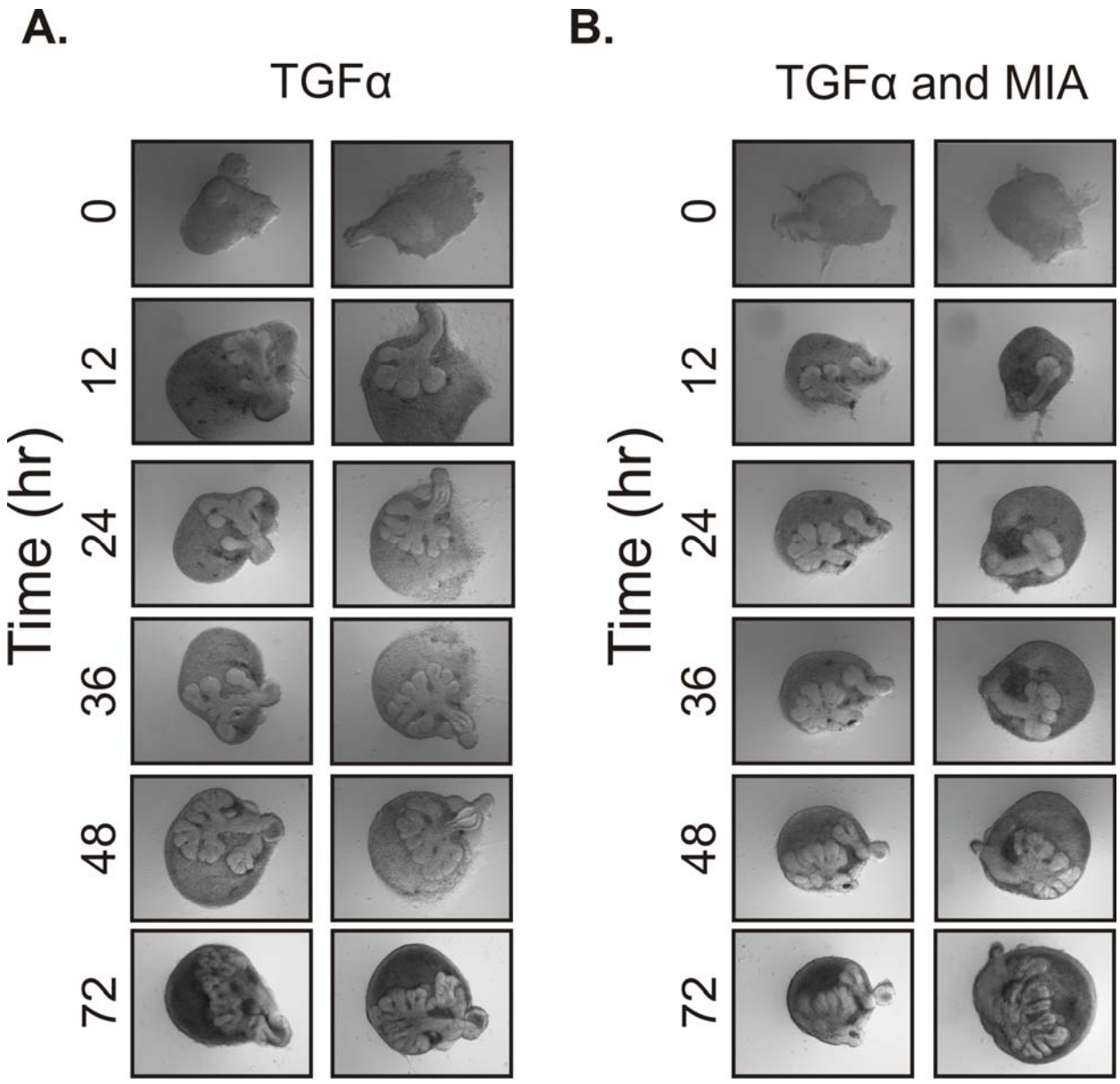

Figure 3: Submandibular gland (SMG) branching morphogenesis in the presence of NHE1 inhibition by 5-N-Methyl N-isobutyl amiloride (MIA) (20 μM). A) SMGs were isolated from embryonic day 13 Balb/C mice (5) (time 0). Left SMGs from each animal were stimulated to branch with 18nM TGFα and were cultured in the absence of NHE1 inhibition. Panel A represents two SMGs from two animals. B) SMGs were isolated from embryonic day 13 Balb/C mice (5) (time 0). Right SMGs from each animal were stimulated to branch with 18nM TGFα and were cultured in the presence of NHE1 inhibition by MIA (50 μM). Panel B represents two SMGs from two animals.

Chapter 4: NHE1 as a potential target for chemotherapy augmentation.

Abstract

In recent years, targeted therapies have vastly improved the effectiveness of treatment for breast cancer patients. Even still, however, cytotoxic chemotherapy with a wide variety of adverse side effects is still included in many treatment regimens. De-regulation of pH is one of the defining characteristics of disease progression in cancer. This has been attributed to the Na+/H+ exchanger subtype 1 (NHE1), a master regulator of intracellular pH. Here, with the human breast cancer cell line MCF7, we explored the therapeutic potential of NHE1 inhibition by combining two commonly prescribed chemotherapeutic agents, cyclophosphamide (CPA) and doxorubicin (DOX), with the NHE 1 inhibitor 5-N-Methyl N-isobutyl amiloride (MIA). We found that NHE1 inhibition at a non-toxic level was able to lower the IC_{50} of CPA by roughly 5 fold. Our results suggest that NHE1 is a good potential target for chemotherapeutic augmentation.

