ROLE OF THE RELEASED FORMS OF L1-CAM IN BREAST CANCER CELL MOTILITY

By

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TABLE OF CONTENTS

LIST OF FIGURES

INTRODUCTION

MATERIALS AND METHODS

RESULTS

LIST OF ABBREVIATIONS

ADAM	A Disintegrin and Metalloproteinase
TEM	Transmission Electron Microscopy
CAMs	Cell adhesion molecules
Ig	Immunoglobulin
NCAM-L1	Neural cell adhesion molecule-L1
ERM	Ezrin-radixin-moesin
CHO	Chinese hamster ovary
MVBs	Multivesicular Bodies
BM	Brain Metastasis
FACS	Fluorescent-activated cell sorting
DMEM	Dulbecco's Modified Eagle's Medium
P/S	Penicillin/Streptomycin
BGS	Bovine growth serum
FBS	Fetal bovine serum
ATCC	American Type Culture Collection
HRP	Horseradish peroxidase
H-I serum	Heat inactivated serum
PBS	Phosphate buffered saline
PBST	PBS + Tween 20 solution
PTA	Phosphotungstic acid
kDa	Kilodalton

BLAST	Basic Local Alignment Search Tool
CE	Cell extract
ECM	Extracellular matrix
TX-100	Triton X-100
CO_2	Carbon dioxide
µg	Microgram
µl	Microliter
ml	Milliliter
M	Molar
mM	Millimolar
$CaCl_2$	Calcium chloride
$MgCl_2$	Magnesium chloride
NaCl	Sodium chloride
BBS	BES-buffered saline
$CaPO_4$	Calcium phosphate
RIPA	RadioImmuno precipitation assay
SDS	Sodium Dodecyl Sulfate-PolyAcrylamide Gel Electrophoresis
PVDF	PolyVinylidene Fluoride
EDTA	Ethylenediaminetetraacetic
dNTP	Deoxyribonucleotide triphosphate
1xTAE	Tris acetate and EDTA

ABSTRACT

L1-CAM (L1) is a neural cell adhesion protein which plays a major role in neural development. Recently, L1 is found to be abnormally expressed in several cancers and has been shown to be a biomarker indicating poor outcome and bad prognosis of the cancer. L1 is a transmembrane protein which is abnormally shed in cancer cells to release the cleaved ectodomain. The L1 ectodomain stimulates cell motility and migratory ability of ovarian, colon and other cancerous cells. L1 is also released by the cancer cells in the form of exosomes. Expression of L1 was found in few breast cancer cell lines and was also associated with aggressive nature of the breast cancer disease. However, the molecular mechanisms of L1 in breast cancer are not known. Breast cancer metastasis to the brain leads to very quick death. The extracellular environment in the brain contains L1 protein and the dissemination and metastasis of breast cancer cells in the brain could depend on the L1 expressed by the breast cancer cells. In order to understand the role of L1, I have characterized the presence of L1 in three breast cancer cell lines MDA-MB-231, MDA-MB-435 and MDA-MB-468. The amount of L1 expressed by the cell lines was correlated with their known metastatic potential. Having found L1 in breast cancer cells, I have performed experiments to find the role of the two released forms of L1, the soluble L1 ectodomain and the L1 released in the form of exosomes.

Overexpression of L1 ectodomain caused the MDA-MB-468 cells to flatten out more and they developed long cytoplasmic extensions. L1 ectodomain overexpression also led to an increase in the motility of both MDA-MB-468 and the MDA-MB-231 cells. Random motility analysis of MDA-MB-468 cells

overexpressing L1 ectodomain showed a statistically significant increase in the average velocity of about 45%, and MDA-MB-231 cells overexpressing L1 ectodomain showed a significant increase in the average velocity of about 18%. As MDA-MB-468 cells have very less expression of L1 protein, they have shown a greater change in cell motility due to overexpression of L1. However, scratch assay of MDA-MB-468 cells with L1 ectodomain overexpression showed an increase of only about 10% and there seemed to be a subpopulation of cells which were highly motile compared to the other cells along the scratch. These results show that L1 ectodomain can stimulate breast cancer cell motility. Attenuation of L1 expression using short hairpin RNA in MDA-MB-231 cells reduced the cell motility and random motility analysis showed a statistically significant decrease of 25% in the average cell velocity.

Exosomes were isolated from the breast cancer cells and they were confirmed as exosomes based on the presence of the exosomal markers TSG101 or CD9. To establish the role of exosomes in cell motility, exosomes from 231shL1 cells containing little L1 or exosomes from 231Ctrl cells were incubated with 231shL1 cells. Random motility analysis of 231shL1 showed a very high increase of about 80-100% increase in cell velocity with exosomes from 231Ctrl cells compared to the exosomes from 231shL1 cells containing lesser L1. This shows the importance of L1 in exosomes in stimulating breast cancer cell motility.

Overall, this study shows the importance of the two released forms of L1, soluble L1 ectodomain and the exosomal L1, in stimulating cell motility of breast cancer cells. It also unravels the importance of intact exosomes containing L1 in cancer cell motility, which has not yet been demonstrated in any of the

other types of cancerous cells releasing L1 in the form of exosomes. Therapeutic antibodies to L1 have already been tested in *in vivo* models showing reduced tumor growth in the case of ovarian cancer. With my current study of L1 in breast cancer, understanding the role of soluble L1 ectodomain and the exosomal L1 in breast cancer would take us a step further to possibly developing therapeutics antibodies to L1 to prevent breast cancer metastasis, in the near future.

CHAPTER 1

INTRODUCTION

Cell motility and migration are some of the key processes involved in cancer metastasis. Metastasis is the leading cause of death by cancer, but the molecular mechanisms of cancer metastasis are not clearly understood. During metastasis, cancer cells interact with the extracellular matrix to promote mechanisms by which they become highly motile and invade other tissues. L1-CAM is a cell adhesion molecule which has been found to be one of the molecular biomarkers associated with tumor progression and poor survival rates of several cancers including colon, ovarian, glioma, melanoma, lung and several others. This study aims at understanding the role of L1 in breast cancer metastasis and breast cancer cell motility.

1.1. L1: A neural cell adhesion molecule

L1-CAM (or L1) is the founding member of the L1 family of proteins. The four members of the vertebrate L1 family being: L1, Close homolog of L1 (CHL1), NrCAM and Neurofascin, NgCAM (chicken) (Schmid & Maness, 2008). The general structure of the different vertebrate and invertebrate proteins of the L1 family is shown below (**Figure 1**). The striking feature of the L1 family is the highly conserved short cytoplasmic domain of about 110 amino acid residues (Brummendorf et al., 1998).

