Abstract

Title of Thesis: The Role of Kinesin Motor Proteins in Mammary Epithelial And Breast Tumor Cell Microtentacles

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Breast cancer is the second leading cause of cancer deaths in women in the US with nearly 90% of solid tumors arising as epithelial carcinomas. Primary tumor metastasis often leads to aggressive secondary tumors starting from micrometastases that may lie dormant years after reattachment. In recent studies, detached mammary epithelial and breast tumor cells were found to produce long, dynamic protrusions composed of detyrosinated tubulin and vimentin that promote homotypic aggregation and reattachment to surfaces. These protrusions are termed microtentacles (McTNs) to distinguish them from actin-based filopodia/invadopodia and tubulin-based cilia. It is thought that McTNs function as a cell survival response to facilitate reattachment when adhesion to extracellular matrix is lost. Our goal is to elucidate the mechanisms that regulate the formation and function of McTNs by identifying key components involved in McTNs. We focused on kinesin motors that traffic cellular membranes, vesicles, and proteins along microtubules. Our long-term goal is to understand McTN structure and function to develop effective treatments that reduce the reattachment of circulating tumor cells in distant tissues. We tested the central hypothesis: kinesin motor proteins play a key role in the formation and function of McTNs. This hypothesis is based on the following evidence. 1) Circulating tumor cells bind blood vessels via a cytoskeletal mechanism consistent with McTNs. 2) Highly metastatic tumor lines display increased
McTN frequencies. 3) McTNs are composed of coordinated vimentin and detyrosinated microtubules. 4) Detyrosinated microtubules and vimentin are likely cross-linked by kinesin proteins. 5) Kinesin-1 preferentially binds and traffics on detyrosinated microtubules in vivo. Using the anesthetics/kinesin inhibitors lidocaine and tetracaine, we inhibited kinesin function within cells as observed with GFP-fusion kinesin proteins, reduced McTN frequency, and decreased attachment efficiency in an effective, nontoxic concentration. Lidocaine and tetracaine destabilized vimentin filament support from α-tubulin as observed by immunofluorescence. Utilization of GFP:Kinesin1-wildtype and GFP:Kinesin1-mutant overexpression systems did not increase McTN frequency but increased McTN length, tubulin stability, and attachment efficiency. Partial inhibition with Kinesin-1 siRNA reduced attachment efficiency. In conclusion, Kinesin-1 is a candidate regulator of McTN formation and function and a possible therapeutic target to reduce reattachment of circulating tumor cells.
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<tr>
<td>ACS</td>
<td>American Cancer Society</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine tri-phosphate</td>
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<tr>
<td>BTC</td>
<td>breast tumor cell</td>
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<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<td>CTC</td>
<td>circulating tumor cell</td>
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<tr>
<td>C-terminal</td>
<td>carboxyl terminal</td>
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<tr>
<td>CCD</td>
<td>charge-coupled device</td>
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<td>DCIS</td>
<td>ductal carcinoma in situ</td>
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<tr>
<td>DMEM/F12</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
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<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
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<td>EGTA</td>
<td>ethylene glycol tetraacetic acid</td>
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<td>EMT</td>
<td>epithelial mesenchymal transition</td>
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<td>E-plates</td>
<td>electronic microtiter plates</td>
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<td>FAAT</td>
<td>fast anterograde axoplasmic transport</td>
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<td>GFP</td>
<td>green fluorescent protein</td>
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<td>GFP&lt;sup&gt;kinesin1WT&lt;/sup&gt;</td>
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<td>GFP-Mem</td>
<td>green fluorescent protein membrane</td>
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<tr>
<td>Glu</td>
<td>detyrosinated tubulin</td>
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<tr>
<td>HB-EGF</td>
<td>heparin binding epithelial growth factor</td>
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<td>H&lt;sub&gt;2&lt;/sub&gt;O</td>
<td>dihydromonoxide</td>
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<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
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<tr>
<td>IDC</td>
<td>invasive ductal carcinoma</td>
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<tr>
<td>IF</td>
<td>intermediate filament</td>
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<tr>
<td>IgG</td>
<td>immunoglobulin</td>
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<tr>
<td>KIF</td>
<td>kinesin superfamily</td>
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<td>KHC</td>
<td>kinesin heavy chain</td>
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<tr>
<td>KSP</td>
<td>kinesin spindle protein</td>
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<tr>
<td>LA</td>
<td>Latrunculin-A</td>
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<tr>
<td>McTNs</td>
<td>microtentacles</td>
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<tr>
<td>MEC</td>
<td>mammary epithelial cell</td>
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<tr>
<td>MgCl&lt;sub&gt;2&lt;/sub&gt;</td>
<td>magnesium chloride</td>
</tr>
<tr>
<td>MSTB</td>
<td>microtubule stabilizing buffer</td>
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<tr>
<td>NaBH&lt;sub&gt;4&lt;/sub&gt;</td>
<td>sodium borohydride</td>
</tr>
<tr>
<td>NaCl</td>
<td>sodium chloride</td>
</tr>
<tr>
<td>NIH</td>
<td>National Institutes of Health</td>
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<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartic acid</td>
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<tr>
<td>NP-40</td>
<td>nonylphenoxypolyethoxylethanol</td>
</tr>
<tr>
<td>N-terminal</td>
<td>amine terminal</td>
</tr>
<tr>
<td>PARP</td>
<td>poly-(ADP-ribose)-polymerase</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PEI</td>
<td>polyethyleneimine</td>
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<tr>
<td>PF-DMEM</td>
<td>phenol-free Dulbecco’s Modified Eagle’s Medium</td>
</tr>
<tr>
<td>PI</td>
<td>propidium iodide</td>
</tr>
<tr>
<td>PIPES</td>
<td>piperazine-N,N'-bis[2-ethanesulfonic acid]</td>
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PMS – phenazine methosulfate
PVDF – polyvinylidene fluoride
RIPA – radioimmunoprecipitation assay
ROI – Region of Interest
RPMI-7951
siRNA – silencing RNA
TBS – Tris buffered saline
TBST – tris-buffered saline tween-20
TRAIL – (TNF)-related apoptosis-inducing ligand
Tris-HCl – Tris-hydrochloride
TTX – Tetrodotoxin
TWEEN-20 – Polysorbate 20
XTT - Sodium 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide
I. INTRODUCTION

A. Cancer Prevalence in the United States

Cancer is the second leading cause of deaths in the US with nearly 90% of human solid tumors arising as epithelial carcinomas [1]. It was estimated that 28% of all new cancer cases and 15% of all cancer deaths were due to breast cancer alone in 2010 (Fig. 1.1, ACS). While advancements in research has allowed for earlier detection, better diagnosis and treatment, it is clear that improved methods are still needed for the enhancement of cancer prevention and care.

Figure 1.1 2010 Estimates on leading sites of new cancer cases and deaths
ACS 2010 Cancer Statistics

Cancer begins when mutations occur in normally functioning cells causing uncontrolled growth. This in turn can progress into primary tumors that are often benign and surgical excision is an effective treatment option at this early stage. However,
primary tumors often go undetected and can develop the capacity to form new blood vessels to sustain tumor expansion. These blood vessels additionally provide the tumor cells an escape route from their site of origin into the vascular or lymphatic system. Generally, the majority of cells that disseminate from a primary tumor will die by apoptosis or death due to shear force within the capillaries (Fig. 1.2).

![Figure 1.2 Model of three common tumor cell fates after intravasation into the circulatory system. 1. Apoptosis. 2. Fragmentation due to shear force. 3. Successful metastasis through reattachment and extravasation into a distant secondary site.](image)

Nevertheless, a small percentage of cells survive the arduous journey through the circulatory and/or lymphatic system and come to rest at a secondary tissue site. These cells progress to invade and grow into more aggressive secondary tumors resulting in higher mortality rates for cancer patients. The transition from a benign primary tumor into an aggressive secondary tumor occurs as the cells acquire mutations to migrate from the tissue of origin, survive the harsh environment of detachment, and reattach.
B. Breast Cancer and Microtentacles

1. Breast Cancer and Women

Breast cancer is the leading cause of death in women with approximately 1 in 8 women developing invasive breast cancer in the United States (ACS). The two most common breast cancers are ductal carcinoma in situ (DCIS) and invasive ductal carcinoma (IDC). DCIS is noninvasive and accounts for approximately 1 out of 5 new breast cancer cases diagnosed each year [2]. Detection of DCIS has improved since the initiation of mammograms, yet; the majority of breast cancers are still discovered at the late stages of invasive breast cancer when tumor masses are more easily detected and likely to have metastasized [2]. Disseminated breast tumor cells will primarily metastasize to the lungs, bone, liver, and spleen (Fig 1.3) [3].

**Figure 1.3 Common sites of breast cancer metastases**
Breast tumor cells that disseminate from the primary tumor are carried by blood flow through the heart and to the capillary beds of the lungs. Cancers cells that pass through the lungs may come to rest in other remote organ sites such as the bone, liver, and spleen. [4]
2. The Structure and Formation of Microtentacle Protrusions in Mammary Epithelial and Breast Tumor Cells

Disseminated tumor cells will follow the pattern of blood flow and usually come to rest at the first extensive capillary bed that they encounter. It was generally believed that tumor cells, starting from micrometastases, undergo a long period of dormancy of months to decades before invasion and growth occurs to form a secondary metastatic tumor. However, results from our laboratory indicate that these cells may not be as dormant as previously suspected. It has been observed in mammary epithelial cells (MECs) and breast tumor cells (BTCs) that detached cells produce long, dynamic protrusions that actively promote reattachment to each other and to other surfaces (Fig. 1.4) [5].

Figure 1.4 Microtubule protrusions are required for efficient cell–cell attachment. Transient transfections of green fluorescent protein (GFP)-memb into MCF10A cells show protrusions extending around adjacent cells (white arrows), +/- the actin depolymerization, Latrunculin-A (LA). Protrusions that have not yet contacted adjacent cells continue dynamic movement (white arrowheads). [5]
The initial studies tested the hypothesis that these membrane protrusions produced by the MEC and BTCs were actin-based. The actin depolymerizers, Latrunculin A and Cytochalasin D, known inhibitors of actin-based structures, such as lamellipodia and filopodia, were used to test the structures of the MEC and BTC protrusions. However, when these actin depolymerizers were applied to suspended MECs and BTCs, instead of a reduction in the membrane protrusions as expected if these protrusions were actin-based, there was a strong enhancement in the lengths and frequencies. Suspended MECs and BTCs were then treated with Colchicine, a microtubule depolymerizer, and the long protrusions were inhibited [5]. Based on these findings, it was revealed that these novel protrusions were tubulin-based and not actin-based, as first suspected (Fig 1.5).

**Figure 1.5 Membrane protrusions are microtubule-based.**
EpH4 cells were suspended in either DMEM (control) 5 μM Latrunculin-A (LA), or 1 μM Colchicine for 30 min. Cells were fluorescently stained for α-tubulin (green) and polymerized actin (red). [5]
A study by Korb et al. provided supporting evidence showing a role for tubulin-based protrusions during tumor metastasis [6]. In this study, the role of cytoskeletal structures in the early steps of extravasation was investigated using the cytoskeletal inhibitors Nocodazole and Cytochalasin D. Fluorescently labeled HT-20 cells were pretreated with 2.5 ug/mL Nocodazole or 1 uM Cytochalasin D and compared to untreated cells. Cells were injected into the portal veins of rats and observed for tumor cell adhesion and migration into the hepatic parenchyma (Fig. 1.6) [6]. Untreated cells showed an average of ~40 adherent cells. Cells treated with Cytochalasin D displayed ~50% increase in adherent cells while Nocodazole-treated cells had a marked decrease in adherent cells compared to the controls (Fig. 1.7). The results from this study revealed similarities to the mechanisms observed in the protrusions of MEC and BTCs (Fig. 1.5) suggesting a possible role of the membrane protrusions in suspended MEC and BTCs.

**Figure 1.6 Intravital observations of CalceinAM-labeled HT-20 cells within the liver microcirculation.** [6] White arrows indicate a cell that has reattached to the vascular wall (+) and a cell that has migrated into the tissue [6].
Figure 1.7 Effects of cytoskeleton disruption on metastatic tumor cell adhesion in vivo. HT-20 cells were fluorescently labeled and either left (A) untreated or pretreated with (B) Nocodazole or (C) Cytochalasin D. Cells were observed in a standardized microscopic field for 30 min after injection at 5 minute interval time points. n=8 [6]

When cells are attached, a counterbalance of microtubule extensions from the microtubule organizing center and the contractile forces generated by the actin cytoskeleton is established to maintain its cellular shape [7], [8]. When a cell becomes detached, cell death is induced by the disruption of this balance of forces [9] and cell survival depends on 1) reattachment to a surface and/or 2) apoptotic insensitivity [10]. Given the harsh environment of the bloodstream for most solid tumor cells, the ability of metastatic cells to reattach to surfaces is essential for tumor cell survival. One possible mechanism that may facilitate the reattachment process may be the development of long, dynamic protrusions of the plasma membrane [5]. Actin-based protrusions, such as
lamellipodia and filopodia, have been extensively studied for their roles in cellular migration and motility of adherent cells [11], [12]. However, the cytoskeletal dynamics after release from the extracellular matrix have largely been overlooked.

3. Microtentacles are composed of Glu-tubulin and Vimentin Intermediate Filaments, which are markers of increased metastatic potential in Breast Tumor Cells

Looking closer at these membrane protrusions, it was discovered that the protrusions were specifically enriched in detyrosinated (Glu)-tubulin [5], a post-translationally modified form of α-tubulin. Glu-tubulin is characterized by a c-terminal glutamic acid that is exposed by the removal of the terminal tyrosine residue. While microtubules composed of full-length α-tubulin have a half-life of minutes in cells, microtubules enriched in Glu-tubulin can persist for up to 16-20 hours in vivo [13]. When MECs and BTCs were suspended, levels of Glu-tubulin were found to increase and localize within these protrusions [5]. Interestingly, Glu-tubulin is a clinical marker of poor prognosis in breast cancer patients [14], but the mechanism by which tubulin detyrosination affects tumor aggressiveness remains unclear. One possible explanation of the increase in Glu-tubulin may be due to the adaptation of Glu-tubulin based protrusions for selective advantage. The discovery of these Glu-tubulin enriched protrusions that facilitate reattachment indicates a cell survival mechanism that is inherited through selection during the metastatic progression. Delving further into the structural components of the microtubule protrusions, it was discovered that the Glu-tubulin based protrusions were also enriched with vimentin intermediate filaments (IF) (Fig. 1.8).
Vimentin, a marker of an epithelial to mesenchymal transition (EMT) in breast cancers also correlate with the metastatic potential and invasiveness of tumors [16]. Vimentin levels correlate with microtubule protrusion frequencies in BTCs, which are enhanced with treatment with the actin-depolymerizer, Latrunculin A (Fig. 1.9A, B) [17]. Live-cell membrane protrusion counts correlate with vimentin-expressing, invasive cell
lines with a statistically-significant, increase in the frequencies in membrane protrusions than non-vimentin expressing, non-invasive cell lines (P<0.05, t-test, n=3, black asterisks) (Fig. 1.9C). Immunoblot expression profile of Glu-tubulin, α-tubulin, PAN-cytokeratin, and vimentin additionally indicates that cells with higher protrusion frequencies express elevated vimentin, lower cytokeratin, while Glu-tubulin remains largely unchanged (Fig 1.9D) [15].