Introduction

Breast cancer is a disease whereby functional tissue disregards normal polarity and growth regulating pathways and begins to proliferate in a disorganized and dysfunctional manner. Considerable work and resources have been applied to understanding the etiology of this disease, as well as to the discovery of new and more effective methods of treatment. Over the last 20 years, utilizing genetic and molecular techniques, remarkable advances have been made in the development of targeted therapies that have vastly improved the treatment of various cancers, including breast cancer. Drugs such as tamoxifen and transtuzamab (Herceptin), which

target the Estrogen Receptor (ER) and the Human Epidermal Receptor type 2 (HER2) respectively, have become the gold standard of treatment for patients whose breast cancer expresses these targets (Brollo et al., 2013; Fleeman et al., 2011; Jaiyesimi et al., 1995; Tai et al., 2010; Tan and Swain, 2003). Still, due to the genetic heterogeneity of the disease, the current regimens of treatment for many cancers include the use of traditional cytotoxic chemotherapies (NCCN Guidlines 2012). Many of these agents, such as cyclophospamide (CPA) and doxorubicin (DOX), are highly toxic to the patient.

Our previous work has shown that intracellular pH (pHi) regulation by the Na+/H+ exchanger subtype 1 (NHE1), a ubiquitously expressed master regulator of pHi, is necessary for normal processes such as mammary branching morphogenesis and the maintenance of tissue architecture. This exchanger accomplishes pH regulation by catalyzing the electro-neutral exchange of extracellular Na+ for intracellular H+ in a 1:1 stoichiometry. Using both cell culture and *in vitro* tumor systems, normal regulation of pH has been shown to be disrupted in cancer. Deregulation of pH in transformed tissue results in a reversal of the normal pH gradient to a more acidic extracellular pH (pHe) (6.2–6.9 vs. 7.3–7.4) and more alkline pHi (7.12–7.65 vs. 6.99–7.20) compared with normal tissues (Cardone et al., 2005; Gillies et al., 2002). This reversed pH gradient is permissive to aggressive cancer cell behaviors such as uncontrolled cell cycle progression (Doppler et al., 1987), both serum and anchorage independent growth, and increased aerobic glycolytic metabolism (Reshkin et al., 2000). The acidic extracellular micro-environment has even been shown to promote invasion by the activation of acid induced proteases (Kindzelskii et al., 2004).

There is some evidence to suggest that this effect is irrespective of the considerable genotypic heterogeneity observed in cancer, as pH gradient disruption is seen very early in

cancer progression, specifically during transformation. This has been demonstrated with a variety of individual oncogenes (Cardone et al., 2005; Hagag et al., 1987; Kaplan and Boron, 1994; Maly et al., 1988; Ober and Pardee, 1987; Reshkin et al., 2000; Siczkowski et al., 1994). Using an inducible E7 oncogene from HPV16, Reshkin et al. showed that the pH reversal was due specifically to NHE1 activity, as inhibiting NHE1 with 5-N,N-dimethylamiloride (DMA) blocked the phenomenon (Reshkin et al., 2000). NHE1 itself is over-expressed in a number of cancers including: bladder (Sanchez-Carbayo et al., 2006), esophageal (Wang et al., 2006), cervical (Scotto et al., 2008), and in lobular breast cancer (Zhao et al., 2004), however, over-expression is not necessary for NHE1 to effect pH alteration, as it becomes constitutively active in transformed cells (Cardone et al., 2005).

The abnormal cancer cell pH gradient is directly due to the cell's ability to hyper-secrete H+ ions by NHE1 over-activation. When considering the involvement of pH regulation by NHE1 in both normal and cancerous biological processes, it becomes clear that this exchanger is a strong candidate for the treatment of breast cancer as its inhibition has the potential to mitigate many aggressive cancer cell behaviors. We therefore explored NHE1 as a potential target for chemotherapy augmentation in combination with CPA and DOX, two commonly prescribed chemotherapeutic agents, in the human breast cancer cell line MCF7. We found that NHE1 inhibition at non-toxic levels was able to lower the IC_{50} of CPA in MCF7 cells by more than 5 fold. Though our findings are preliminary, we believe that our data suggests that NHE1 is a viable target for chemotherapy augmentation.

Materials and Methods

Cell Culture

The MCF7 cell line was obtained from the American Type Culture Collection (ATCC) and was maintained between 20 and 70% confluence in a 10 cm plate (BDsciences) with basal media ((High Glucose Dulbecco's Modified Eagle's Medium (Lonza), 1x Penicillin / Streptomycin (Gibco)) containing 10% (v/v) fetal calf serum (FCS) (Fisher). Media was removed and replaced with a fresh volume every 48 to 72hrs. The cells were passaged at 70% confluence with 0.25% Trypsin (Cellgro). Cells were cultured with reduced serum (2% FCS) for 24 hrs prior to experimentation.

MTT assays for IC_{50} determination

Equal densities (10,000 cells/well) of MCF7 cells were seeded in a 96 well plate and cultured for 24hrs in basal media containing 2% FCS. After 24hrs, cultures were exposed to a range of either cyclophosphamide (CPA) (0-84 μM), doxorubicin (DOX) (0-103 μM), or the NHE1 inhibitor 5-N-Methyl N-isobutyl amiloride (MIA) (0-100 μM). The 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed after 72 hrs according to the MTT based *in vitro* Toxicology assay kit (Sigma, TOX-1). Absorbance at 570 nm and 690nm was recorded by a Spectra Max 340 PC microplate spectrophotometer (Molecular Device, CA). The data was normalized to the untreated (control) condition within each experiment and graphed in GraphPad Prism 5 for Windows (GraphPad Software, San Diego California USA). IC_{50} values were calculated in Prism using a least squares fit non-linear regression.

Results

IC_{50} determination for MIA:

5-N-Methyl N-isobutyl amiloride (MIA) is a specific inhibitor of NHE1. It is a more potent, chemically modified, analogue Amiloride-HCl (Midamore®), an NHE1 inhibitor used clinically as a diuretic for treatment of hypertension and currently under investigation to be used to guard against ischemia after cardiac reperfusion (Haist et al., 2003; Karmazyn, 1999)(ClincialTrials.gov #NCT01228214). We chose to use this inhibitor to inhibit NHE1 in MCF7 breast cancer cells and found that the IC_{50} of MIA on MCF-7 cells was 21.1 μM (Confidence Interval (CI): 20.54 - 21.76 μM) as determined by MTT analysis (Table 1, Figure 1B). By brightfield microscopy, we noted a morphological change consistent with apoptosis in the cells at 22 μM, coinciding with the measured IC_{50} (Figure 1A).