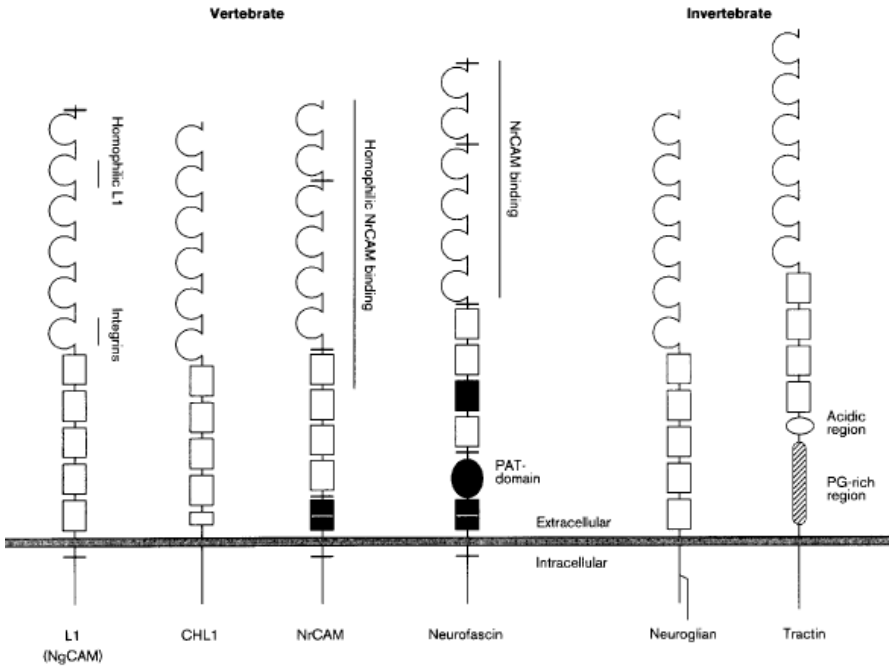

Figure 1 Schematic representation of L1 and other members of the L1 subfamily.

Vertebrate proteins on the left and invertebrate proteins on the right. Ig-like domains are indicated by horse-shoe shapes, and FNIII-like repeats by boxes. Amino termini are at the top. Horizontal bars and dark gray areas indicate alternatively spliced small segments and domains, respectively. PAT, proline/alanine/threonine-rich; PG, proline/glycine-rich (Brummendorf et al., 1998).

1.1.1. Structure and function of L1

L1CAM is a 200-220 kDa transmembrane protein. L1 has 6 extracellular Ig domains followed by 5 FN Type III repeats and a highly conserved cytoplasmic tail of about 110 amino acids (Hortsch, 1996; **Figure 2**). The different domains of L1 have different significant binding partners. The first Ig domain is essential for binding to neurocan, another cell adhesion molecule necessary for axon guidance and neurite outgrowth (Oleszewski et

al., 1999). The homophilic binding of L1 with other L1 molecules occurs via the second immunoglobulin domain. It is found that L1 promotes cell binding to integrins via the RGD sequence on the sixth immunoglobulin domain. Integrins α5β1, αvβ3, αvβ3 and others interact with the RGD domain (Oleszewski et al., 1999). Of all the fibronectin (FN) domains, the third FN domain spontaneously homomultimerizes to form complexes, which support RGD-independent interactions of L1 with the integrin $\alpha_9\beta_1$ (Silletti et al., 2000). L1 interacts with the actin cytoskeleton by the direct association of the L1 cytoplasmic domain with ankyrin, a spectrin-binding protein (Davis & Bennett, 1994).

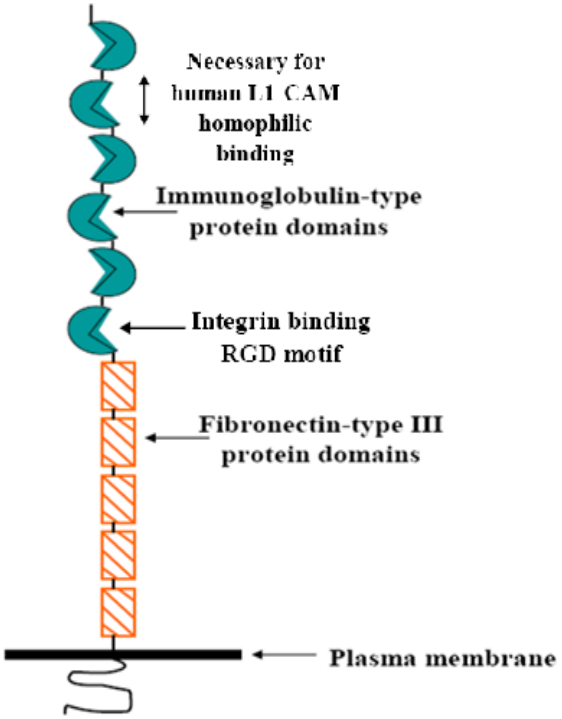

Figure 2 Structure of L1 (adapted from Hortsch et al. 1996)

The function of L1 during nervous system development is well characterized. L1 plays an important role in axonal guidance and neuronal outgrowth, cell-cell and cell-substrate adhesion, fasciculation and myelination (Hortsch, 1996; Schmid & Maness, 2008). Interaction of L1 with other neural cell adhesion molecules like NCAM is necessary for this process (Maness & Schachner, 2007). L1 is essential for learning and memory (Fransen et al., 1998) and also plays an important role in axonal regeneration and neural repair (Zhang et al., 2008). The full length L1 molecule, along with the extracellular ectodomain fragment of L1, are both involved in stimulating axon outgrowth. Limited proteolysis of L1 at the growth cones release L1 ectodomain to enhance the homeophilic L1-L1 interactions for promoting intercellular interactions and cell adhesion on the axonal surface.

1.1.2. Expression of L1

L1-CAM is primarily expressed in the peripheral and central nervous during development (Hortsch, 1996). L1 is expressed on the surface of axons and growth cones and also expressed by Schwann cells during development (Fransen et al., 1998). L1 is also expressed outside the nervous system in leukocytes (Kowitz, 1992), epithelial cells of the small intestine during development (Probstmeier et al., 1990) and non-proliferating cells of the urogenital tract (Kujat et al., 1995). Recently evidence suggests that L1 is overexpressed in several types of cancers in the tissues where L1 is normally not expressed. Types of cancers include glioma (Yang et al., 2009), colon cancer (Gavert et al., 2005), ovarian cancer (Euer et al., 2005), lung cancer

(Katayama et al., 1997), breast cancer (Shtutman et al., 2006) and others as well.

Figure 3 Splicing variants of L1. Exon 2 and exon 27 are spliced out in non-neuronal isoform (adapted from Shtutman et al., 2006).

L1 is expressed in two different splice variants: the neuronal and the non-neuronal form. Exon 2 in the transmembrane region and Exon 27 in the cytoplasmic domain are spliced out in the non-neuronal form (Jouet et al., 1995; **Figure 3**). Exon 27 has the RSLE sequence which is part of the tyrosine based sorting signal YRSLE which is present in the neuronal form and is required for the sorting of the protein to the axonal growth cone (Kamiguchi & Lemmon, 1998).

1.1.3. L1 Syndrome

The locus of L1CAM gene is present on the X-chromosome at locus Xq28 (**Figure 4)** and hence diseases related to L1 show an X-linked inheritance pattern. Mutations in L1 are known to cause four X-linked neurological disorders: X-linked hydrocephalus (HSAS), MASA syndrome, complicated spastic paraplegia type 1 (SP-1), and X-linked agenesis of the corpus callosum (Wong et al., 1995; Weller & Gärtner, 2001). They were combined into an acronym called CRASH syndrome based on the clinical symptoms, which are Corpus callosum hypoplasia, Retardation, Adducted thumbs, Spastic

paraplegia and Hydrocephalus (Fransen et al., 1995). The name CRASH syndrome seemed to be offensive and was changed to L1 Syndrome (Schrander-Stumpel, 1998). It is interesting to know that the severity of the L1 disease correlates with the domain in which the mutation is present. The mutations in the extracellular domain of L1 in the Ig or FN domains cause a severe phenotype including massive hydrocephalus causing prenatal death whereas most of the cytoplasmic mutations showed a mild phenotype like mild mental retardation (Fransen et al., 1998). Most severe is the premature truncation of the ectodomain which might be viewed as mimicking uncontrolled proteolysis of the ectodomain observed in several cancerous cells expressing L1.