Figure 1.9 Microtentacles frequency and length increases with metastatic potential. (A) Panel of human MEC (MCF10A) and BTC lines transfected with GFP-membrane (GFP-Mem) display morphologically different membrane protrusions (black arrows). Invasive cell lines were (HCC1395, Hs578t, MDA-MB-436, MDA-MB-157) and non-invasive lines were (MCF10A, ZR75-1, Bt-20, SkBr3, MDA-MB-468). (B) MCF10A, SkBr3, and MDA-MB-436 treated with Latrunculin-A (LA, 5μM) showed enhancement of protrusions (C) Live-cell membrane protrusion counts of vimentin expressing and non-expressing cell lines (P<0.05, t-test, n=3, black asterisks). (D) Immunoblot expression profile of Glu-tubulin, α-tubulin, PAN-cytokeratin, and vimentin in vimentin expressing and non-expressing cell lines [15].
Glu-tubulin is not intrinsically stable on its own but obtains increased stability due to co-alignment with the more flexible and resilient IF, vimentin [18]. *In vitro* preparations of fully polymerized IFs showed elastic properties that showed considerable resistance to deformation and breakage due to mechanical strain [19], [20]. It is suggested that Glu-tubulin is a signal for the recruitment of vimentin to microtubules as shown by preferential colocalization of vimentin to newly formed Glu-microtubules by immunostaining [21]. To differentiate between actin-based structures such as lamellipodia/filopodia from these novel microtubule-based protrusions, we have termed these dynamic protrusions *microtentacles* (McTNs).

**C. Kinesin Involvement in the Microtentacle Structure and Function**

1. **Introduction to Kinesins**

Members of the kinesin superfamily (KIF) are motor proteins that traffic cellular organelles, vesicles, proteins, and various molecular cargo along microtubules in an ATP-dependent fashion [22], [23]. Kinesins were first discovered in studies of axonal transport in the giant squid and termed conventional kinesins/kinesin heavy chain (KHC) before the discovery of related kinesin proteins that shared a high degree of conserved homology (Fig. 1.10) [23].
The globular N-terminal head domain of the kinesin heavy chains share a highly conserved motor domain (~345 amino acid) that contains an ATP binding site and a microtubule binding interface (Figure 1.10). The C-terminus contains the stalk/tail region that interacts with cargo or adaptor proteins [25]. In between the head and tail is the neck region that determines the directionality of the motors along the microtubules [26]. Kinesins that contain the motor domain at the N-terminus typically move towards the plus-end while kinesins with C-terminus motor domains move towards the minus end [27]. Kinesins possessing centralized motor domains regulate microtubules by acting as a
microtubule depolymerizer [27], [28]. Though kinesins share a central catalytic core, the adjacent neck allows for family specific features that provide directionality and regulation [28]. The motor-neck region is conserved for each family with variability occurring in the stalk/tail region and allows for diverse functional applications (Fig. 1.11).

![Figure 1.11 Typical kinesin structures](http://www.ncbi.nlm.nih.gov/pubmed?term=svoboda%201993)

Kinesins are generally found in their folded inactive state diffusely within the cytoplasm [29]. A smaller amount is bound to microtubules in the unfolded active state, exposing the adenosine triphosphate (ATP) binding region allowing for processivity to occur along microtubules [30]. Current classifications have standardized kinesin nomenclature and have renamed the conventional kinesin, Kinesin-1, in a system that contains 14 classes of kinesins (Figure 1.12) [31].
Generally, non-mitotic kinesins are the transporters of organelles and vesicular cargo and play an important role in the regulation of microtubules and cellular packaging. For example, in attached cells, kinesins are required for the delivery of components.
required for focal adhesion regulation along microtubule tracks to the microtubule caps along the periphery [32]. Kinesins also have roles in microtubule bundling, sliding, and crosslinking using a second microtubule binding site on the tail which confers flexibility and dynamic motion [23], [33], [34], [35]. Anterograde microtubule transport mediated by microtubule-based motor proteins play a dominant role in microtubule bending and act to regulate the spatial distribution of microtubules in living cells [36]. One of the defining characteristics of McTNs is their dynamic motion, possibly due to the forces generated by kinesin motion.

In addition to trafficking materials and cargo along microtubules, kinesins have many diverse roles from chromosomal separation and spindle movements during mitosis and meiosis to involvement in cellular mobility and synaptic transmission [37], [23], [38], [39]. Scientific studies have mostly focused on kinesin involvement in axonal transport and cellular mitosis. However, due to kinesin’s function in cellular proliferation, it is of great interest in the field of cancer research and a key target of cancer chemotherapies that inhibit uncontrolled proliferation of cancerous tumors. Promising cancer treatments come from kinesin spindle protein (KSP) inhibitors which are used to inhibit selective mitotic kinesins. KSP inhibitors are not expected to produce the same neurological toxicities as the first generation mitotic inhibitors that broadly targets microtubules [40]. (S)-Monastrol, a potent and selective Eg5 inhibitor, is the best known KSP inhibitor and has found success in treating paclitaxel resistant tumors [41]. Though much focus is put on mitotic kinesin, non-mitotic conventional kinesins are also an ideal candidate for targeted therapies.
2. Kinesin Targeting in Microtentacles

Tumor cell targeting after the initial release from the primary tumor may be possible by focusing on McTN inhibition before reattachment has been initiated. Reduction of McTN generation would increase the possibility of death due to apoptosis and cell fragmentation by decreasing the number of potential adherent cells to the blood vessel wall. This in turn, would effectively reduce the number of micrometastases formed in the distant secondary sites, which is the stage of increased patient mortality. One possible target protein in the structure and function of McTNs may come from the non-mitotic members of the KIFs, due to their variability in structure and involvement in cargo trafficking along microtubules [23], [38]. Kinesin motor proteins traffic cargo along a complex network of microtubule roadways transporting essential proteins, organelles, and packages to their correct destinations. Of particular interest is the kinesin motor protein’s ability to transport vimentin filaments, squiggles, and particles along microtubules [42]. In addition, Kinesin-1 preferentially binds to Glu-microtubules and is thought to play a role in cross-linking Glu-tubulin and vimentin IFs [21]. Targeting of McTN specific, non-mitotic kinesins opens the potential for a focused treatment during a critical window of tumor progression.

D. Central Hypothesis and Aims

We investigated Kinesin-1 involvement in McTNs and tested the central hypothesis: Kinesin motor proteins play a key role in the formation and function of McTNs. In this thesis, we focused on two aims. Aim1: The local anesthetic lidocaine
and tetracaine inhibits metastatic potential due to inhibitory effects on kinesin and thus McTN protrusions. We tested this hypothesis by investigating the effects of the kinesin inhibitors, lidocaine and tetracaine, on McTN frequency, cellular viability, kinesin inhibition in cells, homotypic aggregation, cytoskeletal integrity, and attachment efficiency in MECs and BTCs. In chapter IV, we address **Aim 2: Genetic targeting of kinesins affect McTN function and the reattachment process of breast tumor cells.**

Here we used genetic targeting to elucidate the specific roles of Kinesin-1 within MECs and BTCs using the overexpression constructs $^{\text{GFP}}$Kinesin1 wildtype and $^{\text{GFP}}$Kinesin1 mutant to investigate McTN generation, reattachment, and protein expression. Additionally, Kinesin-1 siRNA was used to knockdown Kinesin-1 expression to monitor McTN function during reattachment.
II. METHODS

Cell culture and drug treatment

MCF10A, a non-tumorigenic and immortalized mammary epithelial cell line was cultured in 10cm plastic dishes (Nunc/CellStar) with Dulbecco’s modified Eagle’s medium/F12 (Gibco) supplemented with 5% horse serum, insulin (5 µg/ml), EGF (20 ng/ml), hydrocortisone (500 ng/ml), penicillin-streptomycin (100 µg/ml each), and L-glutamine (2 mmol/L). MCF10A-Bcl2 was grown in MCF10A media plus the addition of Puromycin (2 µg/mL) (Sigma). MDA-MB-436 and Hs578t breast tumor cells were grown in DMEM supplemented with penicillin-streptomycin (100 µg/mL), L-glutamine (2 mmol/L) and 5% fetal bovine serum. Lidocaine hydrochloride monohydrate (Sigma) and tetracaine hydrochloride (Sigma) were reconstituted to a stock concentration of 0.5 M solution. Latrunculin A (LA) (Biomol) was reconstituted as a 5 mM stock in ethanol. All treatments of MCF10A and MDA-MB-436 cells with all drugs were performed with serum-free DMEM.

Immunofluorescence

MCF10A cells were grown on glass coverslips in 24-well plastic plates and treated with DMEM or with DMEM plus the corresponding drug concentrations at various time points. The cells were then fixed with ice cold methanol for 10 minutes. Cells were blocked with 5% BSA/0.5% NP-40/PBS for 1 hour before primary antibody incubation with mouse anti-vimentin (1:1000; Zymed), rabbit anti-Glu-tubulin (1:1000; Chemicon), mouse anti-tyrosinated tubulin (1:2000; Sigma), and mouse-anti-α-Tubulin-
FITC (1:500; Sigma) in 2.5% BSA/0.5% NP-40-PBS for overnight. The corresponding secondaries; anti-mouse-Alexa Fluor 594 (1:1000; Molecular Probe), anti-rabbit-Alexa 488 (1:1000; Molecular Probes), and anti-mouse Alexa 647 (1:1000; Molecular Probes) was incubated for 1 hour. The nuclear stain Hoescht 33342 (1:5000; Sigma) was also added during the secondary incubation. All washes were done in 1x PBS. Coverslips were then mounted on glass slides using Fluoromount-G (SouthernBiotech, AL). Epifluorescent imaging was done on an Olympus IX-81 inverted microscope mounted with a CCD camera and 100x lens. Images were acquired using Volocity software (ImproveVision Inc.; Waltham, MA).

Z-stack image acquisitions were acquired using an Olympus CKX41 inverted fluorescent microscope (Allentown, Pa) equipped with an Olympus F-View II 12-bit CCD digital camera system. Series analysis of integrated protein fluorescence was performed using the Olympus MicroSuite 5 imaging software and manually setting the region of interest (ROI). The total integrated fluorescence of Glu-tubulin and α-tubulin protein levels were measured over 1.4 μ with 0.2 μ steps and averaged separately for each ROI. Each cell was normalized to α-tubulin levels with an n=3.

**Immunoblotting**

MCF10A, MCF10A1-Bcl2, and MDA-MB-436 cells were grown to confluency in 6-well dishes and treated with DMEM or DMEM with the corresponding concentrations of lidocaine, tetracaine, or LA for the designated time. Cells were harvested by washing with PBS and gently scraping in ice-cold RIPA lysis buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1mM
phenylmethylsulfonyl fluoride, 1% protease inhibitor cocktail (Sigma, P2714)). To ensure total recovery of cells, all media and washes were collected and retained by through centrifugation for 5 minutes at 300× G. Pellets were lysed in RIPA Buffer and lysates were combined. Lysates were then incubated on ice for 15 minutes with occasional vortexing before being stored at -80°. Protein concentrations were measured using a Lowry-based assay (Bio-Rad, Hercules, CA). 20 µg – 30 µg of total protein was separated on NuPAGE 4-12% Bis-Tris gels, 1.5 mm x 15 wells (Invitrogen) then transferred to Immuno-Blot PVDF membranes (Bio-Rad, Hercules, CA). All membranes were blocked in 5% milk in Tris-buffered saline (TBS) with 0.1% Tween for 1 hour at room temperature followed by an overnight incubation at 4°C of corresponding antibodies separately. Rabbit-PARP (1:1000; H-250, Santa Cruz), Mouse-Vimentin (1:1000; C-20, Santa Cruz), Mouse-Bcl2 (1:1000, BD Transduction), Mouse-β-actin (1:1000: Sigma), Mouse H1 (1:1000, Millipore), Mouse H2 (1:1000, Santa Cruz), Rabbit anti-UKHC (1:1000, Santa Cruz), mouse anti-GFP (1:1000; Santa Cruz), mouse anti-tyrosinated tubulin (1:1000; Sigma) in 1% milk TBS plus 0.1% Tween 20 (TBST). Corresponding secondary antibodies to IgG conjugated to horseradish peroxidase (HRP) were used (1:5000; GE Healthcare, Piscataway, NJ) and visualized using ECL plus chemiluminescent detection kit.

**Homotypic Aggregation Assay**

Media containing 1% Methylcellulose (Sigma M0512) was prepared in phenol red free DMEM (17-205CV Mediatech Inc). Cells were treated with growth media containing Hoechst (1:5000) for 15-30 minutes. 0.4% methylcellulose media was treated
with $2\times$ the concentrations of lidocaine or tetracaine. Cells were then trypsinized and resuspended in phenol free DMEM at $\sim 5 \times 10^5$ cells/mL and mixed with equal volume of drug treated methylcellulose media. Cells were then transferred to an ultra-low attachment, 24-well plate (Costar; Corning, NY) using a 1 mL 25G 5/8 latex-free syringe (309626 BD Syringe). Wells were read at 365 nm and monitored at 15 minute intervals with a Hamamatsu CCD camera (Hamamatsu Photonics; Hamamatsu City, Japan) using Labworks EpiChemi3 image analysis and acquisition software (UVP, Inc.; Upland CA).

All images were processed by a background flattening algorithm in UVP Labworks, Image Acquisition and Analysis Software, v. 4.6, and exported to ImageJ (NIH, Bethesda, MD) for individual background normalization. Areas of interest were selected based on background-subtracted threshold values. Pixels with intensity values above this threshold were quantified as integrated density, a product of selected area and mean intensity value, and normalized to 0 minutes vehicle control cells to show fold increase in signal over time.

**XTT Viability Assay**

Cells were grown up to $\sim 80\%$ confluency in clear bottomed 96 well plates (Costar; Corning, NY). Growth media was changed with 100 µl phenol red free media treated with DMEM or DMEM treated with either lidocaine or tetracaine and incubated at 37°C for the corresponding time point. XTT salt solutions were made using 1mg/mL XTT salt (X-4626; Sigma) in phenol free DMEM $+ 20 \mu l$ 0.383 mg/ml phenazine methosulfate (PMS) to each ml of XTT solution. 25 µl of XTT/PMS solution was added
to each well and incubated at 37°C for 4 hours. Observations were made using a BioTek plate spectrophotometer at 450 nm to quantify color change.

**Real-Time Cell Attachment Assay**

Cell-substratum attachment was assessed utilizing the xCelligence RTCA SP real-time cell sensing device (Roche Applied Science). MCF10A, MCF10A-Bcl2, and MDA-MB-436 cell lines were grown in their respective growth media, trypsinized, counted, and diluted to a concentration of 1 x 10^6 cells/mL. Approximately 100,000 cells were aliquoted in duplicate and mixed to 96-well electronic microtiter plates (E-plates) prepped as a blank, vehicle control, with 6 mM lidocaine, or with 0.5 mM tetracaine in growth media. Raw CI values from each measured time point were normalized to the maximum CI attained for the parental control cell line at 1 hour. Test cell lines were then represented as a percentage attachment of parental control.

MCF10A and MDA-MB-436 cells were used in the genetic targeting experiments. Cells were transfected with the GFP\textsuperscript{Kineins1} wildtype, GFP\textsuperscript{Kinein1} mutant, GFP-N1, and KIF5B siRNA were and 40,000/well were counted and plated into the 96-well E-plates with proper control and measure every 5 minutes of the time course of 3 hours following the protocol as stated (Fig. 2.1).
xCELLigence Impedance System

Microtentacle Scoring and live cell imaging

MCF10A and/or MDA-MB-436 cells were grown up to ~50% confluency and transfected with ~1 µg/µl of AcGFP1-Mem (Clontech, Mountain View, CA), a green fluorescent protein membrane plasmid that translocates to the cellular membrane using either the Fugene6 transfection reagent (11814443001; Roche) or Exgen 500 (R0511; Fermentas). Cells were then allowed to grow overnight, trypsinized, resuspended with DMEM or DMEM plus lidocaine or tetracaine +/- 5µM Latrunculin A, transferred to a low attachment plate, and incubated at 37°C for 15 minutes before counting. Cells were scored positive under conditions in which the cell exhibited two or more McTNs that


Figure 2.1 Schematic of the xCELLigence Impedance System
were greater than the radius of the cell body. Populations of 100 or more GFP-cells were counted for each trial. Cell images were collected using an Olympus CKX41 inverted fluorescent microscope (Allentown, Pa) equipped with an Olympus F-View II 12-bit CCD digital camera system. Image acquisition and analysis was performed using the Olympus MicroSuite 5 imaging software. Movies of GFP-vimentin transfected cells were captured at 100× magnification and captured at one frame every two seconds and are shown with a 5× acceleration. MCF10A and Hs578t cells were grown up to ~50% confluency and transfected with ~2 µg/µl of pGFP-KIF5C DNA, kindly provided by Dr. Michelle Peckham (Institute for Molecular and Cellular Biology, University of Leeds, Leeds, UK). Movies of GFP-kinesin transfected cells were captured at 100× magnification in an humidified, temperature controlled chamber at 37°C and captured in a Z-stack of 0.5 um slices at approximately one frame per 2-5 seconds using an Olympus IX81 inverted microscope. Analysis and particle tracking were done using the Volocity software (ImproVision Inc.; Waltham, MA).