IC_{50} determination for DOX:

Doxorubicin hydrochloride (DOX) is a cytotoxic anthracycline antibiotic. It functions by interalating DNA stands, thus killing mitotically active cells by disrupting DNA replication and transcription, as well as inducing double stranded DNA breaks. Though potent against cancer cells, DOX has long been known to be highly cardiotoxic (Saltiel and McGuire, 1983). We found the IC_{50} of DOX on MCF-7 cells was 1.6 μM (CI: 0.86 – 3.00 μM), as determined by MTT analysis (Table 1, Figure 2B). By brightfeild microscopy, we noted a morphological change consistent with apoptosis in the cells at 1.7 μM, coinciding with the measured IC_{50} (Figure 2A).

IC_{50} determination for CPA:

Cyclophosphamide (CPA) is one of the oldest and widely used chemotherapeutic agents

for the treatment of breast and other cancers. It is a mustard gas derivative that functions by alkylating DNA on the guanine base thus inducing both inter- and intra-strand cross-linking leading to cell death. The side effects of this agent are considerable and include acute myeloid leukemia, bladder cancer, and permanent infertility as well as vomiting, diarrhea, and gastro-intestinal discomfort. It is also known to be more potent against cancer cells when in an acidic environment (Mahoney et al., 2003). MCF7 cells were cultured and treated as described above (see material and methods). We found the IC50 of CPA was 11.15 μM (CI: 10.74 – 11.57 μM) in MCF7 cells, as determined by MTT analysis (Table 1, Figure 3B). By brightfeild microscopy, we noted a morphological response (rounding of cells and membrane ruffling) to CPA between 10 and 30 μM which is consistent with the IC_{50} we measured by MTT assay (Figure 3A).

MIA in combination with either CPA or DOX:

By MTT analysis of MIA on MCF7 viability, we determined the IC_{50} to be 21.1 μM. We chose to use 1.7 μM MIA for combination experiments because this concentration did not have an obvious effect on MCF7 cell viability (Figure 1A and B). We therefore repeated the range of drug concentration for CPA and DOX described above in the presence of 1.7 μM MIA. We found that the presence of MIA did not affect DOX activity as determined by MTT or brightfield analysis (1.4 μM (CI: 0.62 - 3.39 μM) vs. 1.6 μM (CI: 0.86 – 3.00 μM)) (Table 1; Figure 4A and B), however, the IC_{50} of CPA was lowered from 11.15 μM (CI: 10.74 – 11.57 μM) to 2.14 μM (CI: 1.57 - 2.7 μM) by the addition of MIA (1.7 μM) (Table 1; Figure 5B).

Discussion

The American Cancer Society estimates that there will be 296,980 new cases of breast cancer in 2013. A large portion of these women will choose to undergo chemotherapy, either in the neo-adjuvant or adjuvant setting. The potential adverse side effects of chemotherapy treatment include neutropenia, anemia, and neuropathy (Scialdone, 2012; Stubblefield et al., 2012). These side effects compound the already substantially negative impact of breast cancer on patient health. Here, we used the human breast cancer cell line MCF7 to explore the use of a novel target, NHE1, to lower the effective concentration of two cytotoxic chemotherapeutic agents, doxorubicin (DOX) and cyclophosphamide (CPA). We found that DOX activity was not enhanced by NHE1 inhibition by MIA, but CPA effectiveness was increased more than 5 fold (Table 1; Figure 6).

NHE1 inhibition reduces the cell's ability to recover from an acidic pH (Jenkins et al., 2012; Morgan et al., 2011; Wu and Vaughan-Jones, 1994). Additionally, it has been shown that CPA is more effective in an acidic environment (Mahoney et al., 2003).While further work into the mechanism of our finding has yet to be accomplished, it is tempting to speculate that CPA activity is augmented by NHE1 inhibition by a mechanism involving decreased intracellular pH. Interestingly, it has also been reported that DOX is less effective in an acidic pH, opposite to the response of CPA to an acidic environment (Mahoney et al., 2003).

While we were unable to show that NHE1 inhibition could directly augment the activity of DOX, it may yet ameliorate treatment *in vivo* not only with DOX, but other chemotherapeutic agents with similar chemical and mechanistic profiles such as Daunorubicin and Mitroantrone. There has already been a call to explore NHE1 as a target for the treatment of cancer (Harguindey et al., 2005) and ample evidence to suggest that NHE1 inhibition could mitigate

many aspects of cancer progression (Cardone et al., 2005). Our findings support these ideas and suggest that NHE1 is a viable target for chemotherapy augmentation.

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	IC_{50} (μM)	Confidence Interval (μM)	R^2 of non-linear regression	N
5-N-Methyl N-isobutyl amiloride (MIA)	21.1	20.54 - 21.76	0.80	3
Doxorubicin	1.6	0.86 - 3.00	0.90	4
Doxorubicin +1.7 μM MIA	1.4	0.62 - 3.39	0.90	4
Cyclophosphamide	11.15	10.74 - 11.57	0.86	3
Cyclophosphamide +1.7 μM MIA	2.14	1.57 - 2.7	0.76	3

Table 1: IC_{50} values for 5-N-Methyl N-isobutyl amiloride (MIA), Doxorubicin, Doxorubicin +1.7 μM MIA, Cyclophosphamide, and Cyclophosphamide +1.7 μM MIA. IC_{50} values were determined by MTT analysis. MTT absorbance values were entered into GraphPad Prism 5. IC_{50} values were then calculated by applying a three parameter non-linear regression with a Hill Slope of -1 and a least squares fit. N represents the number of replicates per condition.