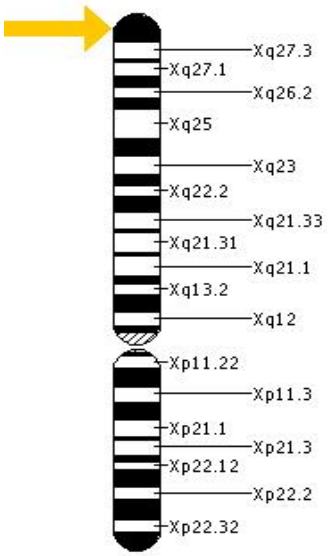

Figure 4 **Gene locus of L1 (arrow-Xq28) on X chromosome. (http://ghr.nlm.nih.gov/gene=l1cam).**

1.1.4. L1 Signaling

L1 interacts with various binding partners: homophilically and heterophilically with various ligands like - L1 itself, axonin-I/TAG-l, DM-GRASP, integrins, NCAM and phosphacan. Some of them are shown in **Figure 5**. Downstream signaling of L1 involves activation of the Src pathway (Maness & Schachner, 2007), sometimes mediated via integrins. L1 signaling activates the MAP kinase signaling pathway at the level of Raf and it was shown that inhibition of FAK, Ras, Raf and MEK impairs NCAM- and L1-dependent neurite growth (Kolkova et al., 2000).

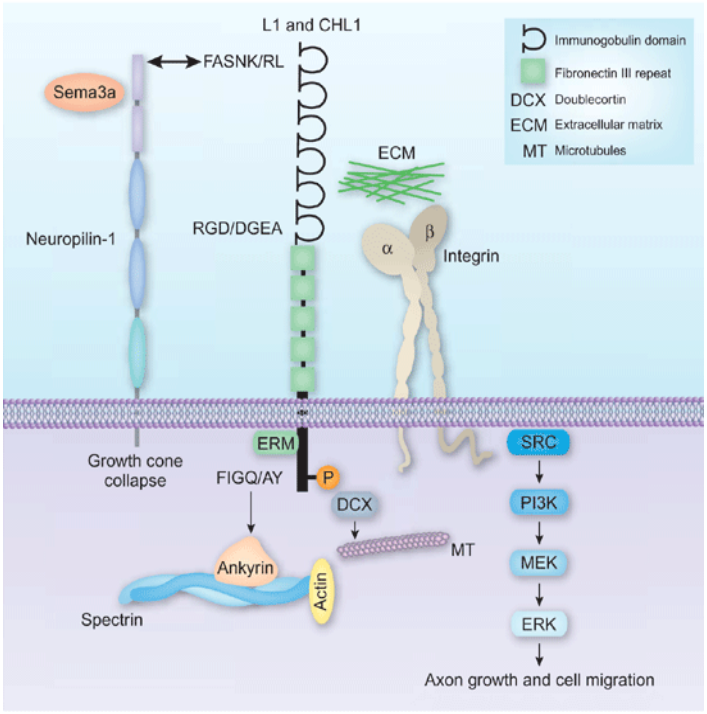

Figure 5 L1 downstream signaling (Maness & Schachner, 2007).

The cytoplasmic domain interacts with the actin cytoskeleton and the microtubules when the conserved motif (FIGQY) is tyrosine

phosphorylated. L1 is also shown to interact with FGFR via its third FN domain (Kulahin et al., 2008) and L1-mediated activation of the neuronal FGFRs induces neurite outgrowth (Walsh & Doherty, 1997).

1.2. L1-CAM in Cancer

L1CAM is overexpressed in several cancers including renal cancer (Allory et al., 2005), ovarian cancer (Zecchini et al., 2008), glioma (Yang et al., 2009) colorectal cancer (Gavert et al., 2005), abdominal-pelvic cancer of unknown primary site (Ben-Arie et al., 2008), lung cancer (Katayama et al., 1997), melanoma (Fogel et al., 2003) breast cancer (Shtutman et al., 2006). The expression of L1 in various cancers is associated with bad outcome and poor prognosis and corresponding to the more advanced stages of the disease and the same is the case in breast cancer as well (Raveh et al., 2009; Schröder et al., 2009).

The normal function of L1 is involved in cell adhesion, neuronal migration and axon outgrowth and fasciculation in the developing brain and PNS. In cancer, L1 seems to perform similar roles in cell-adhesion and cell-matrix interactions, which influence the cancer cell migratory abilities and metastasis, although the regulatory mechanisms controlling expression and proteolysis are abnormal. Mutation of the RGD site of L1 reduced cell motility, invasiveness and cell-cell adhesion. (Gast, et al., 2008). L1CAM correlates with the metastasis of renal cancer cells (Allory et al., 2005). Expression of L1 induces cell transformation, tumorigenesis and increased cell motility (Gavert et al., 2005). L1 also reduced apoptosis and increased cell proliferation and chemoresistance of ovarian cancer cells (Zecchini et al., 2008; Stoeck et al.,

2007). L1-CAM in cancerous cells promotes cell motility and inhibit apoptosis through ERK activation and thereby contributing to tumorigenesis (

Figure 6).

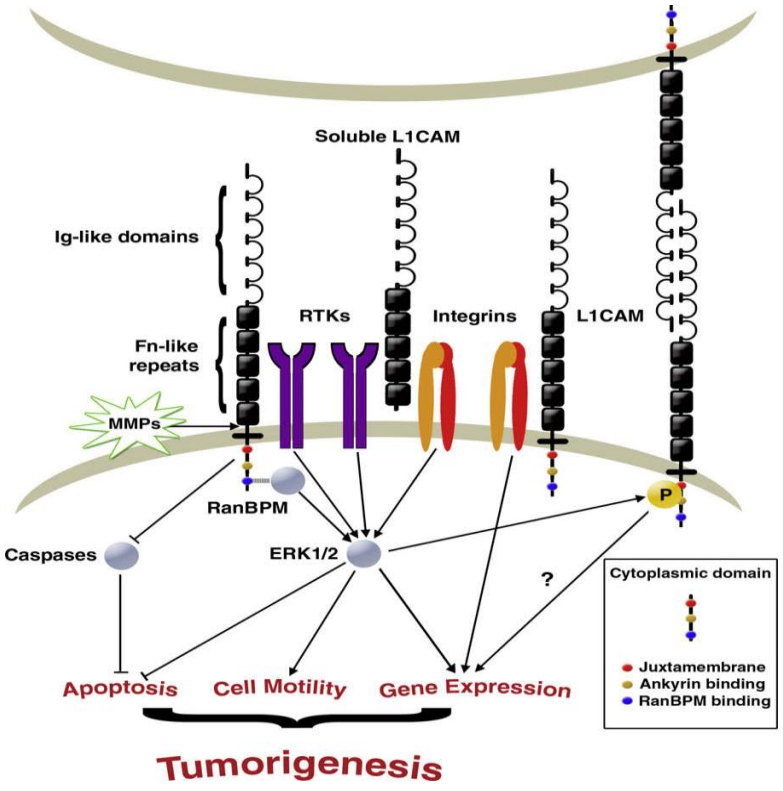

Figure 6 Functions of L1 in cancerous cells (adapted from Raveh et al., 2009).