Cells stained with CellMask Plasma Membrane Stain (Molecular Probes) for McTN counts were counted using the established live-cell McTN count protocol as previously stated. Cells were media aspirated, washed in PBS, and incubated in serum-free, PF-DMEM with (1:2000) CellMask for approximately 1-3 minutes. Cells were washed 2-3x thoroughly before trypsinized and suspended in serum-free, PF-DMEM for incubation and McTN counts.

Propidium iodide (PI) (Sigma; 1:3000) staining was performed using Phenol Red Free-DMEM treated with PI to resuspended, trypsinized cells for 20 minutes at culture
conditions. Imaging was performed on an Olympus CKX41 inverted fluorescent microscope. Counts were scored positive upon absorption of the PI stain.

**Transfections**

GFP-Kinesin1 wildtype, GFP-Kinesin1 mutant [43], and GFP-N1 constructs were transfected using Fugene6 for MCF10As (2 ug DNA/3 ul transfection reagent) [43] and ExGen 500 for MDA-MB-436 cells (2 ug DNA/5.3 ul transfection reagent) as previously stated. Cells were observed after 16-24 hours of incubation for GFP expression.

KIF5B siRNA (Santa Cruz) and conjugated non-silencing control (NS-control) was transfected using Hyperfect Transfecting reagent (Qiagen) in the ratios listed in Table 1 in IMEM (Gibco).

**Table 1. siRNA Protocol**

<table>
<thead>
<tr>
<th>Culture Format</th>
<th>Media Volume (ul)</th>
<th>siRNA (ng)</th>
<th>Vol. of 10uM siRNA stock (ul)</th>
<th>Final siRNA volume (ul)</th>
<th>Vol. of Hyperfect (ul)</th>
<th>Final siRNA cc (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>24-well</td>
<td>500</td>
<td>37.5</td>
<td>.6</td>
<td>100</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>6-well</td>
<td>2300</td>
<td>150</td>
<td>1.2</td>
<td>100</td>
<td>12</td>
<td>5</td>
</tr>
</tbody>
</table>

Cells were observed after 16-24 hours of incubation for GFP expression in the NS-control. MCF10A cells were incubated for 2 days and MDA-MB-436 for 3 days before collection. Protein samples for immunoblotting, attachment assays, and live-cell McTN count were prepared as previously described.

**Microtentacle Length Measurements**
MCF10A cells were transfected with GFP-Kinesin1 wildtype, GFP-Kinesin1 mutant constructs [43], and GFP-N1 and stained with CellMask Plasma Membrane Stain as previously stated. Cells were allowed to incubate for 15 minutes at 37°C over a 24-well, low-attachment plate and multiple single plane images captured per well using the Olympus IX81 inverted microscope at 20x with the Volocity software. McTN lengths were measured using the ImageJ software (opensource).
III. LOCAL ANESTHETICS INHIBIT KINESIN MOBILITY AND MICROTENTACLE PROTRUSION IN HUMAN EPITHELIAL AND BREAST TUMOR CELLS

A. Abstract

Detached breast tumor cells produce dynamic microtubule protrusions that promote reattachment of cells and have been termed tubulin McTNs due to their mechanistic distinctions from actin-based filopodia/invadopodia and tubulin-based cilia. McTNs are enriched with vimentin and detyrosinated α-tubulin (Glu-tubulin). Evidence shows that vimentin and Glu-tubulin are cross-linked by kinesin motor proteins. Using known kinesin inhibitors, lidocaine and tetracaine, the roles of kinesins in McTN formation and function were tested. Live-cell McTN counts, adhesion assays, immunofluorescence, and video microscopy were performed to visualize inhibitor effects on McTNs. Viability and apoptosis assays confirmed the non-toxicity of the inhibitors. Treatments of human nontumorigenic mammary epithelial and breast tumor cells with lidocaine or tetracaine caused rapid collapse of vimentin filaments. Live-cell video microscopy demonstrated that tetracaine reduces motility of intracellular GFP-kinesin and causes centripetal collapse of McTNs. Treatment with tetracaine inhibited the extension of McTNs and their ability to promote tumor cell aggregation and attachment. Lidocaine showed similar effects but to a lesser degree. Our current data supports a model in which inhibition of kinesin motor proteins by tetracaine leads to the reductions in McTNs, and provides a novel mechanism for the ability of this anesthetic to reduce metastasis.
B. Introduction

Local anesthetics induce their pain relieving effects through a variety of actions on cellular functions, many of which inhibit cargo transport and motility [18], [44], [45], [46], [47]. The main proteins involved in cellular trafficking are the highly conserved ATP-dependent motor proteins, kinesin and dynein. Kinesins consist of a large family of motor proteins that generally walk along microtubules towards the plus end or away from the centrosomes [27]. Dyneins walk along microtubules towards the minus end or towards the centrosome. It was discovered that the local anesthetics lidocaine and tetracaine inhibited fast anterograde axoplasmic transport (FAAT), which is powered by kinesins [44], [45], [46], [47]. Lidocaine and tetracaine’s dose-dependent, reversible, inhibitory action on kinesins were later confirmed using a modified in vitro motility assay (Fig. 3.1) [48].

Figure 3.1 Tetracaine and lidocaine are effective kinesin inhibitors
a) Tetracaine (5 mM) and b) lidocaine (50 mM) effectively inhibits kinesin motility as graded by the % of gliding microtubules. This effect was reversed when the drugs were washed out. [21]
Kinesins transport vimentin, a key component of McTNs, along microtubules. Microinjection of antibodies directed against conventional kinesins result in the collapse and perinuclear accumulation of IFs without microtubule disruption [49], [50]. Immunofluorescence confirm vimentin particles, squiggles, and mature IFs associated with kinesins and microtubules [21], [49]. It is possible that kinesins may have a key role in the construction and stabilization of the McTN protrusions that develop in metastatic BTCs when detached from their extracellular matrix. An in vivo study using metastatic mouse melanoma cells (B16-F1, B16-F10) treated with tetracaine successfully prevented reattachment and colonization of the lung [51]. Tetracaine reversibly disrupted transmembrane cytoskeletal control, which then induces alterations in cell shape, loss of stable adhesive interactions, and a decrease in blood-borne arrest and metastatic properties without affecting surface protein composition [51]. Lidocaine was also able to inhibit LM8 cells from metastasizing to the lungs of mice in another study [52]. Despite the tremendous novelty of these findings, the specific mechanism underlying the anti-metastatic effect of anesthetics was never pursued and is still not completely understood. However, given the discovery of McTNs in MEC and BTC lines, there is a connection between anesthetics, kinesins, and the reattachment process of metastatic cells [5, 15]. In order to elucidate this role, we examined the effects of inhibiting kinesin activity on McTN extension. We investigated the effects of the two anesthetics, lidocaine and tetracaine, on the frequency of McTNs in a concentration range that is non-toxic. We also investigated the effects of the kinesin inhibitors on vimentin filaments and on the motility of the conventional kinesin-1 using a fluorescently tagged kinesin (GFP-KIF5C) within intact cells.
C. Results

1. Inhibition of micotentacles by the local anesthetics lidocaine and tetracaine.

To visualize McTNs, MCF10A and MDA-MB-436 cells were first transfected with a membrane-localizing green fluorescent protein (GFP-Mem) \cite{5}, \cite{15} and suspended over a low-attachment plates for approximately 15 minutes to allow for McTN generation before counting. McTNs were blindly scored positive, under the criteria, if two of more McTNs extended longer than the radius of the cell body (Fig. 3.2A-C). Latrunculin-A, an actin depolymerizer, increased the frequency of McTNs in MCF10A and MDA-MB-436 cells as did another actin depolymerizer, Cytochalasin-D (Fig. 3.2D,E) \cite{53}, \cite{54}. In MCF10A cells, lidocaine and tetracaine was both effective in reducing McTNs compared to the control. However, in MDA-MB-436 cells, 1mM lidocaine did not significantly reduce McTNs (Fig. 3.2C). It is possible that MDA-MB-436 cells possess a greater resistance to lidocaine due to the large endogenous levels of vimentin filaments that may confer additional stability to the McTN structures (Fig. 3.3). Tetracaine, however; a more potent inhibitor of kinesins, reduced McTN frequencies for both cell lines, with and without the presence of the actin depolymerizers LA (Fig. 3.2D,E) \cite{48}.
Figure 3.2 Tetracaine is an effective inhibitor of McTN protrusions in MCF10A and MDA-MB-436 cells. MCF10A human MECs and MDA-MB-436 human BTCs were transfected with GFP-Membrane and scored for McTN generation. A-C) Representation of McTN types counted and not counted A) DMEM (positive) B) 5 µM LA (positive) C) 0.25 mM tetracaine + LA (negative) D) MCF10A n=4 E) MDA-MB-436 n=4 McTN counts D,E) MCF10A and MDA-MB-436 cells either under No Treatment, 5 µM LA, 1 mM lidocaine +/- 5 µM LA, or 0.25 mM tetracaine +/- 5 µM LA. Cells were blindly scored positive when exhibiting two or more McTNs that extended greater than the radius of the cell body. * indicate significant differences compared to that of No Treatment. ** indicates significant differences compared to that of 5 µM LA treatment. ANOVA test, p<0.05. n=3
Concentrations of lidocaine and tetracaine that inhibit microtentacles are non-toxic to human mammary epithelial cells and breast tumor cell lines.

Figure 3.3 Lidocaine and tetracaine do not affect cellular viability or induce PARP cleavage at the concentrations that inhibit microtentacle protrusions. A,B) XTT Viability Assay of MCF10A, MCF10A-Bcl2, and MDA-MB-436 cells for treated with 1 mM lidocaine and 0.25mM tetracaine for 4 hours. (n=3) C) Western Blot: MCF10A, MCF10A-Bcl2, and MDA-MB-436 samples with/without lidocaine and tetracaine treatments blotted for PARP cleavage, Vimentin, Bcl2, and actin. MDA-MB-436 with 1μg/ml TRAIL for two hours as a positive control for cleaved PARP.
The effects of lidocaine and tetracaine on the viability of human MECs and BTCs have not been well-established. A tetrazolium hydroxide viability assays was performed to confirm the anesthetics were non-toxic in the concentrations used for McTN inhibition. While McTNs are affected after 15 minutes of treatment with either 1mM lidocaine or 0.25mM tetracaine, no toxicity is observed at the concentrations for human MECs (MCF10A, MCF10A-Bcl2) and a human BTC line (MDA-MB-436) even after four hours of exposure (Fig. 3.2; Fig 3.3A,B). As MCF10A cells are highly sensitive to apoptotic cell death, particularly in response to changes in cell shape [9], the resistance conferred by Bcl-2 expression show that the anesthetics were not toxic to apoptotically-resistant cells. To determine if treatments with anesthetics would induce short-term apoptosis in MCF10A cells, a western blot was performed to assay cleavage of Poly-(ADP-ribose)-polymerase (PARP) (Fig. 3.3C). Treatment with the anesthetics did not induce PARP cleavage at 4 hours, even though the apoptotic pathway is functionally active as shown by a positive control treatment with tumor necrosis (TNF)-related apoptosis-inducing ligand (TRAIL). Additionally, a propidium iodide (PI) exclusion assay was conducted under the same conditions used for live-cell McTN counts. This confirms the maintenance of cellular membrane integrity in MCF10A and MDA-MB-436 cells under lidocaine and tetracaine drug treatment (Fig. 3.4).
Figure 3.4 The plasma membrane integrity is maintained in concentrations and conditions that are effective in inhibiting McTNs. A propidium iodide exclusion assay was conducted on MCF10A and MDA-MB-436 cells in DMEM, 1 mM lidocaine, and 0.25 mM tetracaine under the same conditions of live-cell McTN counts. A) Panels show representative cells in DMEM, 1 mM lidocaine, and 0.25 mM tetracaine with PI treatment. B) PI counts in MCF10A cells. C) PI counts in MDA-MB-436.

Lidocaine and tetracaine cause a centripetal collapse of vimentin intermediate filaments and inhibit vimentin trafficking.

The effects of lidocaine and tetracaine on the cytoskeleton components α-tubulin and vimentin were analyzed via immunofluorescence of attached MCF10A (Fig. 3.5A-H) and MDA-MB-436 (Fig. 3.5I-P) cells. Filaments of α-tubulin proteins (Fig. 3.5A, I) and
vimentin IFs (Fig. 3.5B, J) were found ubiquitously throughout the cytoplasm before treatment with lidocaine and tetracaine. After 30 minutes of incubation in 1 mM lidocaine or 0.25 mM tetracaine, vimentin filaments in MCF10A and MDA-MB-436 cells collapse to a perinuclear position (Fig. 3.5F, H, N, P).

![Figure 3.5 Anesthetic treatment causes collapse of vimentin intermediate filaments. A-P) MCF10A and MDA-MB-436 cells stained with Hoechst, α-tubulin, and vimentin (A,B) MCF10A T=0 in DMEM C-H) MCF10A T=30 minutes in DMEM or DMEM +/- 1 mM lidocaine or 0.25 mM tetracaine. I,J) MDA-MB-436 T=0 in DMEM K-P) MDA-MB-436 T=30 minutes in DMEM or DMEM +/- 1 mM lidocaine or 0.25 mM tetracaine. Arrows point to areas of vimentin collapse at T=30 minutes with 1 mM lidocaine or 0.25 mM tetracaine treatment with corresponding arrows in α-Tb. G) Arrowheads point to focal points of α-Tb collapse seen in MCF10A cells. Hoescht stain in top, right insets.

This is consistent with the vimentin collapse observed in fibroblasts when the anterograde movement of kinesins is inhibited via microinjection of an antibody against conventional kinesins [50], [49]. However, α-tubulin microtubules remained filamentous
with little differences between drug treated and DMEM control (Fig. 3.5C, E, G; K, M, O) though foci of α-tubulin can be seen in MCF10A cells at 30 minutes (Fig. 3.5G white arrows).

We further investigated the effects of tetracaine on the vimentin and α-tubulin networks of suspended MDA-MB-436 cells (Fig. 3.6). Control cells displayed McTNs composed of filamentous vimentin and α-tubulin [15]. Cells treated with 0.25mM tetracaine, however, displayed a marked decrease in McTNs as seen in the McTN counts (Fig. 3.2).

Figure 3.6 Anesthetic treatment of suspended MDA-MB-436 cells induces the collapse of vimentin and α-tubulin. Suspended MDA-MD-436 cells were left untreated (A,B) or treated with 0.25mM tetracaine (C,D) fixed and stained with vimentin (A,C) or α–tubulin (B,D) mAbs after 15 minutes of suspension. Arrows point to McTNs with vimentin and α–tubulin staining in the control cells (A, B). Suspended cells treated with tetracaine show decreased McTN protrusions (C, D).
Local anesthetics attenuate reattachment of suspended mammary epithelial cells and breast tumor cell lines.