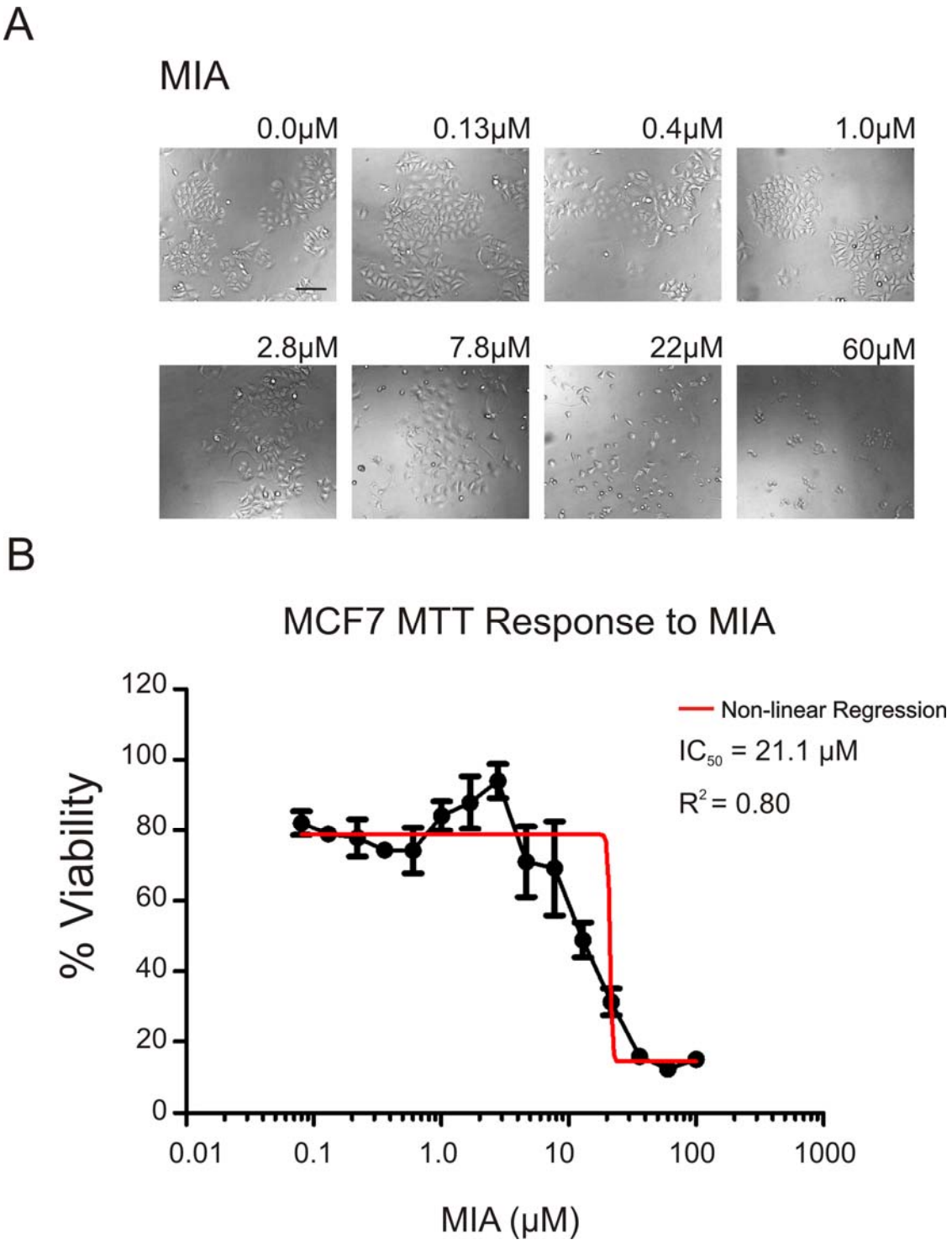

Figure 1: IC_{50} of 5-N-Methyl N-isobutyl amiloride (MIA). A) MCF7 cells were grown in 2D and exposed to a range of concentrations of MIA (0-100 μM). Brightfield images were selected from that range to demonstrate the morphological response of MCF7 cells to MIA. Cell rounding can be seen between 2.8 and 7.8 μM. B) MTT results from MCF7 cells exposed to MIA. The data was normalized to the untreated (control) condition fit with a least squares non-linear regression (red line). The IC_{50} of MIA on MCF7 cells was 21.1 μM (Confidence Interval: 20.54 - 21.76 μM). Scale bar= 50μm.