L1-CAM interacts with the receptor tyrosine kinases (RTKs) and integrins, leading to ERK1/2 activation. L1CAM is often cleaved by MMPs and the shed, soluble L1CAM form binds to integrins, RTKs and L1CAM on the surface of the cells. The intracellular domain of L1CAM (insert) contains sub-domainsl: the juxtamembrane domain, the ankyrin binding domain and the Ran binding protein M (RanBPM) domain which interact with various partners to activate ERK, thereby promoting progression through the cell cycle, and inhibition of apoptosis via the caspases, or Erk pathways.

1.2.1. L1 Shedding

L1 is a transmembrane protein that is cleaved by ADAM10 (Mechtersheimer et al., 2001), which is a metalloproteinase. Cleavage by ADAM10 releases a 200kDa ectodomain, and a short cytoplasmic fragment is found in the membrane (**Figure** 7; Gutwein et al., 2003). The released ectodomain is one of the soluble forms of L1 that is free to leave the cell membrane and interact with the ECM or cell surface receptors. The process of cleavage of surface L1 is referred to as 'L1 shedding', which is an important phenomenon taking place in cancer cells.

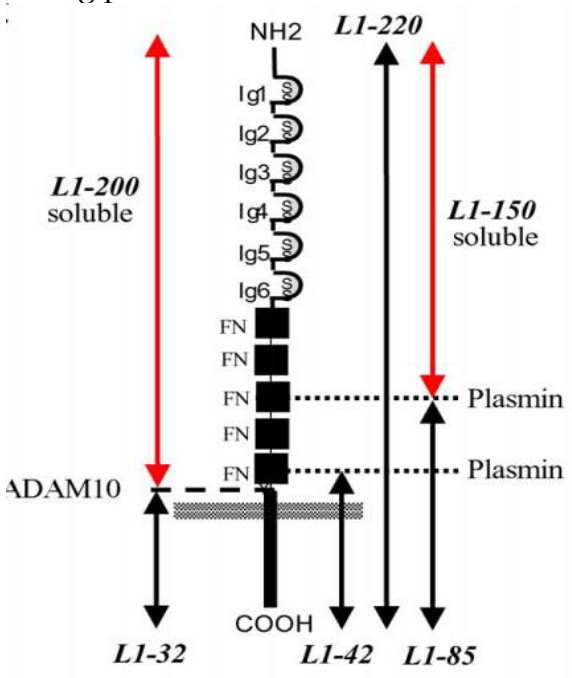

Figure 7 Cleavage fragments of L1 (adapted from Gutwein et al., 2003)

Altering the surface proteins is a way in which cells can communicate to the extracellular environment and interact with other cells. Membrane-proximal cleavage of L1 leaving a 32-kDa cytoplasmic tail in the membrane was detected in AR and SKBR3 breast cancer cells (Gutwein et al., 2000). Soluble L1 could stimulate L1-transfected CHO cell migration, which

was blocked by blocking with antibodies to integrins. Hence, the soluble ectodomain might stimulate cell migration by autocrine-paracrine binding to the integrins (Mechtersheimer et al, 2001).

1.2.2. L1 released in the form of exosomes

Another released form of L1 found in cancerous cells is the L1 in exosomes. Exosomes are tiny membranous vesicles of the size of about 40-100 nm released by different kinds of cells like reticulocytes (Pan et al., 1985), oligodendrocytes (Krämer-Albers et al., 2007), cancer cells including prostate cancer cells (Lehmann et al., 2008), breast cancer cells (Zhang et al., 2007) and many other cell types. Exosomes are formed by the invaginations of the membranes of the late endosomal multivesuclar bodies (MVB) (Sharples et al., 2008). It is proposed that exosomes play a very important role in intercellular communication (Niel et al., 2006). The endosomal sorting complex (ESCRT) and other chaperone proteins are involved in the packaging of the cargo proteins in the membranous vesicles and the protein composition varies based on the cell type (Niel et al., 2006; **Figure 8**). Tsg101, CD9, hsp70 and Alix are some of the packaging & chaperone proteins present on most kinds of exosomes and can be used as exosomal biomarkers (Guescini et al., 2010; Sharples et al., 2008).

Exosomes containing L1 were detected in ovarian cancer cells and ADAM10-mediated cleavage of L1 occurs in these vesicles (Keller et al., 2009; Gutwein et al., 2003). Similarly, exosomes released by glioma cells also showed the presence of full-length L1 and cleaved L1 ectodomain (Yang et al., 2009).

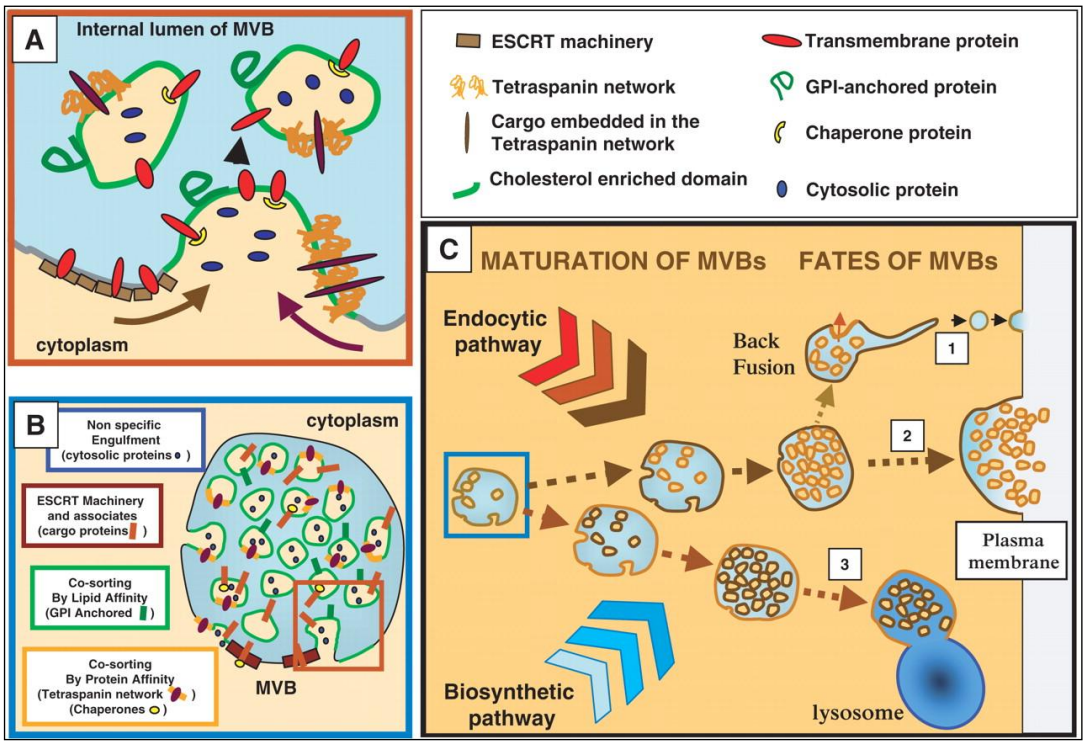

Figure 8 **Schematic representation of the biogenesis, maturation and release of exosomes (adapted from Niel et al., 2006).**

A: Sorting of transmembrane proteins, chaperones and cytosolic proteins occurs at the limiting membrane of Multivesicular Bodies (MVBs). **B**: Heterogeneity in the population of the inwardly budding vesicles in single MVBs is introduced by the presence of several sorting mechanisms acting separately on different domains of the limiting membrane. **C**: Different fates may be conferred on different subpopulations of MVBs depending on their composition of lipids and proteins: (1) Back fusion of the vesicles with the limiting membrane of the MVBs, in which molecules previously sequestered on the vesicles are recycled back to the limiting membrane and to the cytosol. This also allows plasma membrane expression of endosomal proteins. (2) Fusion of MVBs with the plasma membrane and consequent release of the vesicles in the extracellular medium as exosomes. (3) Fusion of MVBs with lysosomes leading to the degradation of the molecules sorted on the internal vesicles.