Tetracaine modifies microfilament organization and decreases cell-adhesion characteristics in endothelial cell adhesion assays [51]. We showed McTN inhibition in MCF10A and MDA-MB-436 using tetracaine and McTN inhibition with lidocaine in MCF10A cells (Fig 3.2D, E). Due to the implication of the role of McTNs in reattachment after detachment, we assessed tetracaine and lidocaine’s effect on cell-substratum adhesion properties of MCF10A, MCF10A-Bcl2, and MDA-MB-436 cell lines using the xCelligence RTCA SP real-time cell sensing system. Lidocaine concentrations were increased from 1mM to 3mM after preliminary studies determined improved inhibition of reattachment in the xCelligence system. In all cell lines, reattachment was greatly attenuated with drug treatments up to 2 hours. While MCF10A-Bcl2 cells attach more quickly than MCF10A cells, they are still inhibited by lidocaine and tetracaine (Fig. 3.7B). Overall, reattachment of both MCF10A MECs and MDA-MB-436 BTCs was significantly (P<0.05, t-test) inhibited by treatment with lidocaine and tetracaine (Fig. 3.7A-C).
Figure 3.7 Lidocaine and tetracaine impede reattachment of human MECs and BTCs. Human mammary epithelial cells (A) MCF10A and (B) MCF10A-Bcl2 were measured for reattachment through impedance of electrical current using the xCelligence RTCA SP real-time cell sensing instrument in the presence of lidocaine (3 mM), tetracaine (0.25 mM), or vehicle control (H2O) as indicated.
Figure 3.7 Lidocaine and tetracaine impede reattachment of human MECs and BTCs. Breast tumor cells (C) MDA-MB-436 were measured for reattachment through impedance of electrical current using the xCelligence RTCA SP real-time cell sensing instrument in the presence of lidocaine (3 mM), tetracaine (0.25 mM), or vehicle control (H2O) as indicated.

Tetracaine causes the rapid centripetal collapse of microtentacles.

Next, we used live-cell microscopy to directly observe the effects of tetracaine on McTNs, on a single cell, during tetracaine drug treatment. MDA-MB-436 cells were suspended and allowed to partially reattach over a bovine serum coated glass coverslip. In the DMEM vehicle control, dynamic McTN motion persisted for extended periods of time up to 12 minutes as depicted in the panels above (Fig. 3.8). Upon addition of 0.125mM tetracaine, a rapid reduction in McTN length and frequency was observed. Complete retraction of all protrusions was obtained by 12 minutes similar to the observations made with compounds targeting vimentin assembly in McTNs [15].
Figure 3.8 Tetracaine causes the reabsorption of microtentacle protrusions. Still, time-lapse images of suspended MDA-MD-436 cells in Phenol red-free DMEM. Cells have been allowed to partially attach to the bottom of a glass culture dish and filmed using DIC microscopy. In control cells, microtentacles persist up to 12 minutes but when treated with 0.125 mM Tetracaine, microtentacles collapse and are reabsorbed completely by 12 minutes.

Recent studies have shown that McTNs facilitate efficient formation of cell-cell and cell-matrix attachments [5]. To determine the effects of lidocaine and tetracaine on McTN function, we tested the rate of homotypic aggregation in suspended populations of MCF10A, MCF10A-Bcl2, and MDA-MB-436 cells in the presence of lidocaine or tetracaine. A single-cell suspension was prepared using a 0.2% methylcellulose medium, with or without appropriate drug. A 25-gauge syringe was used to separate cells into a single-cell suspension. Aggregation was observed 30-60 minutes after suspension in all cell lines (Fig. 3.9). Tetracaine impeded the rate of aggregation of suspended cells compared to DMEM untreated wells. Lidocaine did not significantly reduce the aggregation rate (Fig. 3.9A-C). At 90 minutes, the differences between the integrated
densities of each treatment for each cell line can be seen with the greatest separation between tetracaine and DMEM treatments in MDA-MB-436 cells (Fig. 3.9D-F).

**Figure 3.9 Tetracaine is a more effective inhibitor of homotypic aggregation than lidocaine.**  
A-C) Hoechst stained MCF10A, MCF10A-Bcl2, and MDA-MB-436 cells were separated using a 25 gauge syringe and mixed in a 0.2% methylcellulose PF-free media with or without 1 mM lidocaine or 0.25 mM tetracaine solution. Images were analyzed through quantifications of integrated density taken every 15 minutes.  
D-E) Imaged wells of MCF10A, MCF10A-Bcl2, and MDA-MB-436 in 1 mM lidocaine and 0.25 mM tetracaine in bas relief, at 0 and 90 minutes.
**Tetracaine and lidocaine inhibits intracellular kinesin motor motility.**

To specifically examine the effect of tetracaine and lidocaine on kinesins within the cell body, MCF10A cells were transfected with GFP-KIF5C, a fusion protein of the wildtype kinesin-1 protein with a GFP tag. MCF10A cells transfected with the GFP-KF5C construct were analyzed for small GFP-particle movements and trafficking. As shown by Peckham et al., GFP-KIF5C particles were visualized in MCF10A cells as speckles moving along filaments, most likely to be microtubules, at varying velocities and directions with intermittent stops [43]. In order to observe the impact of the anesthetics on kinesin motility, time-lapse movies were taken before and after drug treatment with tetracaine and lidocaine. Clear, motile GFP particles were tracked and recorded at 5 frames/minute for vehicle control and drug treatment. Movies were started after treatment to record the time frame in which inhibition is noted. GFP particles within cells with DMEM treatment alone moved deliberately along the filamentous tracks (Fig. 3.10A, F). Cells treated with tetracaine or lidocaine; however, displayed particles that can slowed over time with almost complete inhibition of movement after approximately 10 minutes of treatment with lidocaine and complete inhibition at 4 minutes with tetracaine (Fig. 3.10B, C, E, F).
Figure 3.10 GFP-KIF5C forward motion is inhibited by anesthetics. MCF10A cells before lidocaine treatment (A) and after lidocaine treatment (B) MCF10A cells before tetracaine treatment (D) and after tetracaine treatment (E) where velocities of selected GFP speckles were observed and tracked. (n=3, P<0.05, t-test) (C, F).
**D. Discussion**

We investigated the effects of the local anesthetics, lidocaine and tetracaine, on the cytoskeletal function of McTN protrusions. Lidocaine and tetracaine were found to be effective, nontoxic inhibitors of McTNs in MEC cells (Fig. 3.2D; Fig 3.3A). In live-cell counts of suspended MCF10A and MDA-MB-436 cells, application of lidocaine and tetracaine showed significant reduction in most McTN counts. Lidocaine had no significant effect on the tumorigenic MDA-MB-436 cell line in the concentration used (Fig. 3.2E). This was postulated as being due in part to the large quantity of vimentin IFs expressed in the MDA-MB-436 cytoskeletal system (Fig. 3.4C). In Fig. 3.5, we showed the destabilizing effect of lidocaine and tetracaine on the vimentin support along α-tubulin filaments with the greatest reduction in filaments in MCF10A cells than MDA-MB-436 cells (FIG. 3.5). Live-cell movies of GFP-KIF5C motility additionally demonstrated the effects of lidocaine and tetracaine inhibition on kinesin movement within intact, live-cell MCF10As (Fig. 3.10). Inhibition of kinesin motility and McTNs generation translated to a reduction in reattachment as test with the xCelligence real-time, cell-sensing instrument (Fig.3.7) and reduction in homotypic aggregation (Fig. 3.9).

The vimentin filament network is a dynamic system as demonstrated by time-lapse studies of GFP-vimentin expressing cells [42], [15]. In these studies, vimentin filaments were observed assembling, disassembling, shortening, and elongating in the cytoplasm [55], [56]. GFP-vimentin studies in fibroblasts also confirmed bidirectionality along microtubules with majority of the movements directed in the anterograde direction [57], [49]. This anterograde movement is due to the tight vimentin association with conventional kinesins [57], [49]. Since it is likely vimentin structural support is important
to maintain McTN dynamics in suspended cells, kinesin inhibition would affect vimentin dynamics and thus McTNs. Microinjection studies support this hypothesis where vimentin filaments collapsed perinuclearly after kinesin antibody injections [50], [49]. We see a similar effect on vimentin filaments in the lidocaine and tetracaine immunofluorescence studies where vimentin filaments collapse perinuclearly while microtubule structures stay relatively filamentous (Fig. 3.5).

In addition to vimentin trafficking, kinesins also act as crosslinkers in microtubule bundles allowing for sliding and movement to occur [58]. F-actin retrograde flow and microtubule polymerization forces oppose each other causing compression in microtubules that result in microtubule bending. When the F-actin cortex is disassembled by detachment or by actin depolymerizers, such as Latrunculin A or Cytochalasin D, the retrograde force is dissipated allowing for outward growth of microtubules [53]. This is observed in suspended MEC and BTCs in the form of McTN protrusions and is further enhanced by treatments with actin depolymerizers [5]. The dynamic movement and growth of the McTNs is likely due to the continual forces generated by kinesins and dyneins that travel along microtubule filaments. In the absence of kinesin anterograde movement, the retrograde movement of dynein motor proteins along microtubules would facilitate the disassembly of the microtubule filaments as they move cargo to the opposite minus end. In Fig. 3.8, the gradual collapse and reabsorption of McTNs is observed over the time course of 12 minutes with tetracaine treatment, as captured with DIC microscopy. This reduction in McTNs with tetracaine and lidocaine treatment translates to a reduction in attachment of suspended MCF10A and MDA-MB-436 cells.
It is thought that the inhibition of kinesin motion by lidocaine and tetracaine is through the interactions of the charged form of the kinesin inhibitors with the neck region of the kinesin motor proteins inhibiting the rotational process of the free kinesin head [21]. During the forward stepping process, the kinesin neck region unwinds, allowing the rotation of the microtubule-free head after ATP hydrolysis [59], [60]. Lidocaine and tetracaine were not found to compete for kinesin binding to microtubules or impede ATP hydrolysis. Instead, both drugs uncoupled the ATP hydrolysis from kinesin movement effectively inhibiting anterograde motion along microtubules (Fig. 3.10, 3.11) [48].

![Diagram of kinesin movement](http://www.ncbi.nlm.nih.gov/books/NBK22572/figure/A4890/?report=objectonly)

**Figure 3.11 Lidocaine and tetracaine inhibition of kinesin movement**

Overall, tetracaine displayed greater inhibition of kinesins, McTNs, and attachment in the MCF10A MEC and MDA-MB-436 BTCs than lidocaine. In a previous study by Nicolson et al., melanoma cells pretreated with tetracaine were injected into mice, resulting in the reduction of tumor cells retained in the lung by 2-5 fold while also decreasing overall metastasis compared to that of the control [51]. It was proposed that the effects of the local anesthetics on cell morphology, agglutination, receptor distribution, and adhesion explained the decrease in lung tumor colonies [51]. However, the underlying mechanisms for this action were never clarified. Though lidocaine was not
effective in reducing McTN frequency in MDA-MB-436 cells in the 1mM concentration used in live-cell McTN counts (Fig. 3.2), lidocaine was observed to inhibit the invasiveness of the human cancer cells HT1080, HOS, and RPMI-7951 in the concentration range of 5-20 mM [52]. In addition, murine osteosarcoma cells (LM 8), treated with 5-30 mM of lidocaine also showed reduction in the occurrence of pulmonary metastatic tumors by 50-90% [52].

The coordination of cytoskeletal filaments underlying McTNs provides a target to bring together these compelling in vivo studies with the more recent in vitro observations that anesthetics reduce kinesin motility. Our data showing that tetracaine inhibits kinesin motility within intact cells and induces collapse of the vimentin filaments that support McTNs provide a novel molecular mechanism for how tetracaine reduces the metastatic efficiency of CTCs (Fig. 3.5; 3.10) [51].
IV. GENETIC TARGETING OF KINESIN FUNCTION REDUCES MICROVENTACLES AND REDUCES THE REATTACHMENT OF HUMAN BREAST TUMOR CELLS

A. Abstract

The kinesin superfamily of motor proteins consists of 14 classes of kinesins that play various roles from chromosomal separation in mitosis to cargo trafficking along microtubules. Due to kinesin involvement in cell proliferation, development of inhibitors of mitotic kinesins has become an interest in the area of cancer research. In recent years, the development of dynamic, microtubule-based membrane protrusions in suspended mammary epithelial and breast tumor cells have been discovered. These microtactacles (McTNs) consist structurally of vimentin intermediate filaments and detyrosinated tubulin filaments. Kinesin-1, an anterograde trafficking kinesin, preferentially binds to detyrosinated microfilaments and traffics vimentin filaments. The local anesthetics and kinesin inhibitors, tetracaine and lidocaine, have been shown to inhibit kinesin motility in vitro in mammary epithelial and breast tumor cells. In addition, lidocaine and tetracaine have inhibitory actions on McTNs, attachment, and metastatic potential in mice. However, lidocaine and tetracaine have multiple actions upon cellular systems. We focused on the specific effects of inhibiting through genetic targeting to reduce KIF5B and over-express KIF5C, which are isoforms of Kinesin-1. In this study, we find that the overexpression of two KIF5C proteins increased the lengths and stability of McTNs as well as the tumor cell reattachment rate without increasing average McTN frequencies. Glu-tubulin levels were dramatically elevated in GFP-Kinesin1MUT transfected mammary epithelial cells as determined with immunofluorescence. However, KIF5B siRNA was only able to decrease reattachment of mammary epithelial cells by approximately 15%. 
Although only partial reduction in attachment was obtained through siRNA, overexpression of Kinesin-1 showed dramatic morphological changes in McTN lengths and stability supporting the role of kinesins within McTNs.

**B. Introduction**

The kinesin superfamily consists of a wide variety of highly conserved yet structurally diverse motor proteins that consume ATP for processivity along microtubules [27], [61]. Kinesin-1 was the first to be discovered in giant squid axons [61]. It consists of a tetramer made up of two heavy and two light chains that bind to traverse the length of microtubule filaments in the cytoplasm delivering cargo, providing support, and compartmentalizing organelles to their proper destinations. Microtubule modifications appear to function as molecular traffic signals to target specific kinesins to various subpopulations of microtubules. Microtubules can undergo a multitude of post-translational modifications such as acetylation, tyrosination, detyrosination, D2 modification, polyglutaminylation, polyglycylation, palmitoylation, and phosphorylation [62], [63], [64]. In the fungus *Asperigillus Nidulans*, kinesin-3 UncA motors were shown to move vesicles along the subpopulation of Glu-microtubules [65]. In mice that lacked the ability to form poly-glutamylated tubulin, a decrease in synaptic vesicles trafficked by KIF1 kinesins were shown [66]. In human neurons, acetylated microtubules promoted kinesin-1 binding and increase the speed at which cargo is trafficked [67], [68]. Interestingly, kinesin-1 preferentially binds to and cross-links Glu-tubulin to vimentin filaments, the main components of McTNs, in attached cells [5], [15], [21], [43], [69]. It
is likely that kinesins play a similar role in suspended cells, bundling microtubules and recruiting supportive structures such as vimentin IFs to allow for McTN extension.

Vimentin is trafficked by kinesins and disruption of endogenous vimentin, through dominant negative constructs, decreases McTN frequencies [15]. It is possible that by focusing on a specific plus-end directed kinesin or a subpopulation of kinesins, the anterograde kinesin movements within McTNs that transport vimentin and facilitate growth and stability could be halted. This in turn would effectively inhibit McTN growth and the dynamic motion that kinesins confer to microtubule bundles through sliding. Microinjections of kinesin antibody and anesthetics have already demonstrated the destabilizing effects on vimentin and McTNs when kinesin motility is inhibited [70]. However, broad spectrum kinesin inhibitors will likely produce cellular toxicities due to the key roles kinesins play in many vital cellular functions. Individual kinesin family members, however; possess specific functions that make them ideal for targeted therapies [71]. Kinesin-1 is of particular interest due to its propensity to bind to Glu-microtubules over other modified forms of microtubules and differentially traffics cargo on Glu versus Tyr-microtubules [43]. Kinesin-1 has the ability to perform both fast and slow transport, as well as, having the capacity to bind multiple adaptor proteins for specific cargo [72]. Kinesin-1 possesses three isoforms (KIF5A, KIF5B, and KIF5C) that vary slightly in length due to variations in the end cargo binding domain [27], [73]. Each isoform encompasses the ability to homodimerize and heterodimerize with one another increasing variability of function [73, 74]. KIF5A and KIF5C are mainly neuronal while KIF5B is ubiquitously expressed in a broad range of cells [73, 74]. The three KIF5 isoforms have a high degree of homology and appear to have functional redundancies [73, 74].
Supporting evidence comes from KIF5C knockout mice which displayed decreases in motor neurons, but retained viability as well as normal levels of kinesin isoforms within specific brain structures [73, 74]. In another experiment, abnormal mitochondria aggregation in *kif5b-* cell lines were rescued with the transfections of exogenous KIF5A, KIF5B, and KIF5C [73, 74].