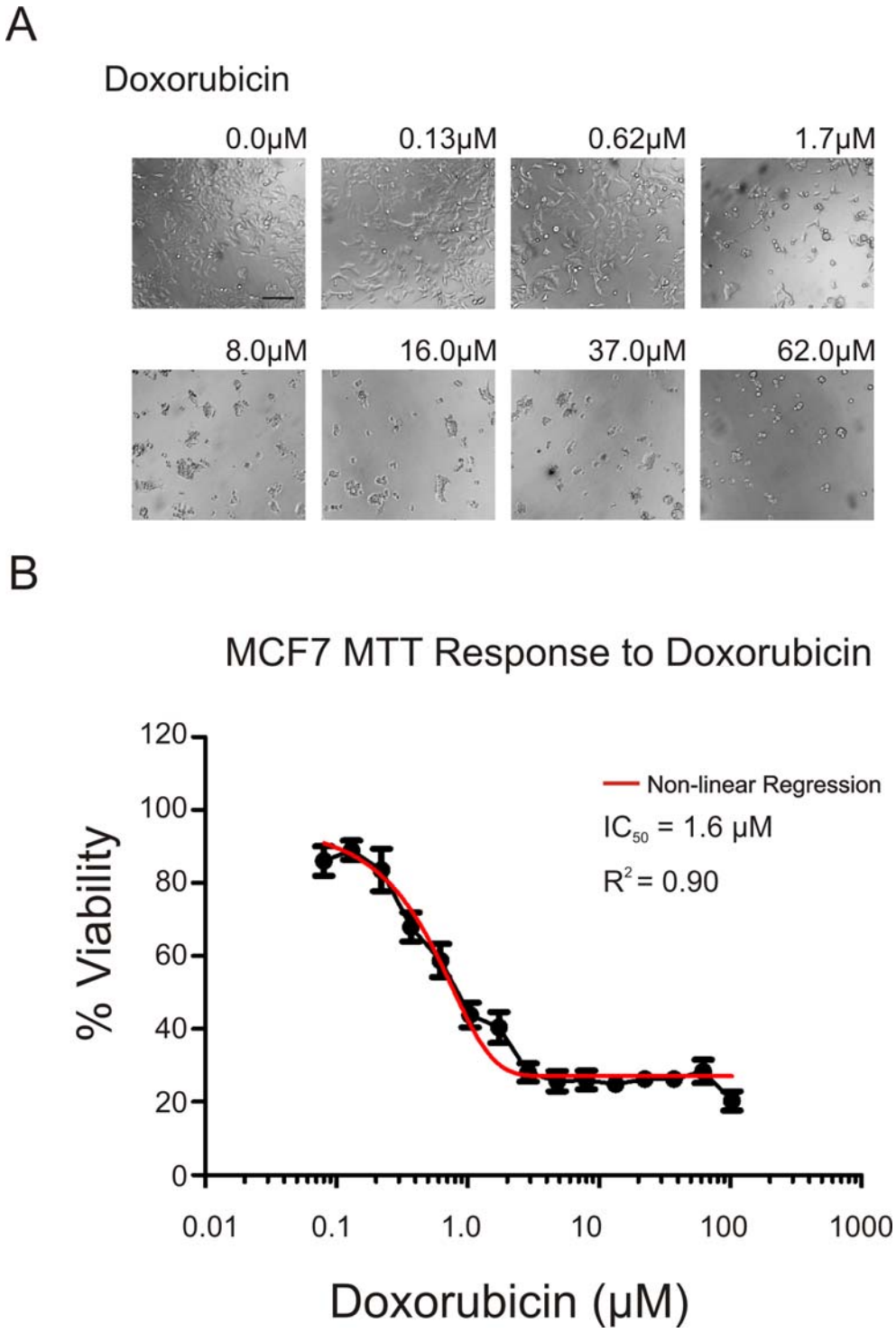

Figure 2: IC_{50} of Doxorubicin (DOX). A) MCF7 cells were grown in 2D and exposed to a range of concentrations of DOX (0-103 μM). Brightfield images were selected from that range to demonstrate the morphological response of MCF7 cells to DOX. Cell rounding consistent with cell death can be seen between 0.62 and 1.7 μM. B) MTT results from MCF7 cells exposed to DOX. The data was normalized to the untreated (control) condition and fit with a least squares non-linear regression (red line). The IC_{50} of DOX on MCF7 cells was 1.6 μM (Confidence Interval: 0.86 – 3.00 μM). Scale bar= 50μm.