1.2.3. Previous work of L1 in breast cancer

L1 is not expressed in the normal breast tissue, neither in the epithelial nor the ductal cells (Huszar et al., 2006; **Figure 9**). Whereas in breast cancer cell lines like MDA-MB-435 (Huszar et al., 2006), AR breast tumor cells (Gutwein et al., 2003) and MCF7 cells (Shtutman et al., 2006), L1 is detected. The full length transmembrane L1 is found to increase the cell motility and scattering of the MCF7 breast tumor cells, whereas the soluble ectodomain did not stimulate motility in that study (Shtutman et al., 2006).

Table 1 L1 expression in normal tissues

		L1
Tissue		
Skin (20)	Epidermis	–
	Melanocytes	–
	Eccrine sweat glands: acini and ducts	–
	Apocrine sweat glands: acini and ducts	–
	Sebaceous glands and hair follicle	–
Breast (25)	Myoepithelial cells	–
	Ductal cells	–
Gastrointestinal tract		
Stomach (7)	Mucosa	–
Small intestine (5)	Lining epithelium	–
	Intestinal glands	–

Figure 9 L1Expression in normal tissues- breast has no L1 expression (adapted from Huszar et al., 2006).

L1 is present in 14-15% of the primary breast cancer cases, and high expression of L1CAM correlates with shorter disease- free and overall survival (Schröder, 2009). Expression of L1 was detected in the breast cancer cell lines MDA-MB-231 and MDA-MB-435, and L1 was shed in these cells and the 32

kDa cytoplasmic segment was detected using a western blot analysis (Adla, 2007). Although it is known that L1CAM could play a significant role in breast cancer like in other cancers, the molecular mechanisms are still unexplored.

1. 3.Breast cancer metastasis to the brain

Based on the recent Cancer Facts and Figures 2009, published by the American Cancer Society (ACS), breast cancer is the second leading cause of death by cancer, lung cancer being the primary cause. About 40,000 cancer deaths are predicted this year due to breast cancer. There is a possible diagnosis of 192,370 new cases of invasive breast cancer in women in the U.S., and 62,280 new cases of non-invasive (in situ) breast cancer. Brain metastasis of breast cancer occurs in 10-15% of the patients suffering from breast cancer (Tsukada et al., 1983; Boogerd, 1996). The survival of brain metastasis patients is very short and is in the range of 2-16 months (Weil et al., 2005). In about 20% of patients, a mean 1 year survival is seen from the time of diagnosis (Palmieri et al., 2007). This is a very dismal situation.

As mentioned earlier, L1 is present in about 14-15% of the breast carcinomas. But the significant mechanisms related to the role of L1 in breast cancer are still unknown. Metastasis of the breast cancer to the brain can be an interesting venue to explore. It was found 1% of the membrane protein in the brain is L1CAM. In an L1-rich environment, the presence of L1 on the breast cancer cells might influence their interactions with the ECM in the brain or with the L1 along axons and interaction in between cells expressing L1.

1.4. Hypothesis

Based on the background knowledge and previous work of L1 discussed so far, I have come up with the following hypothesis:

"**The shed ectodomain of L1 and the L1 embedded in the exosomal membrane contribute to the cell motility *in vitro* and metastasis *in vivo* in human breast cancer cells.**"

Figure 10 below shows the illustration of the hypothesis.

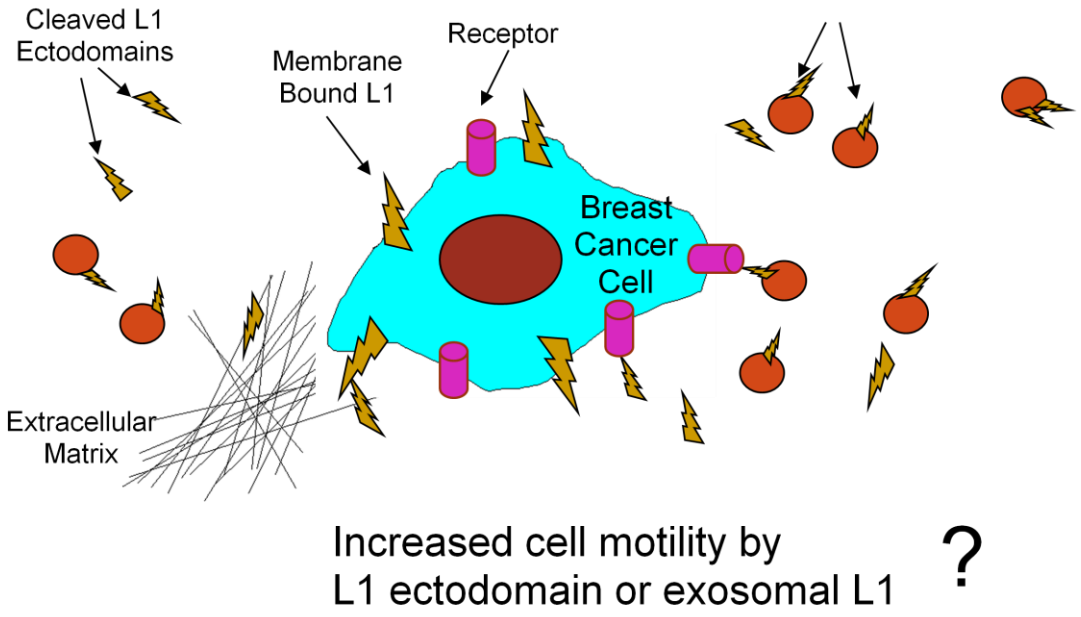

Figure 10 Schematic showing the two released forms of L1 possibly interacting with the receptors on the breast cancer cell.
1. Cleaved L1 ectodomains in the ECM and **2.** Exosomal L1 present on the membranes of exosomes in the ECM.

In order to explain the hypothesis further, L1 is released in two different soluble forms – shed L1 ectodomain and L1 present on the surface of exosomes (also referred to as exosomal L1) which might interact with receptors on the breast cancer cells (**Figure 10**). The goal of this study is to

investigate whether the two different soluble forms of L1 (shed ectodomain, exosomal L1) would contribute to changes in cell motility *in vitro* and if the shed ectodomain can modulate metastasis *in vivo.*

In order to address the hypothesis, these are the aims proposed:

- **Aim 1**: To characterize the function of L1 ectodomain in breast cancer cell motility *in vitro* and metastasis *in vivo.*
- **Aim 2:** To characterize the function of exosomal membrane L1 in breast cancer cell motility *in vitro*.

Specific Aims for Aim1: To characterize the function of L1 ectodomain in breast cancer cell motility *in vitro* and metastasis *in vivo.*

- Characterize L1 expression in highly metastatic MDA-MB-231, MDA-MB-435 and the less metastatic MDA-MB-468 cells.
- Overexpress L1 ectodomain in the MDA-MB-231 and MDA-MB-468 breast cancer cells.
- Attenuate L1 expression in the more metastatic breast cancer cells (MDA-MB-231) using shRNA knockdown lentiviral vector.
- Characterize the new cell lines using western blot analysis and FACS analysis.
- Perform time-lapse assays for cell motility analysis *in vitro*.
- Use chick embryo model to study the metastasis *in vivo.*

Specific Aims for Aim2: To characterize the function of exosomal membrane L1 in breast cancer cell motility *in vitro*.