The inhibitory effect of lidocaine and tetracaine on kinesin motility has been demonstrated in *in vitro* studies as well as in attached MEC and BTC lines [48], [70]. McTNs protrusions occur when cells are suspended and consist of vimentin IFs and Glu-microtubules which are associated with kinesin-1 [5], [15], [21], [43], [69]. McTN frequencies decreased with the application of lidocaine and tetracaine treatment in the MCF10A MECs and MDA-MB-436 BTCs, [70]. McTNs facilitate the reattachment process of suspended MEC and BTCs *in vitro* [5]. However, when lidocaine and tetracaine is applied, this reattachment process is impeded [70]. *In vivo* studies in mice also demonstrate lidocaine and tetracaine's ability to effectively inhibit tumor cell metastasis within the lungs, though the mechanisms in which these anesthetics work remains obscure [52], [51]. One explanation may lie in the inhibitory actions of lidocaine and tetracaine on kinesins and consequently McTN protrusions that facilitate reattachment of suspended cells.

Though lidocaine and tetracaine have been shown to inhibit kinesins and reduce McTNs and attachment, both drugs have many off target effects that have yet to be explored [21], [70]. The anesthetic properties that make lidocaine and tetracaine effective analgesics are the drugs ability to inhibit kinesin trafficking of synaptic cargo, and the ability to perturb ion channels [44], [45], [46], [47], [75], [74]. To exclude these
off target effects, we used genetic targeting of kinesins to specifically target kinesin-1 and clarify its involvement in McTNs. We used two over-expression models, \text{GFP KIF5C wildtype (GFP kinesin1WT)} and \text{GFP KIF5C mutant (GFP kinesin1MUT)} and a KIF5B siRNA to investigate the effects of kinesins on McTN protrusion counts and attachment.

C. Results

\textbf{GFP kinesin1 wildtype and GFP kinesin1 mutant increase McTN length but not McTN frequency.}

The \text{GFP kinesin1WT} and \text{GFP kinesin1MUT} constructs [43] were transfected into MCF10A human mammary epithelial cells and observed for GFP expression (Fig. 4.1).

![Figure 4.1 Schematics of GFP Kinesin1-wildtype and GFP Kinesin1-mutant](image)

\textbf{Figure 4.1 Schematics of GFP Kinesin1-wildtype and GFP Kinesin1-mutant}

\textbf{A.} \text{GFP Kinesin1WT} is an N-terminally tagged, full-length, rat GFP-KIF5C fusion protein.

\textbf{B.} \text{GFP Kinesin1MUT} is the \text{GFP Kinesin1WT} introduced with a T93N mutation in the catalytic motor domain [43]

The \text{GFP kinesin1WT} construct was observed as motile speckles along an intricate microtubule network. \text{GFP kinesin1WT} trafficked to the distal ends along the cell periphery where GFP aggregation can be seen. The diffuse cytoplasmic distribution is likely due to free floating, inactive kinesins (Fig. 4.2). The \text{GFP kinesin1MUT} construct contains a point
mutation (T93N) in the motor domain that impedes forward stepping yet allows for ATP hydrolysis within the active site without impeding attachment to microtubules [76]. GFP\textsuperscript{Kinesin1MUT} transfected MCF10A cells displayed heavy GFP localization along microtubule filaments with very little if any cytoplasmic distribution and no collection at the distal ends (Fig. 4.2) [43].

Figure 4.2 \textsuperscript{GFP} Kinesin\textsuperscript{WT} and \textsuperscript{GFP} Kinesin\textsuperscript{MUT} transfected MCF10A MECs. The A. \textsuperscript{GFP} Kinesin\textsuperscript{WT} displayed GFP localized filaments throughout the cell, collecting at the distal ends of the cell periphery. B. \textsuperscript{GFP} Kinesin\textsuperscript{MUT} decorated filaments heavily and did not collect at the distal ends.

To determine the effects of the Kinesin1 constructs on McTNs, MCF10A cells were separately transfected with \textsuperscript{GFP} Kinesin\textsuperscript{WT}, \textsuperscript{GFP} Kinesin\textsuperscript{MUT}, and GFP-N1 for 24 hours before visualization using CellMask orange plasma membrane stain to facilitate McTN identification for blinded McTN counts. Cells were suspended for 15 minutes before scoring for McTNs. It was predicted that the \textsuperscript{GFP} Kinesin\textsuperscript{WT} would enhance McTN length and frequency while the \textsuperscript{GFP} Kinesin\textsuperscript{MUT} would inhibit the extension of the McTNs due to its immobility and thus reduce the length and frequency of McTNs.
However, no significant differences arose in the average number of cells scored positive for generating McTNs compared to the GFP-N1 control (Fig. 4.3). Surprisingly, differences rose between $^{\text{GFP}}$Kinesin1WT and $^{\text{GFP}}$Kinesin1MUT when comparing the lengths of the McTNs. $^{\text{GFP}}$Kinesin1WT and $^{\text{GFP}}$Kinesin1MUT displayed dramatic increases in the lengths of McTNs in a subpopulation of cells compared to the GFP-N1 control. Interestingly the $^{\text{GFP}}$Kinesin1MUT displayed a greater increase in length even over the $^{\text{GFP}}$Kinesin1WT (Fig 4.3B).

A.

![McTN Counts](image)

B.

![AVG McTN Lengths](image)

Figure 4.3 McTN counts and average McTN lengths of $^{\text{GFP}}$Kinesin1 wildtype and $^{\text{GFP}}$Kinesin1 mutant transfected MCF10A cells  
A) GFP-N1, $^{\text{GFP}}$Kinesin1WT, and $^{\text{GFP}}$Kinesin1MUT transfected MCF10A cells had no significant differences in McTN counts  
B) Differences arose in the increased length of $^{\text{GFP}}$Kinesin1WT, and $^{\text{GFP}}$Kinesin1MUT over the GFP-N1 control
**GFP Kinesin1 wildtype and GFP Kinesin1 mutant increase Glu-tubulin expression.**

Due to the unexpected differences in the \(^{\text{GFP}}\text{Kinesin1WT}\) and \(^{\text{GFP}}\text{Kinesin1MUT}\) McTN lengths, the effects of the constructs on the cytoskeletal system were investigated. However, no significant differences were noted in vimentin, α-tubulin, and pan-kinesin levels when MCF10A cells were transiently transfected with GFP-N1, \(^{\text{GFP}}\text{Kinesin1WT}\), and \(^{\text{GFP}}\text{Kinesin1MUT}\) (not shown). Further probing for Glu-tubulin and Tyrosinated tubulin showed no significant differences between the two protein levels overall following transient transfections with approximately 30-40% efficiencies (Fig. 4.4).

![Image](image.png)

**Figure 4.4** There is no significant difference in the Glu-tubulin and Tyrosinated tubulin levels in GFP-N1, \(^{\text{GFP}}\text{Kinesin1 wildtype},\) and \(^{\text{GFP}}\text{Kinesin1 mutant transfected MCF10A cells.}\) MCF10A cells transfected with GFP-N1, \(^{\text{GFP}}\text{Kinesin1 wildtype,}\) and \(^{\text{GFP}}\text{Kinesin1 mutant constructs and immunoblotted with GFP, Tyrosinated tubulin, Glu-tubulin, and actin.}\)

However, whole population analysis using immunoblot analysis is not optimal for observations of transiently transfected populations. For this reason, we turned to immunofluorescence to investigate individual cells that were positive for GFP-Kinesin1 transfections. MCF10A cells transfected with GFP-N1, \(^{\text{GFP}}\text{KinesinWT,}\) and \(^{\text{GFP}}\text{KinesinMUT}\) probed for Glu-tubulin and α-tubulin (Fig. 4.5). The levels of Glu and α-
tubulin were measured by averaging the total integrated fluorescence of Glu and α-tubulin and normalizing the Glu with α-tubulin levels within the same cell. We then compared the data of the GFP-positive cell with a GFP-negative cell within the same field with an n=3. Cells transfected with GFP-N1, displayed Glu-tubulin diffusely throughout the cell with two localized points indicating the microtubule organizing centers (not shown). In GFP-KinesinWT transfected cells, there was no significant difference in the Glu-tubulin levels compared to untransfected cells in the same field. However, increases in Glu-tubulin expression are pronounced in the GFP-Kinesin1MUT transfected cells where Glu-tubulin stained robustly and concurrently with GFP expression (Fig. 4.5E, H).

**Figure 4.5** Glu-tubulin levels in GFP-Kinesin1 mutant transfected MCF10A cells are elevated compared to GFP-Kinesin1 wildtype transfected MCF10A cells. MCF10A cells were transfected with GFP-Kinesin1WT (A-D) and stained for Glu-tubulin and α-tubulin.
**Glu-tubulin**

**α-tubulin**

**GFP**

Figure 4.5 Glu-tubulin levels in **GFP** Kinesin1 mutant transfected MCF10A cells are elevated compared to **GFP** Kinesin1 wildtype transfected MCF10A cells. MCF10A cells were transfected with **GFP** Kinesin1MUT (E-H) and stained for Glu-tubulin and α-tubulin. The intensities of cells expressing and not expressing GFP-N1 and the **GFP** Kinesin1 constructs were averaged and graphically represented (D, H). n=3

**GFP** Kinesin1 wildtype and **GFP** Kinesin1 mutant increase attachment efficiency in suspended mammary epithelial cells.

We next tested the physiological effects that altered Glu-tubulin levels and McTN length had on the attachment efficiency of MCF10A cells transfected with **GFP** Kinesin1WT and **GFP** Kinesin1MUT. We implemented the RTCA xCELLigence system to monitor attachment. Cells were suspended and equal cell volumes were plated into the RTCA e-plate wells and monitored every 5 minutes for a change in impedance.
over 3 hours. The GFP-N1 control cells began attachment at approximately 20 minutes after suspension. GFPKinesin1WT and GFPKinesin1MUT transfected cells displayed increased attachment efficiency compared to the control with the greatest increase in attachment in the GFPKinesin1MUT followed by GFPKinesin1WT and GFP-N1 transfected cell (Fig 4.6). The difference in attachment efficiency between GFPKinesin1MUT, GFPKinesin1WT, and GFP-N1 correspond in the pattern seen with the increased lengths in McTNs (Fig. 4.3).

Figure 4.6 Overexpression of GFPKinesin1MUT and GFPKinesin1WT increases reattachment of human MECs. GFP-N1 (●), GFPKinesin1WT (△), and GFPKinesin1MUT (x) were transfected into MCF10A for 24hours. 40,000 cells were then suspended, plated, and measured for attachment through impedance of the electrical current using the xCelligence RTCA SP real-time cell sensing instrument.

Partial knockdown of Kinesin-1 with siRNA decrease attachment efficiency in suspended mammary epithelial cells.
We further tested Kinesin-1 function through selective knockdown of the Kinesin-1 isoform KIF5B due to its ubiquitous expression in non-neuronal cells using siRNA. MCF10A cells were transfected and collected at 0, 24, 48, and 72 hours. Immunoblot analysis of KIF5B showed a partial knockdown of the ubiquitous isoform of Kinesin-1 compared to the nonsilencing control at 72 hours (Fig. 4.7). Attachment assays using the RTCA xCELLigence system reflect the partial knockdown with approximately a 15% decrease in attachment efficiency compared to the control. Additional siRNA treatments were tested with MDA-MB-436 cells and a complete knockdown of Kinesin-1 was obtained. However, attachment assays again showed only a 22% decrease in the attachment efficiency even with a more complete knockdown of Kinesin-1 (Fig 4.8).

Figure 4.7 Partial inhibition of Kinesin1 using siRNA reduces reattachment of MCF10A human mammary epithelial cells.
MCF10A cells were transfected with non-silencing control siRNA (●) and Kinesin-1 siRNA (○) were carried out for 72 hours before 40,000 cells were suspended, plated, and measured for attachment through impedance of the electrical current using the xCelligence RTCA SP real-time cell sensing instrument.
Figure 4.8 Partial inhibition of Kinesin1 using siRNA reduces reattachment of MDA-MB-436 human breast tumor cells.

A. MDA-MB-436 cells transfected with non-silencing control siRNA (●) and Kinesin-1 siRNA (○) were carried out for 48 hours before 40,000 cells were suspended, plated, and measured for attachment through impedance of the electrical current using the xCelligence RTCA SP real-time cell sensing instrument. B. Immunoblot analysis of MDA-MB-436 cells treated for 48 hours with Kinesin-1 siRNA shows a decrease in Kinesin-1 levels.

D. Discussion

The kinesin superfamily displays a wide range of functionality ranging from involvement in mitotic spindle formation, organelle positioning, microtubule bundling and sliding, cellular motility, and synaptic vesicle transport [39]. In the area of cancer research, kinesin studies have mainly focused on the role of mitotic kinesins during cellular divisions of actively dividing tumor cells [41]. Current chemotherapies in general focus on dividing solid tumors during the primary or secondary tumor stage. However, metastatic tumor cells that come to rest and either become dormant or in an antiproliferative state, will bypass the majority of these chemotherapies [77].
In this study, we showed the effects of targeted kinesin motor protein in MCF10A MECs. We utilized two Kinesin-1 overexpression constructs and showed an increase in Kinesin-1 functionality would increase McTN extension and cellular reattachment. However, Glu-tubulin stabilization was also a factor in McTN length and stability as demonstrated by the \textsuperscript{GFP}Kinesin1MUT rigor construct. Both the \textsuperscript{GFP}Kinesin1WT and \textsuperscript{GFP}Kinesin1MUT constructs showed an increase in McTN lengths and reattachment. However, only the \textsuperscript{GFP}Kinesin1MUT displayed dramatic increases in Glu-tubulin levels that corresponded to \textsuperscript{GFP}Kinesin1MUT. We additionally used Kinesin-1 siRNA to partially knockdown Kinesin-1 levels but only obtained partial reduction in reattachment even with an almost complete knockdown in the MDA-MB-436 BTC line.

With the discovery of dynamic, tubulin-based McTNs in suspended MEC and BTCs that facilitate reattachment, chemotherapies targeted for microtubule stabilization of dividing cells may actually increase successful metastases of CTCs to distant sites [78]. In one study, the F-actin depolymerizing drug, jasplakinolide, and the microtubule stabilizing drug, paclitaxel, were used separately and jointly to demonstrate the effects on the actin microfilament and microtubule cytoskeletal network of suspended MEC and BTCs [78]. As expected, jasplakinolide enhanced McTN protrusions while paclitaxel-stabilized McTNs resulting in increased attachment and spreading of the suspended cells over extracellular matrix [78]. A combination of jasplakinolide and paclitaxel augmented the thickness and length in both attached and suspended cells [78]. Similarly, we see an increase in McTN lengths due to microtubule reinforcement with the overexpression of the Kinesin-1 isoform, KIF5C, as well as, increased attachment of suspended MEC and BTCs (Fig. ). These studies indicate the need for better understanding of CTCs and the
effects of current chemotherapeutic drugs on the cytoskeletal system in order to develop better inhibitors to target CTCs.

One potential target may come from non-mitotic members of the KIF family, the plus-end directed conventional kinesins due to their specific roles in cytoskeletal trafficking, maintenance, and stability of microtubules that are not involved in tumor cell proliferation [27]. Deregulation of kinesin mRNA and protein has already been linked to several cancers. For example, KIF5B mRNA up regulation has been reported in bladder, stomach, skin, and breast cancers as well as increased KIF5B protein expression [79]. In another study, MDA-MB-231 and MDA-MB-468 tumor cells that overexpressed the kinesins KIFC3, KIFC1, KIF1A, and KIF5A became resistant to docetaxel and the apoptotic effects of these drugs [80]. This effect is similar to the effects seen with Tau overexpression which also confers resistance to chemotherapeutic agents such as paclitaxel and ixabepilone [81-83]. Interestingly, Tau also increases McTN frequencies as well as metastatic efficiency of detached tumor through the stimulation of McTN formation [84]. EMT and Glu-tubulin further provides evidence that microtubule stabilization occurs in human tumors during progression/invasion and is associated with increased metastasis. These studies suggest non-mitotic kinesins as a promising target for novel cancer therapies. Already, the local anesthetics, lidocaine and tetracaine, have demonstrated their ability to inhibit kinesin motility using in vitro motility assays and GFP-kinesins in live-cell time-lapse microscopy experiments [21], [70]. Lidocaine and tetracaine reduces McTN frequency and the reattachment of suspended MEC and BTCs [70]. Evidence from in vivo studies additionally show that lidocaine and tetracaine treatment decrease metastases that form when tumor cells are injected intravenously into
mice [52], [51]. However, to exclude off-target effects of these anesthetics and confirm kinesin involvement in the reduction of McTNs and attachment, we used the GFP\textsuperscript{Kinesin1WT} and GFP\textsuperscript{Kinesin1MUT} overexpression system and targeted Kinesin-1 siRNA to genetically test for kinesin involvement in McTN generation and functionality.