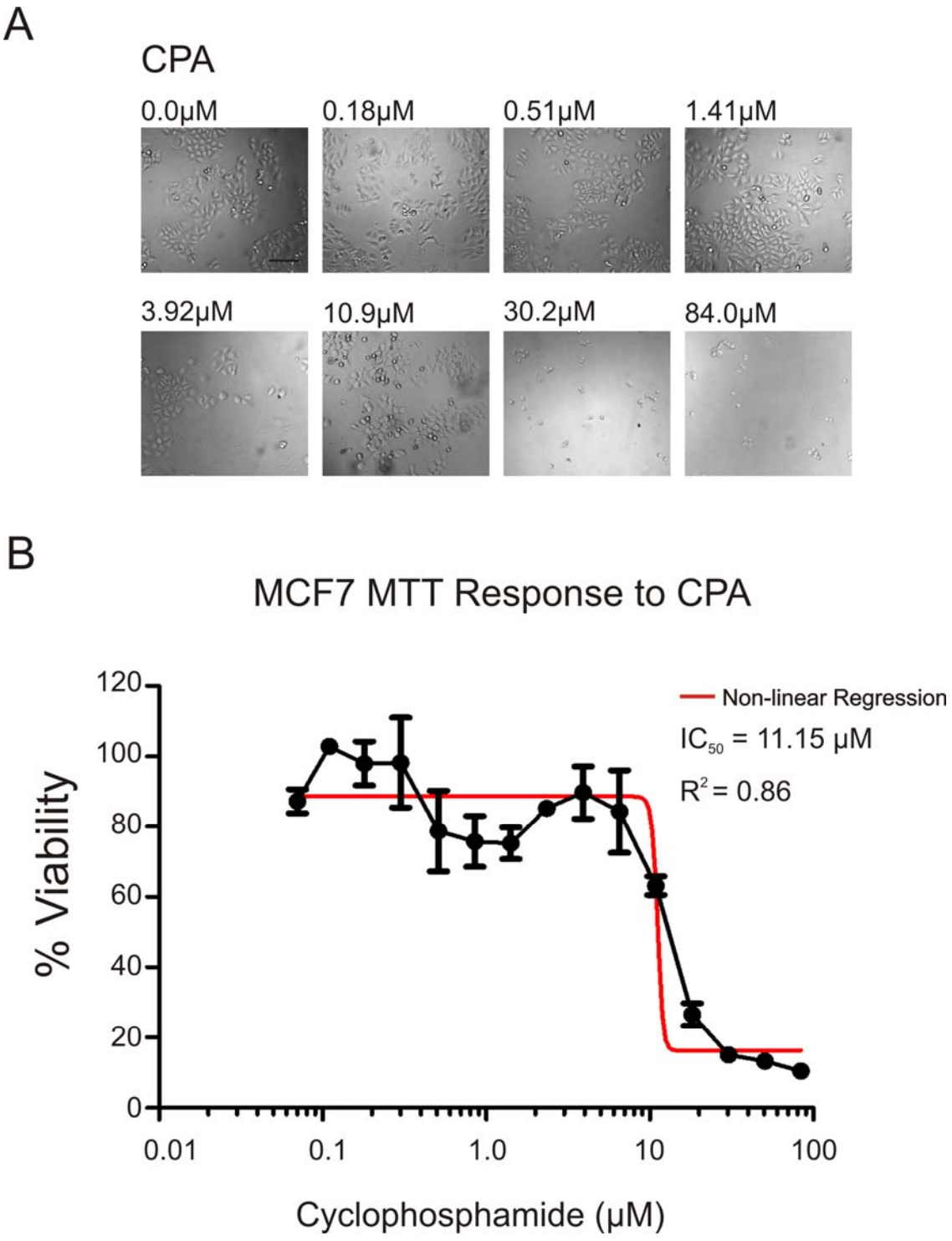

Figure 3: IC_{50} of Cyclophosphamide (CPA). A) MCF7 cells were grown in 2D and exposed to a range of concentrations of CPA (0-84 μM). Brightfield images were selected from that range to demonstrate the morphological response of MCF7 cells to CPA. Cell rounding consistent with cell death can be seen between 3.92 and 10.9 μM. B) MTT results from MCF7 cells exposed to CPA. The data was normalized to the untreated (control) condition fit with a least squares non-linear regression (red line). The IC_{50} of CPA on MCF7 cells was 11.15 μM (Confidence Interval: 10.74 – 11.57 μM). Scale bar= 50μm.

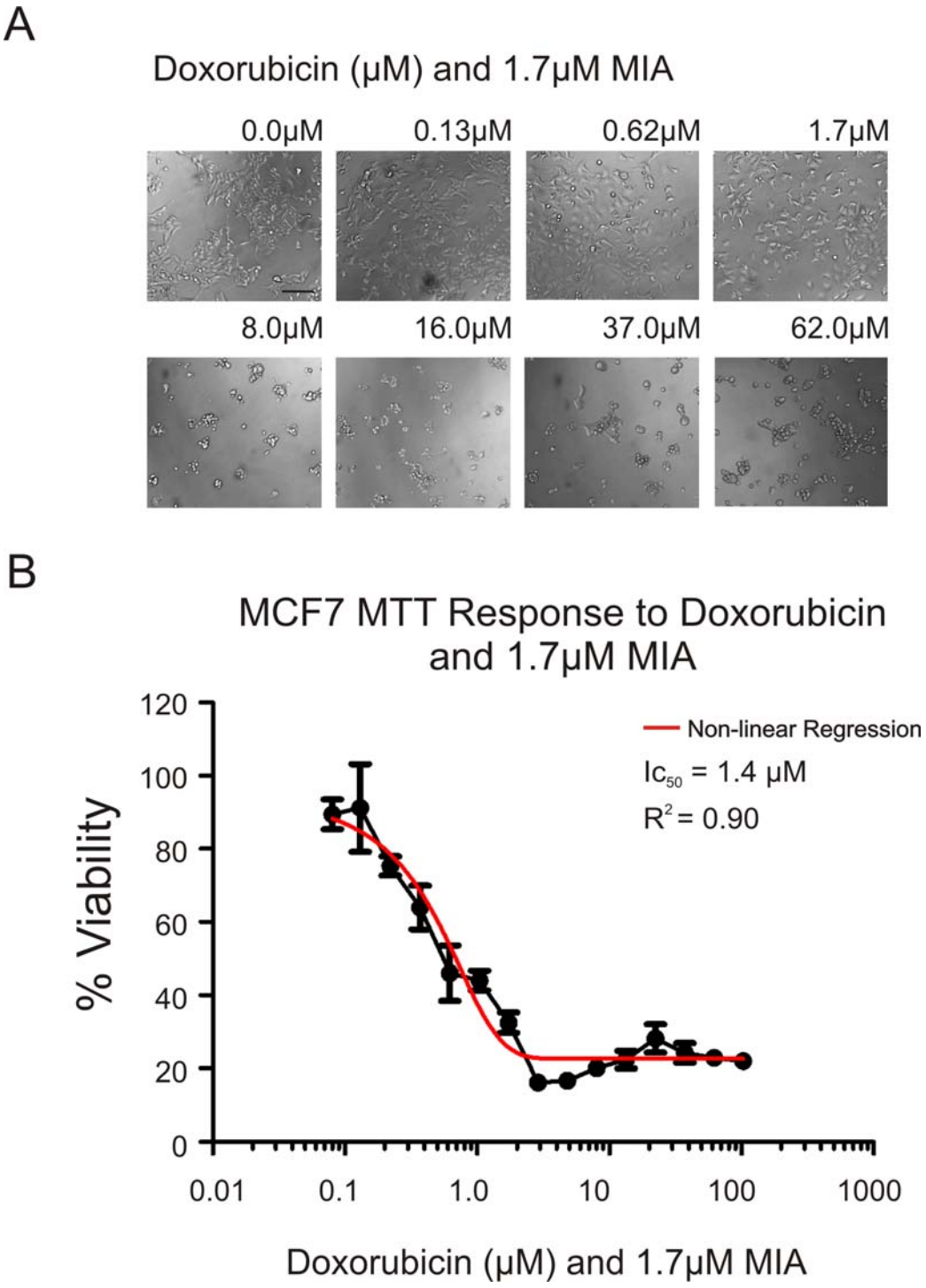

Figure 4: IC_{50} of Doxorubicin (DOX) and 5-N-Methyl N-isobutyl amiloride (MIA). A) MCF7 cells were grown in 2D and exposed to a range of concentrations of DOX (0-103 μM) in the presence of MIA (1.7 μM). Brightfield images were selected from that range to demonstrate the morphological response of MCF7 cells to DOX + MIA (1.7 μM). Cell rounding consistent with cell death can be seen between 1.7 and 8 μM . B) MTT results from MCF7 cells exposed to DOX + MIA (1.7 μM). The data was normalized to the untreated (control) condition and fit with a least squares non-linear regression (red line). The IC_{50} of DOX + MIA (1.7 μM) on MCF7 cells was 1.4 μM (Confidence Interval: 0.62 - 3.39 μM). Scale bar= 50μm.