- Isolate exosomes from the media of the breast cancer cells.

- Characterize the structure of exosomes using Transmission Electron Microscopy.
- Perform time lapse assays on 231shL1 cells after adding exosomal extracts from 231shL1 cells vs 231Ctrl cells.

CHAPTER 2

MATERIALS AND METHODS

2.1. Cell lines and culture

Breast cancer cell lines MDA-MB-231 (231), MDA-MB-435 (435), MDA-MB-468 (468) cell lines were used. The 231 cell line was obtained from Dr.Ulhas Naik (University of Delaware), who originally purchased it from ATCC (No. HTB-26). The 435 cell line was a gift from Dr. Danny Welch (Univ. of Alabama, Birmingham). The 468 cell line was obtained from Leslie J. Krueger (A.I. duPont Hospital for Children). HEK293T17 was obtained from Dr. Robert Sikes (Univ. of Delaware). All the cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM; MT-10-017-CV, Mediatech, Cellgro) with 10% BGS (Hyclone), 1% L-Glutamine stock (Cellgro; 2mM L-Glutamine), 1% penicillin/streptomycin stock solution (pen/strep; 10,000 IU/mL penicillin, 10,000 µg/mL streptomycin, CellGro).

2.2. Lentiviral Vector Construction

Vector 1879 was obtained from Dr. John Kappes (Univ. of Alabama, Birmingham). It consists of a Multiple Cloning Site (MCS) BamHI-SpeI-BclI-NheI-MluI-XbaI-XhoI. This vector also has the puromycin selection marker expressed from an internal ribosome entry site (IRES) (

Figure 11). The vector construction was done along with Yupei Li, another Masters student in the Galileo Lab. The L1 ectodomain (before the start of the transmembrane region) was inserted into the MCS by restriction

digestion to construct the "L1LE" vector. The primers with restriction enzyme cleavage site for SpeI or XhoI (NEB, Ipswich, MA) were used to amplify the L1 ectodomain fragment (3350bp) from pcDNA3.1-HL1 plasmid provided by Vance Lemmon (Univ. of Miami) (Sense: 5'-GAAACTAGTCGCCGGGAAAG-3'; Antisense: L1ED, 5'- GCCTCGAGGAGGGAGCC- 3').

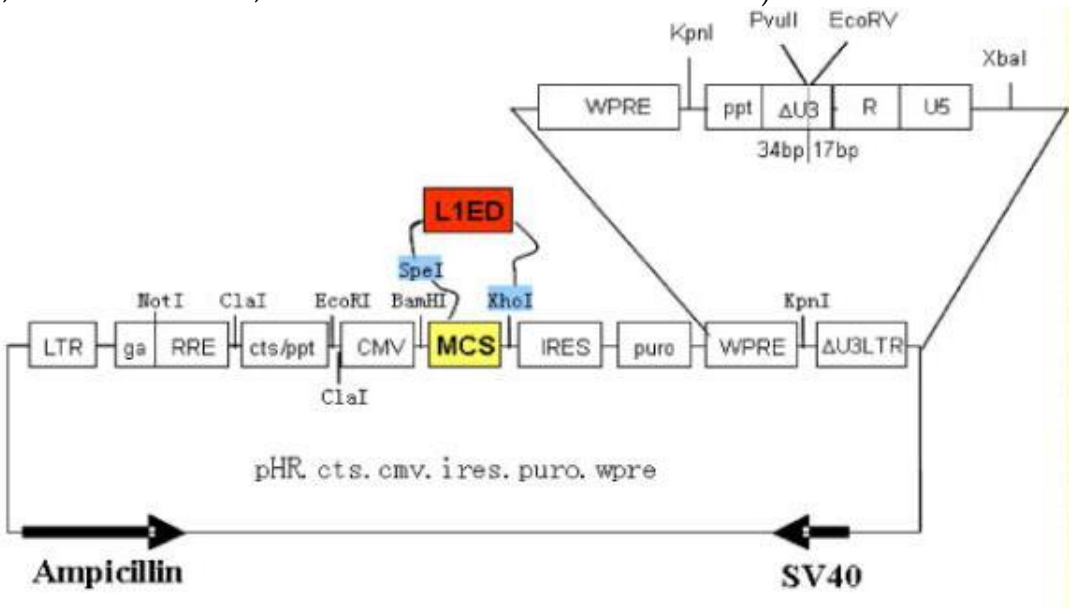

Figure 11 Lentiviral Vector L1LE (Li, 2010).

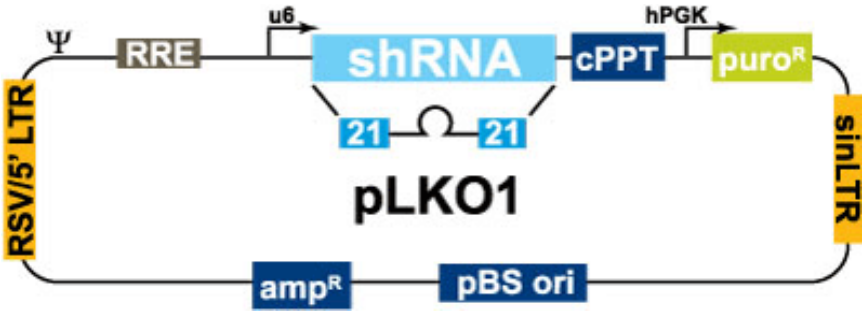

Figure 12 Lentiviral vector pLKO1 with L1 shRNA from Open Biosystems.

For L1 attenuation using short hairpin RNA, the L1-shRNA vector was used (**Figure 12)**. TRC shRNAs (Lenti) targeting human L1CAM, TRCN0000063917 (cat No. RHS3979-97052304) and the non-target shRNA control in the vector pLKO.1 were obtained from the company Open Biosystems (Huntsville, AL).

2.3. Lentiviral Infection

293T cells were grown to about 70% confluence on 10 cm dish in the incubator at 37^{o} C, 5% CO2. 293T cells were transfected with the vector construct (LE or the shL1 vector or the control 1879 vector) along with the helper plasmid pCMVΔR8.2 and the envelope plasmid pMDG in the proportion of 4:3:1 (20µg:15µg:5µg for cells in 10cm dish) via the CaPO4 transfection protocol modified from the procedure of Chen and Okayama, 1987). The plasmid DNA was added to 500µl of $CaCl_2$ (0.25 M), and then 500 µl of 2X BBS (pH 7.01) was added dropwise. The precipitate would turn cloudy after 8-10 minutes and then it was added to the cells. Fresh media is added to the cells the next day. Cells were allowed to recover for 24-48 hours before extracting the virus. 293 cell supernatant was collected and filtered through the 0.45 µm filter (which allowed viral particles to pass through). The viral supernatant was then added to the cell line to be stably infected. For this process of viral infection, polybrene is added to the viral supernatant to obtain a final concentration of 10 µg/ml. About 4 to 5 ml of the viral supernatant was used to infect a 10cm dish. The cells were incubated with the virus overnight and fresh media is added the next day. The following day, the infected cells were selected for puromycin resistance with 1µg/ml puromycin.