We began the studies with the GFP\textsuperscript{Kinesin1} constructs to demonstrate the importance of the kinesin-mediated interactions of vimentin and Glu-tubulin for the formation of a stable microtubule network. GFP\textsuperscript{Kinesin1WT} was predicted to function in the extension of the McTNs while GFP\textsuperscript{Kinesin1MUT} was predicted to inhibit McTN extension due to its lack of motor activity and mobility. However, transfections of both constructs showed no significant change in average McTN counts (Fig. 4.3A). Differences arose in the dramatic increase in the lengths of the McTNs produced by the GFP\textsuperscript{Kinesin1WT} and GFP\textsuperscript{Kinesin1MUT} transfected cells. GFP\textsuperscript{Kinesin1MUT} transfected cells displayed increased lengths of McTNs that exceeded even the GFP\textsuperscript{Kinesin1WT} transfected cells (Fig. 4.3B). Protein immunoblot analysis did not confirm an increase in Glu-tubulin in GFP\textsuperscript{Kinesin1WT} and GFP\textsuperscript{Kinesin1MUT} transfected cells (Fig 4.4). However, transient transfection efficiency of the GFP\textsuperscript{Kinesin1WT} and GFP\textsuperscript{Kinesin1MUT} constructs were less than 40% and individual cell increases in protein levels cannot be quantified using immunoblotting techniques. We turned to IF analysis of GFP-positive cells and confirmed dramatic increase in the Glu-tubulin levels of GFP\textsuperscript{Kinesin1MUT} over \(\alpha\)-tubulin levels compared directly to adjacent untransfected cells (Fig. 4.5). From the increased McTN lengths and Glu-tubulin expression observed in the GFP\textsuperscript{Kinesin1} constructs, two hypotheses were formed. For the GFP\textsuperscript{Kinesin1WT} construct, GFP\textsuperscript{Kinesin1WT} extends McTN lengths through increased trafficking of stabilizing
components, such as vimentin filaments, along Glu-microtubules. While the $\text{GFP}^{\text{Kinesin1MUT}}$ construct is immobile, it strongly cross-links vimentin and Glu-tubulin leading to greater microtubule stabilization and pushes microtubule kinetics toward growth, resulting in greater McTN lengths. From these studies, it was concluded that the structural reinforcement of microtubules through cross-linking to vimentin has a greater contribution to McTN length and stability than kinesin-mediated cargo trafficking.

Next we tested the effects of increased structural integrity on the function of McTNs to promote the reattachment of suspended cells. We utilized the RTCA xCelligence attachment system to monitor real-time attachment of $\text{GFP}^{\text{Kinesin1WT}}$ and $\text{GFP}^{\text{Kinesin1MUT}}$ transfected MCF10A cells. Both the $\text{GFP}^{\text{Kinesin1WT}}$ and $\text{GFP}^{\text{Kinesin1MUT}}$ increased attachment over the control with $\text{GFP}^{\text{Kinesin1MUT}}$ having the greatest increase even compared to $\text{GFP}^{\text{Kinesin1WT}}$ (Fig. 4.6) which mimics the differences in McTN lengths of the two systems.

We then tested the opposite effect by utilizing KIF5B siRNA to partially knockdown the most prevalent Kinesin-1 isoform. However, only a partial reduction in attachment was observed. (Fig. 4.7) A more complete knockdown of KIF5B in the MDA-MB-436 only decreased attachment a maximum of 22% compared to the non-silencing control (Fig. 4.8). By comparison, overexpression of the $\text{GFP}^{\text{Kinesin1WT}}$ increased reattachment more than 100% and the $\text{GFP}^{\text{Kinesin1MUT}}$ increased reattachment more than 300%. These results indicate that the $\text{GFP}^{\text{Kinesin1}}$ expression constructs likely have a dominant effect. The comparatively lesser effect of the siRNA is likely due to redundant function with other kinesin family members and the incomplete knockdown of endogenous kinesin-1 protein.
Vimentin siRNA experiments demonstrate the ability of cells to adapt to intermediate filament down regulation to continue McTN formation. In this study, reduction of vimentin using siRNA was obtained yet McTN counts did not coincide with this decrease in vimentin expression [15]. Instead, the expression levels of a different IF inversely became elevated in response to vimentin depletion [15]. However, disruption of endogenous vimentin filaments with a dominant-negative GFP-vimentin mutant successfully reduced McTN counts in the invasive BTC lines Hs578t, HCC1395, and MDA-MB- [15]. This suggests the possibility that Kinesin-1 is not the only kinesin to function in McTN but is only one of several distinct kinesin populations regulating and functioning in McTNs.

Difficulties in kinesin targeting arise from the nature of kinesins themselves. Due to their roles in important cellular functions involving growth and metabolism, disruption of kinesins can be problematic as mutations that alter kinesin functions manifest as neurological diseases and cancers [85], [86], [87], [88], [89]. Mitotic kinesins are targeted due to their role in cellular proliferation effects. However, inhibition of mitotic regulators also have the propensity to cause chromosomal misalignment, spindle defects, and incorrect cytokinesis which in turn can cause cancer [90], [91]. Another example comes from the finding that Kinesin-2 is a regulator of the von Hippel-Lindau tumor suppressor protein (pVHL30) [92], [79], which inhibits metastasis through the inhibition of HIF-factors and chemokine expression [93]. Loss of Kinesin-2 could translate to a loss of VHL function which has been shown to result in renal cell carcinomas and central nervous system hemangioblastomas [94]. Loss of KIF1B expression also results in resistance against apoptosis leading to neuroblastomas and pheochromocytoma [95].
We focused on Kinesin-1 through genetic targeting of two isoforms, KIF5B and KIF5C. We discovered that the overexpression of the KIF5C proteins, \( \text{GFP} \text{Kinesin1WT} \) and \( \text{GFP} \text{Kinesin1MUT} \), increased the lengths and stability of McTNs as well as the tumor cell reattachment rate without increasing average McTN frequencies. Glu-tubulin levels were dramatically elevated in \( \text{GFP} \text{Kinesin1MUT} \) transfected mammary epithelial cells. Reduction of KIF5B with siRNA only achieved partial decrease in reattachment of mammary epithelial cells by approximately 15%, which is likely due to the incomplete knockdown of endogenous Kinesin-1 protein as well as redundant function of other kinesin members. However, the Kinesin-1 overexpression system shows a dominant morphological effect on the McTN length and stability supporting the role of kinesins within McTNs. Further investigation is required to generate the next generation of effective yet non-toxic kinesin inhibitors.
V. SUMMARY

The kinesin super family provides a wide variety of functions within cells, one of which is the transport of organelles and structures over a complex network of microtubules. In particular, Kinesin-1, a ubiquitously expressed form of kinesin, has been shown to preferential traffic vimentin intermediate filaments on Glu-microtubules versus tyrosinated microtubules [43]. Glu-microtubules are not intrinsically stable on their own but obtain increased stability due to close association with IF [13], [96], [97], [69]. With the possibility that kinesins may play a role in McTN dynamics, we tested the central hypothesis that kinesin motor proteins play a key role in the formation and function of McTNs. Supporting evidence shows that the local anesthetics, tetracaine and lidocaine, were effective inhibitors of kinesin motility in an in vitro motility assay [21] and in in vivo studies, where lidocaine and tetracaine reduced the metastatic capacity of CTCs in mice [51], [52].

We addressed the first hypothesis: The local anesthetic tetracaine inhibits metastatic potential due to inhibitory effects on kinesin and thus McTN protrusions, in Chapter III. From the evidence collected, it was concluded that kinesins are involved in McTN formation and function [70]. We proceeded to test the effects of the kinesin inhibitors, lidocaine and tetracaine, on McTN frequency using blind-cell McTN counts in nontoxic concentrations. Tetracaine was an effective inhibitor of McTNs in MEC and BTCs, while lidocaine was effective in MEC cells. Ineffectiveness in reducing McTNs in MDA-MB-436 cells was likely due to the abundant amounts of vimentin IFs. Immunofluorescence confirmed with increased time, lidocaine was as effective as tetracaine in destabilizing vimentin filaments while leaving the cytoskeletal integrity of
the tubulin network relatively filamentous. Kinesin inhibition was further examined in MCF10A MECs using GFP-fusion kinesin protein, \(^{\text{GFP-KIF5CWT}}\). Lidocaine and tetracaine were successful in inhibiting kinesin motility within cells and live-cell DIC of a single, partially attached MDA-MB-436 also demonstrated the effective absorption of McTNs back into the cell body upon tetracaine treatment. This reduction in McTNs through the use of the kinesin inhibitors lidocaine and tetracaine translated to the reduction in attachment efficiency of MECs and BTCs as measured by the real-time RTCA xCelligence system.

We addressed the second hypothesis: *Genetic targeting of Kinesins Affect Microtentacle Function and Reduces the Reattachment Process of Breast Tumor Cells*, in chapter IV. We began by using two GFP-kinesin fusion constructs, \(^{\text{GFP-Kinesin1}}\) wildtype and \(^{\text{GFP-Kinesin1}}\) mutant transfected into MECs and BTCs. Live-cell McTN counts were done with the hypothesis that the overexpression of the wildtype kinesin would cause an increase and elongation of McTNs while the expression of the rigor mutant would inhibit McTNs due to their inability to transport material. However, there were no significant differences in the McTN counts. Instead, differences in the length and thicknesses of the McTN could be observed in the two overexpression systems. Visualization of the two constructs by immunofluorescence displayed increases in Glu-tubulin levels in \(^{\text{GFP-Kinesin1}}\) mutant transfected cells but not \(^{\text{GFP-Kinesin1}}\) wildtype transfected cells (Fig 4.6). Attachment assays confirmed the increase in functionality of the longer McTNs in \(^{\text{GFP-Kinesin1}}\) wildtype and \(^{\text{GFP-Kinesin1}}\) mutant transfected MCF10A MECs. All observations corresponded with the greatest increases in length, Glu-expression, and attachment in the \(^{\text{GFP-Kinesin1}}\) mutant, followed by \(^{\text{GFP-Kinesin1}}\) wildtype,
and then the control. Our hypothesis for this effect is that $^{\text{GFP}}$Kinesin1 wildtype extends McTNs through increased trafficking and that $^{\text{GFP}}$Kinesin1 mutant increases McTN length through increased stabilization of Glu-tubulin.

Next, we used KIF5B siRNA to knockdown KIF5B expression, the Kinesin-1 that is expressed ubiquitously in non-neuronal cells. Partial KIF5B knockdown was accomplished in MCF10A MECs and complete knockdown in MDA-MB-436 BTCs. However, attachment assays of both cell lines showed that KIF5B knockdown only had partial reduction in attachment. An increase in siRNA concentrations did not increase the knockdown of KIF5B expression in MCF10A nor did it further reduce attachment. In conclusion, it is likely that KIF5B is not exclusively the kinesin involved in McTNs but may be only one of several. It is also likely that multiple kinesins play redundant roles due to their highly conserved homologies and indispensable functions of kinesins.

In summary, kinesins play a role in McTN function by stabilizing the Glu-tubulin-vimentin association that allow for increased McTNs and attachment of suspended MECs and BTCs. Lidocaine and tetracaine have the ability to inhibit kinesins and therefore, McTNs and attachment. Reduction of KIF5B, the dominant isomer of Kinesin-1 in MEC and BTCs, through siRNA allows for only partial inhibition of attachment (Fig. 5.1).
Central Hypothesis
Kinesin motor proteins play a key role in the formation and function of McTNs.

Aim 1:
-Kinesins are involved in McTN formation and function
J. Yoon et al., BCRT 2010 Nov 11.
↓ Kinesin mobility
↓ Vimentin stability
↓ McTNs frequency

Aim 2:
-Kinesins are involved in extending and stabilizing McTNs
J. Yoon et al., 2011 (in preparation)

Figure 5.1 Summary
VI. CONCLUSIONS

When epithelial cells are detached from a surface and become suspended, the cells must overcome anoikis through adaptive mechanism or quickly reattach to a surface to regain cytoskeletal balance and survive. MTOC alignment is achieved through the balancing forces of microtubule filaments against the actin cortex. However, when cells are detached, the actin cortex loosens reducing the opposing force against the microtubule filaments and allows for extension out of the cell body [98], [99]. These microtubule extensions are reinforced with vimentin IFs and are termed microtentacles [5], [15].

A. Kinesin Redundancies

In this thesis, we targeted kinesin-1 in McTNs due to its ability to preferentially bind Glu-tubulin, transport vimentin, bundle and slide microtubules, and proceed in an anterograde motion along microtubules [23], [86], [36], [58]. The KIF5 family consists of the three members KIF5A, KIF5B, and KIF5C to form the three isoforms of Kinesin-1 [27], [73]. The three KIF5 isoforms are highly conserved and appear to have some functional redundancies due to their roles in important cellular activities [73].

KIF5A and KIF5C are mainly expressed in neurons while KIF5B is ubiquitously expressed throughout the body [73]. The Kinesin-1 isomers have the ability to form hetero- and homodimers with one another and share some compatibility with various interacting proteins that mediate the docking of cargo [100]. The ability of kinesins to
preferentially bind to post-translationally modified microtubules, dimerize with one another, and share interacting proteins allows for increased control and variation [27], [73]. This includes the ability of kinesins to rescue mutations and knockdowns of kinesins with redundant roles. Supporting evidence comes from the ability of some kinesin-1 isoforms to rescue each other. In one experiment, abnormal mitochondria aggregation in kif5b-/- cell lines were rescued with the transfections of exogenous KIF5A, KIF5B, and KIF5C [73]. In our experiments, transfections with KIF5B siRNA showed partial (MCF10A) and full (MDA-MB-436) knockdown of protein expression, however; in the attachment assays, both cell lines displayed only a partial decrease in initial attachment (Figure 4.6, 4.7). This discrepancy between reduction in protein expression and attachment in the MCF10A and MDA-MB-436 cells is likely due to the actions of multiple kinesins that are not targeted by siRNA. Of course, not all kinesin functions can be replaced. The generation of KIF5B -/- null mice was found to be embryonic lethal due to KIF5B’s ubiquitous role in most cellular functions and very little co-expression of KIF5A and KIF5C in non-neuronal cells [73]. However, KIF5C -/- null mice survived and displayed decreases in motor neurons, but retained viability as well as normal levels of kinesin isoforms within specific brain structures [73].

**B. Alternative Actions of lidocaine and tetracaine Effects**

In this thesis, we addressed the possibility of kinesin motor protein involvement in the extension and function of McTNs. We began by using the kinesin inhibitors, lidocaine and tetracaine, to test this hypothesis and confirmed the inhibitory effects of the
anesthetics on kinesins in cells, McTN protrusions, and attachment of suspended MEC and BTCs [70]. However, lidocaine and tetracaine have many off target effects that have yet to be extensively explored.

Lidocaine is most commonly used as a local amide anesthetic to induce analgesic, antihyperalgesic [101], and anti-inflammatory effects [102]. This effect in part is due to lidocaine’s ability to block sodium channels [102], inhibit G-protein-coupled receptors [102], [103], and NMDA receptors [104], [105]. A study by Mammoto et al., suggests that lidocaine’s inhibitory effect on McTNs and attachment of suspended cells was not due to sodium channel blocks but in part to an inhibition of a wounding response. In this study, lidocaine reduced the ability of HT1080, HOS, and RPMI-7951 cancer cells to migrate and invade as tested with an in vitro invasion assay [52]. When a specific sodium channel inhibitor Tetrodotoxin (TTX) was used, TTX had little effect on the invasion ability of HT1080 cells. Interestingly, lidocaine’s ability to inhibit the wound induced shedding heparin binding epithelial growth factor (HB-EGF) in HT1080 cells was discovered.