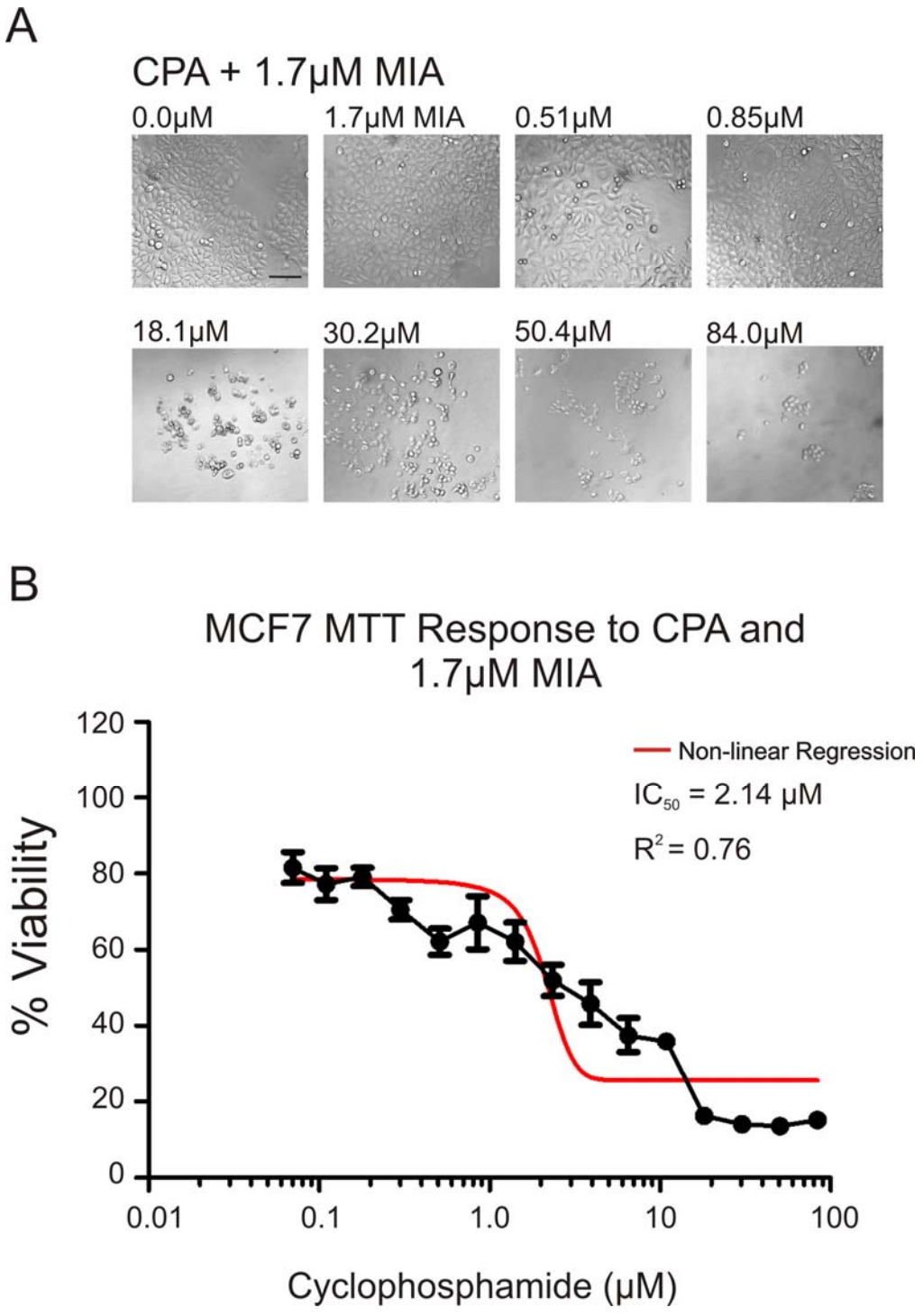

Figure 5: IC_{50} of Cyclophosphamide (CPA) and 5-N-Methyl N-isobutyl amiloride (MIA). A) MCF7 cells were grown in 2D and exposed to a range of concentrations of CPA (0-84 μM) in the presence of MIA (1.7 μM). Brightfield images were selected from that range to demonstrate the morphological response of MCF7 cells to CPA + MIA (1.7 μM). Cell rounding consistent with cell death can be seen between 0.85 and 18.1 μM. B) MTT results from MCF7 cells exposed to CPA + MIA (1.7 μM). The data was normalized to the untreated (control) condition and fit with a least squares non-linear regression (red line). The IC_{50} of CPA + MIA (1.7 μM) on MCF7 cells was 2.14 μM (Confidence Interval: 1.57 - 2.7 μM). Scale bar= 50μm.