For my experiments, 231 and 468 cells were infected with the control 1879 and the L1LE vector to obtain 231Ctrl, 468Ctrl, 231LE and 468LE cell lines respectively. 231 cells were infected with the shL1 vector to obtain 231shL1 cell line.

2.4. Antibodies used in the study

The list of the different antibodies used for western blotting is as follows:

1. **UJ127** (Gene Tex, Catalog no: GTX72362, conc.: 200 µg/ml): A mouse monoclonal antibody raised against the ectodomain of L1. A dilution of 1:1000 was used. HRP-conjugated goat anti-mouse (Jackson Immunoresearch), 1:20,000 dilution was used as the secondary antibody.
2. **CD9** (C-4, Santa Cruz Biotechnology, Catalog no: sc-13118, conc.: 200 µg/ml): A mouse monoclonal antibody which recognizes the 101-210 amino acid region of human CD9 of human origin. A dilution of 1: 500 was used. HRP-conjugated goat anti-mouse (Jackson Immunoresearch), 1:3000 dilution was used as the secondary antibody.
3. **TSG101** (4A10, Catalog no: ab83, conc.: 1mg/ml): A mouse monoclonal antibody which is raised against the amino acids 167-374 of the TSG101 (tumor susceptibility gene) protein of human origin. A dilution of 1:1000 was used. HRP-conjugated goat anti-mouse (Jackson Immunoresearch), 1:20,000 dilution was used as the secondary antibody.

2.5. Western Blot Analysis

Cells were grown to about 80% confluence for protein extraction. Cells were lysed in RIPA Buffer(150 mM NaCl, 1% TX-100, 1% SDS, 0.5% NaDOC, 50 Mm Tris pH 7.5) to which protease inhibitor (PI, Protease Inhibitor Cocktail tablets, 1 tablet dissolved in 10 ml of PBS, Roche) was added, kept on ice for 10 minutes. The extract was subjected to sonication to further disrupt the cells.

Estimation of the protein concentrations was done by using the BCA protein assay kit (Pierce Biotechnology). Samples were prepared by adding equal amounts of protein, then mixed with Invitrogen NuPage 4X LDS sample buffer and the NuPage 10X reducing agent (500 mM dithiothreitol (DTT) and heating them at 70 ^{o} C for 10 min. Samples were centrifuged for 5 min. and then loaded into the NuPage 12-well/17-well 4-12% gradient polyacrylamide gels (Invitrogen). A mix of markers of SeeBlue and Magic Mark (both from Invitrogen) were loaded along with the samples. The gel was run for about one and a half hours at 100-120V in the MOPS Buffer (Invitrogen).

The gel was transferred onto a PVDF membrane overnight at 4^{o}C using a low voltage of 20VDC. The transfer of proteins was checked by using Ponceau stain to visualize bright pink bands. The membrane was kept in blocking solution (5% nonfat dry milk solution in phosphate buffered saline with Tween (PBST: 1X PBS + 0.01% Tween-20) for 2 hours with gentle skaking. The membrane was then incubated in the primary antibody for one hour at room temperature followed by three washes (10 minutes each) with PBST. The membrane was then incubated in a corresponding HRP-conjugated secondary

antibody of the appropriate dilution in the blocking solution for 30 minutes, followed by three washes in PBST (10 minutes each wash).

The chemiluminescence detection system (Pierce Biotechnology, catalog # 32106) was used. The films were exposed for about 30 seconds to 10 minutes in the dark room based on the intensity of the protein bands in the sample.

2.6. Flow cytometry (FACS)

FACS analysis was done on fixed and permeabilized cells. The cells were trypnisized in 0.05% Trypsin/0.2% EDTA and resuspended in DMEM + 10% H-I serum. After splitting each cell type into 3 samples in 1.5 ml centrifuge tubes (for only fixed cells, cells + primary antibody + secondary antibody, cells + secondary antibody only), they were centrifuged at 1000rpm (Beckman Allegra 6R centrifuge) and the cell pellet was resuspended in DMEM + 10% H-I serum.

Cells were fixed in 1% formaldehyde at RT for 30 minutes, followed by two washes with PBS + 5% BGS + 0.03% TX-100 by centrifugation, pelleting the cells and resuspending them in 1 ml of the solution at each step. Cells were incubated in primary antibody (1:100 dilution for UJ127) in PBS + 5%BGS + 0.03% TX-100 for an hour at room temperature. This was followed by two washes and then, the cells were incubated in the Alexa Flour 488 secondary antibody (1:200 dilution, Molecular Probes) in PBS + 5% BGS + 0.03% TX-100 for 45 min at room temperature. Followed by two washes, the cells were finally resuspended in 1 ml of PBS + 5% BGS + 0.03% TX-100. The sample was then added to a 5 ml sieve cap tube to filter out cell aggregates

and analyzed on a Becton Dickinson FACSCalibur flow cytometer for immunofluorescence using Cell Quest software.

2.7. Isolation of exosomes

Cells were incubated for about 18 hours in serum-free media, to which protease inhibitor was added (Protease Inhibitor Tablets, Roche, 1 tablet dissolved in 10 ml of the media). The supernatant was collected and filtered through 0.2 μm filter. The supernatant was processed by a series of differential centrifugation steps to isolate the membranous particles called exosomes. The supernatant was first centrifuged at 3000 x g for 10 minutes to get rid of the remaining cells. This was followed by centrifugation at 10,000 x g for 20minutes, and then an ultracentrifugation at 100,000 x g which was about 28,000 rpm using Beckman Coulter L8-55M Centrifuge for 20 hours at 4˚C using the SW28 rotor.

The exosomes isolated from a 10cm cell culture dish were finally dissolved in 100 μl of RIPA+PI for western blotting analysis and in 100μl of PBS+PI for TEM analysis or for use in the time lapse experiments. The supernatant remaining after the ultracentrifugation, the cell extracts and the exosomal extracts were tested for the presence of L1 using western blotting.

2.8. Transmission Electron Microscopy

Transmission Electron Microscopy is a technique used to visualize the structure of exosomes. The exosomal extracts isolated from MDA-MB-435 cells were spun onto the Cu grids (Electron Microscopy Sciences) using Beckman Coulter L8-55M Centrifuge for 2 hours at 30,000 rpm, 4˚C using the

SW41 rotor. The concentration of the exosomes was too dilute to find enough exosomes using TEM. Spinning onto the grids was done in order to concentrate and improve the adherence of the exosomes onto the grids. Phosphotungstic acid (PTA) is a phospholipid stain which provides negative staining to the exosomes. The grids were stained three times in a drop of PTA, followed by rinsing in PBS. The grids were allowed to dry and then visualized using the TEM microscope (Zeiss CEM 902).

2.9. Time Lapse Imaging

Scratch assays and random motility assays were performed to study the motility of the cells over a time period of about 24 – 48 hours time under the respective treatment.

2.9.1. Scratch Assays

For scratch assays, cells were grown to confluence in a 6-well tissue culture plate. Two scratches were made per well using a 1 ml pipette (blue) tip. In order to get rid of the dislodged cells floating in the dish, cells are washed three times in media with or without serum based on corresponding experiment. For 231 cells, experiments were done in serum-free media whereas 468 cells were tracked in regular media with serum. The reason being, 468 cells were very irresponsive and would not move at all in serum-free media. 3 ml of media was added to the wells after the washes and the dish was incubated in the time lapse microscope apparatus before the tracking of the cells began. The experimental setup is explained below (Section 2.9.3.).