HB-EGF is a member of the epidermal growth factor (EGF) family involved in cancer cell growth, wound healing, and tumor progression in human cancers [106], [107]. In recent years, HB-EGF has risen to be a promising therapeutic target for ovarian cancer [52]. Ovarian, gastric, bladder, breast cancers, malignant melanomas, and glioblastoma cells have high levels of HB-EGF mRNA and secrete large quantities of soluble HB-EGF [108], [109], [107]. Interestingly, suppression of HB-EGF using siRNA reduced the phosphorylation of EGFR and ERK, decreased soluble HB-EGF in the media, and induced apoptosis in MDA-MB-231 BTCs [107].
Increases in intracellular Ca\(^{2+}\) concentrations are also known to stimulate the shedding of HB-EGF from cell surfaces [110]. A Ca\(^{2+}\) ionophores stimulator, A23187, was used to treat HT1080 cells and with the application of A23187, HB-EGF was completely shed from the surface of HT1080 cells. However, with the application of lidocaine, this shedding was only partially reduced [52]. Curiously, lidocaine application had no effect on the induction of HB-EGF activity by tetradecanoylphorbol-13-acetate (TPA), another stimulator of HB-EGF shedding which works through the activation of protein kinase C (PKC) [111], [52]. Lidocaine effects were further tested using LM8, murine osteosarcoma cells, in C3H mice to test for successful metastases to the lung. Twice daily injections of 5-30mM of lidocaine in the inoculation site for four weeks was able to reduce lung metastases 50-90% [52].

Ca\(^{2+}\) is a multifunctional ion that mediates diverse processes within the cell such as the cell cycle, apoptosis, differentiation, motility, muscle contraction, and angiogenesis to name a few [112], [113], [114]. Ca\(^{2+}\) is highly regulated in normal cells but in cancer cells Ca\(^{2+}\) dependent signaling pathways are often hijacked and become implemented in cancer initiation, tumor formation, tumor progression, metastasis, invasion, and angiogenesis [115], [112], [113], [114]. One of tetracaine’s alternative actions is the ability to block Ca\(^{2+}\) channels. In isolated rat ventricular myocytes, low concentrations of tetracaine (0.25-1.25mM) blocked Ca\(^{2+}\) sparks with gradual increase in Ca\(^{2+}\) release in the sarcoplasmic reticulum [75]. High concentrations of tetracaine (>1.25mM) on the other hand inhibited all Ca\(^{2+}\) release [75]. Kinesin-1 itself does not possess Ca\(^{2+}\) binding sites but does appear to use Ca\(^{2+}\) sensors to regulate cargo [116], [117]. One example is
the regulation of mitochondria motility in neurons by coupling the Ca$^{2+}$ sensor, the Miro-Milton complex, to Kinesin-1 [118].

Lidocaine and tetracaine is a promising candidate in the treatment of cancer cell metastases. In addition to the inhibitory action on kinesin motor proteins, studies show lidocaine’s ability to reduce HB-EGF shedding, which has been implicated in cancer cell growth, wound healing, and tumor progression in human cancers [106], [107]. Inhibition of only HB-EGF was partially successful in reducing metastases in mice yet a much higher success rate of 50-90% is seen with lidocaine treatment [52]. Additional effects may come from lidocaine and tetracaine inhibitory effect on ionophore channels that help to regulate the trafficking of kinesin cargo in addition to the inhibitory effect on kinesin motion and HB-EGF shedding.

C. Kinesin Involvement in the Metastatic Process

In the tetracaine studies with suspended MEC and BTCs, tetracaine was a more effective inhibitor of kinesin motility, McTNs, and attachment [70]. This corresponds to tetracaine’s higher potency (LD$_{50}$ = 0.37 (0.08 mM for 24 hours)) than lidocaine (LD$_{50}$ = 9.23 (0.74 mM for 24 hours)), which correlates with the lipophilicity of the anesthetics [119]. Both lidocaine and tetracaine have successfully inhibited secondary metastases in mice when cells are pretreated before injection [51], [52]. Interestingly, lidocaine was only partially successful in inhibiting the ectodomain shedding of HB-EGF which reduced invasion, yet the reduction in the in vivo studies of secondary tumor colonizes in the lung was more effective [52]. This suggests that the mechanism of lidocaine and
likely tetracaine’s metastatic inhibition is due to multiple actions upon the cellular system. We have shown an alternative mechanism of action is the anesthetic’s ability to inhibit kinesin motility, disrupt vimentin coalignment with tubulin, reduce McTN frequency, and reduce attachment efficiency [48, 52], [70].

We attempted to address the effects of kinesin inhibition separate from other anesthetic actions by directing siRNA knockdown on KIF5B, the ubiquitous isoform of Kinesin-1 (Fig. 4.6). Partial knockdown was accomplished in MCF10A cells and complete knockdown in MDA-MB-436 cells (Fig 4.8). However, in the attachment assays, only a partial reduction in attachment is seen for both cell lines. This partial reduction is likely due to the functional redundancies of the Kinesin-1 isoforms and the compensatory mechanisms employed by the cellular machinery and/or incomplete knockdown as in the MCF10As.

With the discovery of dynamic McTNs in suspended MEC and BTCs, the importance of studying the changes that occur during the metastatic progression is highlighted. Cells once thought to passively adhere to the vasculature of the blood vessel walls have now been discovered to play an active role in facilitating the reattachment process of suspended cells [5], [15]. To date, several key components known to enhance metastatic efficiency and predictors of poor patient prognosis have been implicated in McTN structure and function. The main cytoskeletal structure of McTNs is composed of Glu-microtubules and vimentin intermediate filaments [5], [15]. Glu-tubulin, a stable form of tubulin that is not commonly expressed in cells, is a strong marker of patient poor prognosis [14] while vimentin is a marker of the epithelial to mesenchymal transition which leads to increased metastatic potential [17]. Tau is a microtubule stabilizing
protein linked to anti-cancer drug resistance, cancer proliferation, and increased cancer metastasis [81], [82]. Tau expression also correlates with increased McTN frequency, attachment, and metastatic potential in mice [84]. Continuing the kinesin overexpression and kinesin inhibitor study in mice is the likely next step and predicted to find correlation with high kinesin expression with that of high metastatic potential due to McTN expression.
VII. FUTURE DIRECTIONS

A. Additional in vitro studies

In this in vitro study, we would combine the knowledge of lidocaine and tetracaine’s ability to inhibit the shedding of HB-EGF and the genetic targeting of kinesins with siRNA. The effects of lidocaine were only successful in partially inhibiting the ectodomain shedding of HB-EGF, similar to the 50% effect seen with an EGFR neutralizing ligand [52]. However, pretreatment with lidocaine efficiently inhibited the occurrence of secondary metastases (50-90%) in an in vivo study using LM8 cells in mice [52]. Tetracaine was also noted to have similar inhibitory effects on low (F1) and high (F10) lung colonizing melanoma cells (B16) in C57BL mice [51]. Tetracaine has been shown to inhibit kinesin motility in an in vitro motility assay, GFP-fusion kinesins in cells, and inhibited McTNs which reduced attachment [70]. In the genetic targeting experiments with KIF5B siRNA, there was only a partial knockdown of KIF5B expression in MCF10A and a corresponding partial decrease in attachment (Fig. 4.6). However, we see a complete knockdown of KIF5B in MDA-MB-436 BTCs but again, only a partial decrease in attachment (Fig. 4.7). The effects of lidocaine and tetracaine on the MECs and BTCs may be an additive effective. To test this hypothesis, we would combine KIF5B siRNA and the HB-EGF inhibitor, CRM197, to inhibit the shedding on HB-EGF and monitor the reduction of attachment using the RTCA attachment assay.
B. Animal Studies

One possible direction to continue the kinesin studies would be to translate the in vitro studies to in vivo studies using the Xenogen IVIS-200 imaging system. The Xenogen system possesses that ability to detect bioluminescence and fluorescence through the tissue of living animals allowing for short-term studies as well as long-term studies. The Xenogen IVIS-200 system consists of a highly sensitive CCD camera held over a set stage that is enclosed in a chamber to exclude extraneous light. The CCD camera is highly sensitive and can detect up to a single photon within living tissue. Mice are anesthetized using isoflurane gas and up to five mice are able to be imaged on an isoflurane integrated stage (Fig 6.1).

Figure 6.1 Xenogen IVIS-200 Imaging System
In our mouse metastasis model, stable luciferase positive metastatic cells must first be generated using a PGL4 luciferase expression system in metastatic breast cancer cell lines. The cells would then be suspended, counted, and tail vein injected into athymic nude mice. Tail vein injections allow for the quick delivery of the stable, luciferase-expressing cells into the mouse vasculature allowing the cells to come to rest in the first extensive portal network they encounter, the lungs. This initial injection is followed up with an intraperitoneal injection of the substrate, luciferin, to initiate the bioluminescent reaction. Anesthetized mice are imaged within the first 10-15 minutes using the Xenogen system for initial trapping in the lungs after tail-vein injections. Cells within the lungs can then be followed through a time course of hours, days, or months to monitor retention and growth of tumors in live mice (Fig. 6.2).

**Tracking tumor cells with bioluminescence**

![Diagram of bioluminescence tracking](image)

**Figure 6.2 Tracking tumor cells with bioluminescence.**
Stable, luciferase-expressing cells are counted and injected into athymic nude mice. Mice are then anesthetized and injected with a follow up injection of luciferin and placed in the Xenogen system. The Xenogen system measures the bioluminescence given off by the luciferase-expressing cells.
Continuing kinesin inhibitor studies in an *in vivo* system would be an ideal next step to test the efficiency of tetracaine in reducing metastasis in a living model. Lidocaine has already been shown to possess the ability to inhibit HT1080, HOS, and RPMI-7951 cancer cells from invading a secondary target site [52]. Lidocaine was administered twice daily for four weeks to have successful reduction in metastases in the lung [52]. In an older study with tetracaine, successful reduction of lung metastases were seen when mouse melanoma cells were pretreated before tail vein injected into mice [51]. However, subsequent treatments of tetracaine were not administered after the initial drug treatment and extrapulmonary metastases were noted in many of the animals [51].

For our studies, tetracaine will be tested first in the animal model due to the stronger, positive results in the *in vitro* experiments. Preliminary animal studies indicate the need for a constant application of drug treatment as tetracaine is quickly metabolized in the liver. Alzet osmotic pumps are small, infusion pumps that can be implanted subcutaneously to continually deliver drugs at a set rate for up to six weeks (Fig. 6.3). Animals would be dosed prior to and during the initial tail vein injection and monitored for a predetermined time point before sacrificed and any visible tumors collected. Metastatic breast tumor cells treated with tetracaine generate less McTNs which is predicted to reduce initial trapping as well as retention within the lungs over time. Tetracaine is predicted to be an efficient inhibitor of breast tumor metastasis.
Future studies could also continue with the work on the $^{\text{GFP}}$ Kinesin1WT and $^{\text{GFP}}$ Kinesin1MUT overexpression system. This study would follow the format of a previously completed study which used stable, luciferase expressing GFP-MCF7Tau expressing clones. Tau is a microtubule-stabilizing protein with multiple splice variants, as well as; multiple phosphorylation sites used for regulation and affect the rigidity and stability of microtubules. The GFP-MCF7Tau overexpression studies showed a correlation between increased Tau mediated microtubule stabilization with increased McTN frequency, length, and attachment. MCF7Tau-luc and MCF7GFP-luc control cells were trypsinized, suspended in PBS, and tail vein injected into athymic nude mice. Both the MCF7GFP-luc control and MCF7Tau-luc cells showed efficient trapping within the initial scan. However, the MCF7Tau-luc displayed increased efficiency in trapping and retention in the lung capillary beds over the MCF7GFP-luc control population through a time course of eight days (Fig. 6.4) [84].
Tail vein injected MCF7tau-luc cells and $^{\text{GFP}}$MCF7-luc are efficiently trapped in the extensive capillaries of the lung, however the MCF7tau-luc cells show increased retention over the course of 8 days. [84]

The \textit{in vitro} effects on McTNs seen in the $^{\text{GFP}}$Kinesin1 studies are similar to the results seen in the MCF7tau-luc \textit{in vitro} studies which show an increase in McTN length and attachment. It is likely that $^{\text{GFP}}$Kinesin1 animal studies will follow closely with the observations seen in the MCF7tau-luc studies. However, another possible outcome may arise due to kinesin's role as key regulators involved in microtubule sliding and flexibility, as well as, in the trafficking of cargo required for cellular adhesion in the cell periphery. $^{\text{GFP}}$Kinesin1MUT stabilize microtubule filaments, however; and this stabilizing effect
inhibits protein trafficking and microtubule sliding. McTN protrusions exhibit a dynamic range of motion that could increase their ability to promote reattachment. McTNs possess the ability to maneuver in between cell-cell junctions to facilitate the extravasation process into the secondary tissue site [5]. In addition, if overall kinesin trafficking is inhibited by \textsuperscript{GFP}Kinesin1MUT expression, another possible prediction may be that the \textsuperscript{GFP}Kinesin1MUT will have a higher initial trapping of circulating cells over the \textsuperscript{GFP}Kinesin1WT due to the increased length in McTNs, but \textsuperscript{GFP}Kinesin1MUT would display decreased retention and growth over time due to the decrease in attachment efficiency as well as cargo trafficking (Fig 6.5).

Overall, the role of kinesins in McTN formation and function was demonstrated and its involvement during reattachment. Using the drugs, lidocaine and tetracaine, it was shown that kinesin mobility was inhibited, vimentin was destabilized, and McTN frequency was decreased in concentrations that are non-toxic in MEC and BTCs. Kinesin-1 was further targeted and MCF10A cells showed increase in attachment as well as McTN lengths and Glu-tubulin expression of transfected cells. However, siRNA studies demonstrate the conserved nature of kinesins due to their vital roles within the cell, as Kinesin-1 siRNA only partial reduced the attachment rate of suspended cells. Additional studies will need to be done to determine the value of non-mitotic kinesins as a potential therapeutic target in animal models of metastasis.
In future studies, Kinesin1WT and Kinesin1MUT inducible, luciferase expressing stables would be generated for \textit{in vivo} studies in mice.

**Predictions:**

- \textbf{Kinesin1 MUT} 
  - greater retention than Kinesin1 WT

- \textbf{Kinesin1 MUT \textit{in vivo}} 
  - decreased McTN mobility
  - decreased attachment efficiency
  - decreased trafficking of cargo

\textbf{Figure 6.5 Future Studies and predictions} 

In future studies, Kinesin1WT and Kinesin1MUT inducible, luciferase expressing stables would be generated for \textit{in vivo} studies in mice.
VIII. PROTOCOLS

Cell culture

Cell Culture Media

MCF10A Media
1. To 500 mLs of Dulbecco’s modified Eagle’s medium /F12 (Gibco), add:
   a. 5% horse serum
   b. insulin (5 µg/ml)
   c. EGF (20 ng/ml)
   d. hydrocortisone (500 ng/ml)
   e. penicillin-streptomycin (100 µg/ml each)
   f. L-glutamine (2 mmol/L)

MCF10A-Bcl2 Media
1. To MCF10A Media, add Puromycin (2 µg/mL) (Sigma).

CHECK Media
To DMEM, add:
   a. 5% fetal bovine serum
   b. penicillin-streptomycin (100 µg/mL)
   c. L-glutamine (2 mmol/L)

Passaging and Plating of human epithelial and breast tumor cell lines.
1. Remove old media with aspirator.
2. Wash cells with PBS before aspirating PBS.
3. Add 1 mL of Trypsin and incubate for 1 minute in the incubator before aspirating Trypsin.
4. Place plate in incubator and periodically check cells for detachment. To expedite detachment, gently tap on the side of the plate while rotating 90°.
5. Transfer 10 mLs of the corresponding media into a new 10cm plastic dish (Nunc/CellStar).
6. After trypsinized cells have detached from the plate, dilute out cells with fresh media and transfer the volume required for passaging or plating for experiments.