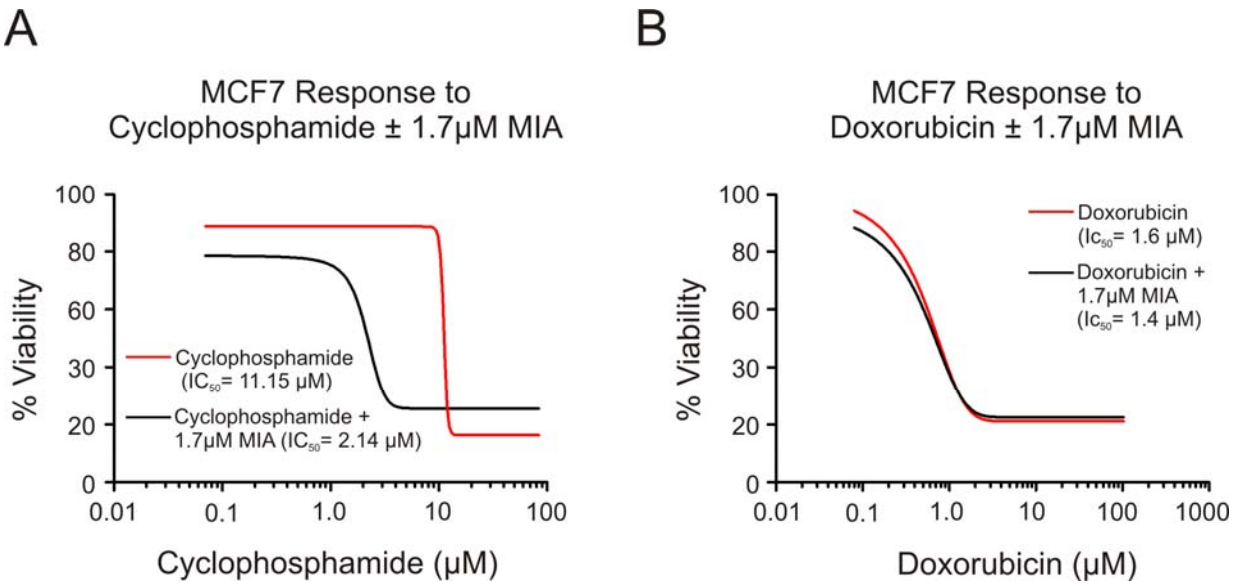

Figure 6: Summary of IC_{50} determinations for Cyclophosphamide (CPA) and Doxorubicin (DOX) ± 5-N-Methyl N-isobutyl amiloride (MIA) (1.7 μM). A) MTT results from MCF7 cells exposed to CPA (red line) or CPA + MIA (1.7 μM) (black line). The data was normalized to the untreated (control) condition in each experiment and fit with a least squares non-linear regression. The IC_{50} of CPA on MCF7 cells was 11.15 μM (Confidence Interval: 10.74 – 11.57 μM). The IC_{50} of CPA + MIA (1.7 μM) on MCF7 cells was 2.14 μM (Confidence Interval: 1.57 - 2.7 μM). B) MTT results from MCF7 cells exposed to DOX (red line) or DOX + MIA (1.7 μM) (black line). The data was normalized to the untreated (control) condition in each experiment and fit with a least squares non-linear regression. The IC_{50} of DOX on MCF7 cells was 1.6 μM (Confidence Interval: 0.86 – 3.00 μM). The IC_{50} of DOX + MIA (1.7 μM) on MCF7 cells was 1.4 μM (Confidence Interval: 0.62 - 3.39 μM).

Concluding Remarks/Future Directions

Branching morphogenesis is a process that is fundamental to the generation of the lung and kidney, as well as the salivary and mammary glands. We have uncovered a novel regulator of both mammary branching morphogenesis and maintenance of branched mammary architecture. While we were not able to conclusively demonstrate that NHE1 is necessary for the development of the lung, kidney, or salivary gland, further work should be done in those systems to uncover the role of pH regulation in their development, as it is likely that NHE1 also plays a pivotal role during their morphogenesis.

Here we used the pH sensitive dye BCECF-AM to gain insight into the function of NHE1 during snapshots of branching morphogenesis. This was achieved using a three-dimensional *ex vivo* model of mammary branching. While this approach has been useful, the next steps in determining pH dynamics during morphogenesis should be taken with *in vivo* imaging of both intracellular and extracellular pH. This can be achieved by magnetic resonance spectroscopy (MRS) using the ^{31}P peak of inorganic phosphate (P_i), or a fluorinated form of vitamin B6 (6-flouropyridoxol), to estimate intracellular pH (pHi). Additionally, an exogenous pH sensitive dye, such as the ^{19}F containing ZK-150471, could be used to simultaneously measure extracellular pH (pHe). These methods have been successfully used in the past to measure the pH in and around tumors *in vivo* (Mason, 1999; Ojugo et al., 1999; Stubbs et al., 1992), and therefore could be applied to understanding pH dynamics during branching morphogenesis.

Elucidation of the relationship between pH homeostasis and maintainance of tissue architecture *in vivo* could provide insight into how pH deregulation in cancer serves to further disease progression by inducing disruption of organized tissue. By inhibiting NHE1 activity on fully branched structures, we uncovered a novel homeostatic pathway involving NHE1 > E-

cadherin > tissue stability. Loss of E-cadherin has been identified as a critcal step in epithelial to mesenchymal transition (EMT) during cancer metastasis (Carneiro et al., 2012; Paredes et al., 2012). Our results implicate pHi regulation by NHE1, potentially through dynamic actin regulation, as a determinant of E-cadherin function. This phenomenon should be explored *in vivo* using time release pellets of the NHE1 inhibitors 5-N-Methyl N-isobutyl amiloride (MIA) or 5-N-Ethyl N-isobutyl amiloride (EIPA), as these can inhibit NHE1 activity in the μM range.

In recent years, NHE1 has been suggested as a potential therapeutic target for the treatment of cancer (Harguindey et al., 2005; Stock and Schwab, 2009). Using the human breast cancer cell line MCF7, we were able to show that NHE1 inhibition at a non-toxic level could increase the effectiveness of cyclophosphamide by more than 5 fold. While encouraging, the potential synergistic effect of cyclophosphamide and NHE1 inhibition needs to be explored more rigorously in cell culture (Greco et al., 1996). Additionally, Mahoney et al. characterized the effects of pH on the activity of a variety of chemotherapeutic agents (Mahoney et al., 2003). Those agents that have been shown to be more active at an acidic pH should be explored in combination with NHE1 inhibition. Ultimately, NHE1 inhibition, either alone or in combination with chemotherapeutic agents, should be explored *in vivo* as a means to mitigate aggressive cancer cell behaviors such as proliferation and invasion, both of which NHE1 as been shown to have roles (Putney and Barber, 2003; Stock et al., 2005).

Cancer is a disease whereby normal processes and regulatory mechanisms become deregulated, thus causing tissue to act in a disorderly and dysfunctional manner. We have shown that NHE1 is necessary for both mammary branching morphogenesis and the maintenance of branched architecture. It has been shown that NHE1 activity becomes abnormal in cancer, thus serving to further progression of the disease (Cardone et al., 2005; Reshkin et al., 2000). Here,

we have shown that its inhibition has the potential to augment chemotherapy in cancer treatment. We believe that the role of pHi regulation by NHE1 should not be underestimated in normal development or cancer progression. Uncovering the regulatory impact of the pH context in which biological processes occur ultimately increases our understanding of the connection between functional form and physiological function.

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Chapter 4

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Concluding Remarks/Future Directions

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