Transfections
Fugene-6 HD (Roche) Transfection 6-well
1. (Day 1) Split cells for ~50% confluency for the following day.
2. (Day 2) Change media into 1.5 mL fresh media without penicillin-streptomycin.
3. In a 1.5mL tube with 97 ul of Opti-Mem (Gibco), add:
   a. 3ul Fugene-6 reagent, vortex
   b. incubate for 5 minutes at RT
   c. Add in 2 ug DNA, vortex
   d. Incubate for 20 minutes at RT
   e. Add the entire content of the tube into 1 well of the 6-well plate in a dropwise manner
   f. Observe in 24-48 hours
ExGen500 (Fermentas) Transfection 6-well  
1. (Day 1) Split cells for ~50% confluency for the following day.  
2. (Day 2) Change media into 1.5 mL fresh media without penicillin-streptomycin.  
3. In a 1.5mL tube with 50ul of Opti-Mem, add:  
   a. 5.3ul Fugene-6 reagent, vortex  
   b. incubate for 5 minutes at RT  
   c. Add in 2 ug DNA, vortex  
   d. Incubate for 10 minutes at RT  
   e. Add the entire content of the tube into 1 well of the 6-well plate in a dropwise manner  
   f. Spin plate at 280xg for 5 minutes  
   g. Observe in 24-48 hours  

siRNA  
6-well  
1. (Day 1) Split cells for ~50% confluency for the following day.  
2. (Day 2) Change media into 2.3 mL fresh media without penicillin-streptomycin.  
3. In a 1.5 mL tube with 150 ul of Opti-Mem (Gibco), add:  
   a. 6 ul Fugene-6 reagent, vortex  
   b. Add in 0.6 ug DNA, vortex  
   c. Incubate for 5-10 minutes at RT  
   d. Add the entire content of the tube into 1 well of the 6-well plate in a dropwise manner  
   e. Spin plate at 280xg for 5 minutes  
   f. Observe in 24-48 hours  

Immunofluorescence  
Attached Immunofluorescence  
1. Grow cells on glass coverslips (12 mm diameter, Fisher cat: 12-545-82) in a 24-well plastic dish.  
   a. If treating with a drug, treat cells in phenol-free DMEM with or without the corresponding drug concentration(s) at the corresponding time point(s).  
2. To fix, incubate cells with 100% methanol for 10 minutes.  
3. Wash once with PBS.  
4. Incubate in 5% BSA/0.5% NP-40/PBS blocking buffer for 1 hour or overnight.  
5. Incubate in primary antibody at the optimal concentration for 1 hour or overnight.  
6. Wash coverslips 3x in PBS  
7. Incubate with secondary for 1 hour  
   Eg. a. mouse-anti-α-Tubulin-FITC (1:500; Sigma)  
      b. mouse-555 (1:1000; Sigma)  
      c. Hoescht 33342 (1:5000; Sigma)  
8. Wash coverslips with 3x PBS  
9. Mount coverslips on glass slides using Fluoromount-G (SouthernBiotech, AL).
Detached Immunofluorescence for Microtentacle Fixation

1. Coat coverslips with PEI (polyethyleneimine)
   a. On top of a small piece of flattened parafilm, place 50 ul droplet of 1% PEI.
   b. Place a coverslip on top of the drop and allow absorption to occur for 30 minutes
   c. Wash coverslips by placing coverslips, PEI-side up, into an ultra-low attachment 24-well tissue culture dish and rinse with PBS 5-7x
2. Transfer detached and separated cells into wells containing the PEI-coated coverslips
3. Add equal amounts of 2x fixation buffer and let stand for 10 minutes in room temperature.
4. Centrifuge the 24-well dish for 5 minutes at 18xG, accelerating and decelerating slowly.
5. Aspirate fixation buffer and deposit in proper waste container.
6. Wash coverslips 3x with PBS for 5 minutes each
7. While coverslips are being washed, prepare a fresh solution of NaBH4 (10 mg/mL) in PBS in the fume hood. Keep on ice. Bubbles should form.
   a. This step is essential to quench the autofluorescence caused by the glutaraldehyde.
   b. 200-250 ul solution is required per well
8. Place the 24-well tissue culture plate on ice and incubate for 7 minutes in the NaBH4 solution.
   a. Gently tap plate to release bubbles to ensure coverslips stay submerged.
9. Remove NaBH4 solution into proper waste containers
   a. Rinse with PBS and transfer PBS into NaBH4 waste container.
   b. Add more PBS before removing from fume hood.
10. Wash wells 3x in PBS for 5 minutes each.
11. Incubate coverslips in blocking buffer at room temperature for 30 minutes.
12. Incubate coverslips in appropriate primary antibody in
13. Wash coverslips 3x in PBS.
14. Incubate coverslips in appropriate secondary antibody for 1 hour in PBS.
   a. Protect the secondary antibody by wrapping all plates and tubes with aluminum foil.
15. Wash coverslips 3x in PBS.
16. Using a fine tweezer, remove coverslips from wells and gently remove excess liquid by blotting or aspirating from the edge.
17. Mount coverslips by placing a drop of Fluoromount G on a glass slide and placing, cell side down, the coverslip on top.
   a. Aspirate excess Fluoromount G from edges without pressing down on coverslips
18. Allow coverslips to set. 30 minutes for dry imaging and overnight for oil immersion imaging.

Solutions Recipes:
   1) Microtubule Stabilizing Buffer (MTSB)
      - 80 mM PIPES
- 5 mM EGTA
- 1 mM MgCl2
- dilute in ddH2O
2) Fixation Buffer:
   - MSTB with 0.3% glutaraldehyde and 0.5% NP-40 added fresh
3) Blocking Solution:
   - 10mg/mL BSA in PBS with 0.5% NP-40.

Epifluorescent imaging was done on an Olympus IX-81 inverted microscope mounted with a CCD camera and 100x lens. Images were acquired using Volocity software (ImproveVision Inc.; Waltham, MA).
Confocal images were done on an Olympus IX-81 inverted microscope mounted with a Fluorview 1000 system. Additional analyses of images were done on ImageJ.

**Westerns**
1. Grow cells to confluency in a 6-well plastic dish with or without treatment in phenol-free DMEM.
2. Harvest cells by washing with PBS and gently scraping in ice-cold RIPA lysis buffer.
   1) RIPA Lysis Buffer
      a. 50 mM Tris-HCl (pH 8.0)
      b. 150 mM NaCl
      c. 1% NP-40
      d. 0.5% sodium deoxycholate
      e. 0.1% SDS
      f. 1 mM phenylmethylsulfonyl fluoride
      g. 1% protease inhibitor cocktail (Sigma, P2714)).
3. To ensure total recovery of cells, collect all media and washes and pellet by centrifugation for 5 minutes at 300×G.
4. Lyse pellets in RIPA Buffer and combine lysates.
5. Incubate lysates on ice for 15 minutes with occasional vortexing before storing at -80° or proceeding with the Lowry-based assay.
6. Spin lysates(s) in a microcentrifuge at 14,000 rpms for 5 minutes at 4°.
   a. Transfer lysates to a new, labeled tube and place on ice. Dispose of pellet.
7. Protein concentrations were measured using a Lowry-based assay (Bio-Rad, Hercules, CA).
8. Load 20 µg – 30 µg of total protein on a polyacrylamide gels, NuPAGE Tris-Glycine or NuPAGE Bis-Tris gel (Invitrogen, Carlsbad, CA).
   a. Fill the reservoir of the gel box with Tris-Glycine running buffer for the Tris-Glycine gel and MES running buffer for the Bis-Tris gels.
   a. Run gel(s) at 125V until blue dye has reached the bottom of the gel.
9. Transfer protein onto an Immuno-Blot PVDF membrane (Bio-Rad, Hercules, CA) using a semi-dry, 1 hour at 25V, or a wet transfer apparatus, 75 minutes at 65V.
10. Block membranes in 5% milk in Tris-buffered saline (TBS) with 0.1% Tween for 1 hour at room temperature
11. Incubation membranes in primary antibody at 4°C overnight in 2.5% milk TBS plus 0.1% Tween 20 (TBST).
Eg.  
   a. Rabbit-PARP (1:1000; H-250, Santa Cruz)  
   b. Mouse-Vimentin (1:1000; C-20, Santa Cruz)  
   c. Mouse-Bcl2 (1:1000, BD Transduction)  
   d. Mouse-β-actin (1:1000; Sigma)

12. Wash membranes with TBST 3x  
13. Incubate membranes with the corresponding secondary antibodies.  
   a. IgG conjugated to horseradish peroxidase (HRP) were used (1:5000; GE Healthcare, Piscataway, NJ)

**Homotypic Aggregation Assay**
1. Grow cells to confluency in a 10 cm tissue culture dish.  
2. Treat cells with growth media containing Hoechst (1:5000) for 15-30 minutes.  
3. Trypsinize cells to detach.  
4. While cells are detaching, prepare the 1% methylcellulose media (Sigma M0512).  
   Eg.  
   a. Treat 0.4% methylcellulose media with 2× the concentrations of lidocaine or tetracaine.  
5. Place ~800 ul-1000 ul of the treated or untreated 0.4% methylcellulose media into an ultra-low attachment, 24-well plate (Costar; Corning, NY)  
6. Resuspend trypsinized cells in phenol-free DMEM (17-205CV Mediatech Inc) and count out cells using a hemacytometer.  
   a. Dilute out cells to ~500,000 cells/mL.  
7. Transfer an equal volume of the cell suspension the ultra-low attachment, 24-well plate using a 1mL 25G 5/8 latex-free syringe (309626 BD Syringe).  
8. Read wells at 365nm and monitor at 15 min intervals with a Hamamatsu CCD camera (Hamamatsu Photonics; Hamamatsu City, Japan) using Labworks EpiChemi3 image analysis and acquisition software (UVP, Inc.; Upland CA).

All images were processed by a background flattening algorithm in UVP Labworks, Image Acquisition and Analysis Software, v. 4.6, and exported to ImageJ (NIH, Bethesda, MD) for individual background normalization. Areas of interest were selected based on background-subtracted threshold values. Pixels with intensity values above this threshold were quantified as integrated density, a product of selected area and mean intensity value, and normalized to 0min vehicle control cells to show fold increase in signal over time.

**Solution Recipe:**
1) 1% Methylcellulose Media  
   a. 1% Methylcellulose (Sigma M0512) into phenol red free DMEM  
   b. Slowly add over low heat until methylcellulose has dissolved completely.

**XTT Viability Assay**
1. Grow cells to ~80% confluency in a clear bottomed 96 well plate (Costar; Corning, NY).  
2. Change media with 100μl phenol red free media treated with DMEM or DMEM treated with drug and incubated at 37°C for the corresponding time point.
3. Add 25 µl of XTT/PMS solution to each well and incubated at 37°C for 4 hours.
4. Observe reactions using a BioTek plate spectrophotometer at 450 nm to quantify color change.

**Solution Recipe:**
1) XTT/PMS solution
   a. 1 mg/mL of XTT salt (X-4626; Sigma) to phenol free DMEM
   b. Warm in the 37° bath until completely dissolved
   b. Before using, add 20µl 0.383mg/ml phenazine methosulfate (PMS) to each ml of XTT solution

**Real-Time Cell Attachment Assay**
Cell-substratum attachment was assessed utilizing the xCelligence RTCA SP real-time cell sensing device (Roche Applied Science).
1. Grow cells in their respective growth media.
2. Trypsinize and count cells to dilute out to a concentration of 1,000,000 cells/mL.
3. Aliquot out 100,000 cells in triplicate and mixed to 96-well electronic microtiter plates (E-plates) prepped as a blank, 2x vehicle control, or with 2x drug treatment.
   Eg. a. 6 mM lidocaine, 0.5mM tetracaine in growth media (2x the concentration).
4. Raw CI values from each measured time point and normalized to the maximum CI attained for the parental control cell line at 1 hour. Test cell lines were then represented as a percentage attachment of parental control.

**Microtentacle Scoring**
1. Grow cells up to ~50% confluency in a 6-well tissue culture plate
2. Transfected cells with ~1 µg/µl of AcGFP1-Mem (Clontech, Mountain View, CA), a green fluorescent protein (GFP) membrane plasmid that translocates to the cellular membrane using Fugene-6 or ExGen.
   a. Fugene-6, per well (11814443001; Roche)
      - 97 ul opti-MEM media
      - 3 ul Fugene-6, incubate for 5 minutes (do not let fugene-6 touch the side of tubes)
      -2 ug DNA, incubate for 20 minutes
      - change media in tissue culture dish with 1.5 mL fresh media and in a dropwise fashion, apply 100 ul of the DNA-Fugene-6 mixture into each well
   b. ExGen, per well (R0511; Fermentas).
      - 42.7 ul
      - 2 ug DNA
      - 5.3 ul ExGen, incubate for 10 minutes
      - change media in tissue culture dish with 1.5 mL fresh media and in a dropwise fashion, apply 100 ul of the DNA-Fugene-6 mixture into each well
3. Grow cells overnight and check for GFP expression the next day
4. Trypsinize cells and resuspended with DMEM or DMEM drug before transferring to a low attachment plate
5. Incubate cells at 37°C for 15 minutes before starting counts.

**Cell Mask orange (Invitrogen) C10045**

Cells are scored positive under conditions in which the cell exhibited two or more McTNs that were greater than the radius of the cell body. Populations of 100 or more GFP-cells were counted for each trial. Cell images were collected using an Olympus CKX41 inverted fluorescent microscope (Allentown, Pa) equipped with an Olympus F-View II 12-bit CCD digital camera system. Image acquisition and analysis was performed using the Olympus MicroSuite 5 imaging software.

**Live cell imaging**

**Live-cell Movies**

1. (Day 1) Split cells for ~50% confluency for the following day.
2. (Day 2) Transfected with ~2 µg/µl of DNA
3. (Day 3) Observe for GFP transfection efficiency
4. Captures movies at 100× magnification in a, humidified, temperature controlled chamber at 37°C in a Z-stack of 0.5 um slices at approximately one frame per 2-5 seconds

pGFP-KIF5C and pGFP-KIF5C mutant DNA was kindly provided by Dr. Michelle Peckham (Institute for Molecular and Cellular Biology, University of Leeds, Leeds, UK). Movies of GFP-particles were captured at 100× magnification and captured at one frame every two seconds and are shown with a 5× acceleration using an Olympus IX81 inverted microscope. Analysis and particle tracking were done using the Volocity software (ImproVision Inc.; Waltham, MA).

**Live-cell Movie w/partial attachment**

1. (Day 1) BSA coat glass bottomed dishes with filtered 2% BSA solution
   a. Remove BSA solution and allow dishes to dry, store in 4°C
2. (Day 2) Trypsinize and suspend cells in PF-DMEM
3. Allow cells to settle and attach for ~20-30 minutes in BSA coated plates
4. Image cells

**Propidium iodide (PI) (Sigma)**

1. Add Phenol Red Free-DMEM treated with PI
   a. (1:3000) Phenol Red Free-DMEM: PI stain
2. Trypsinized cells and resuspend
3. Score cells for positive PI staining

Imaging was performed on an Olympus CKX41 inverted fluorescent microscope. Counts were scored positive upon absorption of the PI stain.
**Drugs**

Tetracaine hydrochloride (Sigma) 0.5M stock solution in ddH2O  
- a. 250 mg of tetracaine powder  
- b. 1.662 ul of ddH2O, vortex

1. Filter solution using a 3 mL Luer-Lok Syringe (BD) fitted with a 0.22 uM filter tip.  
2. Aliquot solutions into 0.5 mL tubes and store in -80°C for no more than two months.

Lidocaine hydrochloride monohydrate (Sigma)  
- a. Weigh out 750 mg of tetracaine powder  
- b. Add in 519.35 ul of ddH2O and vortex

1. Filter solution using a 3 mL Luer-Lok Syringe (BD) fitted with a 0.22 uM filter tip.  
2. Discard unused solution

Latrunculin A (LA) (Biomol)  
5mM stock solution in ethanol.  
1. Add in 237.2 ul ethanol and vortex  
2. Aliquot solutions into 0.5 mL tubes and store in -20°C
IX. REFERENCES