2.9.2. Random Motility Assays

For random motility assays, only 20% of the tissue culture dish should be filled with cells. There should be enough cells to get data from one snapshot of the dish but at the same time, the cells should be far enough apart from each other so that they do not hinder each other's random movements. Hence, about $2X10^5$ cells were coated on each well of the 6 well-dish. Fresh media with or without serum was added to the cells based on the experiment. As mentioned earlier, random motility assays for the 231 cells were done in serum-free media whereas regular media was added to the 468 cells. 3 ml of media was added to the wells after the washes and the dish was incubated in the time lapse microscope setting before the tracking of the cells began.

Random motility assays were done after the exosomes are added to the cells in the 8-well chamber slide (Lab-Tek II Chamber Slide System). About 20% of the surface area ($0.7cm^2$) was to be covered by the cells. Hence, 40000 cells were seeded into each well of the chamber slide the previous night. When experiments were done using the 8-well chamber slides with 0.2 ml media, the lid of the dish was lined with petroleum jelly to prevent evaporation of the media in 24 hours.

2.9.3. Experimental Setup for Time Lapse Microscopy

Cells were tracked using a time-lapse microscope for analyzing their cell motility. The surface of the cell culture dish bottom was coated with a streak of rubber cement on the edges to prevent skidding of the dish on the stage of the microscope during automated stage movement. The culture dish was placed in a built-in chamber on the ProScan II automated stage (Prior

Scientific) on a fully automated Nikon TE-2000E microscope, which maintained a CO2 level of 5% through a gas injection controller (Forma Scientific). As the experiment ran over a period of 24-48 hours, the temperature was maintained through at 37° C warm air temperature controller (Air Therm; World Precision Instruments). The humidity was maintained by inserting two 35 mm dishes filled with water (at the end which was exposed to the warm air flow). The experimental setup is shown below (Fotos et al., 2006; **Figure 13**).

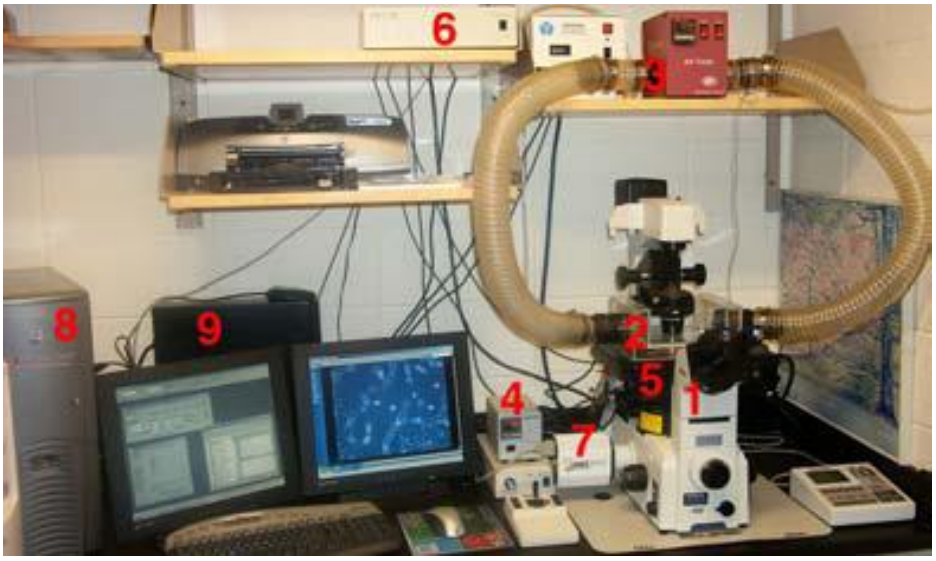

Figure 13 Components of the custom fully automated time-lapse microscopy system.

1) Fully automated Nikon TE-2000E with epifluorescence, 2) incubator chamber, 3) WPI temperature controller, 4) Tokai Hit stage insert warmer controller, 5) Prior ProScan II flat-top automated stage, 6) Prior stage controller, 7) Photometrics CoolSNAP ES CCD camera, 8) custom 3GHz computer with 2 gigabytes of RAM, 2 hard drives, dual monitors, and MetaMorph Premier software, 9) uninterruptible power supply capable of running entire system. Out of view is CO_2 injection system connected to incubator chamber via tubing (Fotos et al., 2006).

Two different spots on each well are selected for capturing the images. MetaMorph Premier Software (Molecular Devices Corporation) was used to create memory lists to store the desired positions on the dish where the images were to be taken every 5 minutes, captured by Photometrics CoolSnap ES CCD camera (Roper Scientific, Inc.). Images were collected using a Nikon CFI Plan ELWD DM 20X C Ph1 (correction collar 0-2 mm; 0.45 NA) objective lens (set at 20X) (Adla, 2007). A green filter was used inline for illumination for capturing the phase contrast images and the exposure time was either 150 or 200 ms. Using the journals in MetaMorph, the required objective, lamp voltage, storage location for the captured images were selected and recorded.

2.9.4. Analysis of Cell motility data

The resolution of the images first was converted to 800 X 600 dpi using XnView software. Movies were made from the stacks of the images. About 15 cells per treatment were selected for tracking the position using 'Track Points' tab in MetaMorph software. The paths of the individual cells were displayed and the data concerning the positions and the velocities of the cell at the various timepoints were stored in an excel datasheet for further analysis of finding out the average velocity, individual cell velocities. The details of the procedure were described in (Fotos et al., 2006).

2.10. Microinjection of Breast Cancer Cells into Chick Embryo Vasculature

For the chick embryo experiemts, fertilized White Leghorn eggs were obtained from the Department of Animal and Food Sciences at the

University of Delaware. The eggs were stored in the refrigerator until use. The day the eggs were placed in a wooden incubator maintained at 37^{o} C and kept humidified, is termed as Day 0 or embryonic day 0 or E0. They were incubated for 5 days and were taken out for injection on E5.

Breast cancer cells to be injected first were trypsinized in 0.05% trypsin/ 0.02% EDTA, counted on the hemacytometer and dissolved in media to obtain a final concentration of 5X10^{6} cells/ml. Fast Green (Sigma; 1% stock solution) was the visualizing dye used to observe the blue color of the cell solution while injecting them into the chick embryo. The volume of the stock dye added was 8% of the cell solution volume. Cells were kept on ice throughout the experiment.

The microinjection setup is shown below (**Figure 14**). The cancer cells were to be injected into the vasculature of the chick embryo. A glass micro pipette was used to inject the cells. The cells were filled into the tip using a pressurize microinjector (PV830 Picopump; World Precision Instruments) attached to a nanomanipulator (NM3D-25VP; Discovery Technology International). The joystick was used to move the nanomanipulator in the left-right, up-down or above-below (XYZ) directions. A "jabbing" knob was also present, which was used to puncture the blood vessel, if required. The pipette tip was broken using a forceps and it was usually made pointed to make it easy to puncture a hole in the blood vessel.

Nanomanipulator used to maneuver syringe attached to pressurized microinjector for injection into chick embryo vasculature. The

joystick is used to control movement of the syringe, which is pointing into the air hole at the top of an egg containing an E5 chick embryo (Texeira, 2009).

Eggs were sprayed with alcohol and then the area of the empty space inside the shell was marked by looking at the egg under a light. The top of the egg shell was cut off, a drop of media was added to make it easy for removing the top layer covering the vasculature without destroying any of the blood vessels. About 50,000 cells i.e. 10µl of the cell solution was injected into the blood vessel (**Figure 15**).

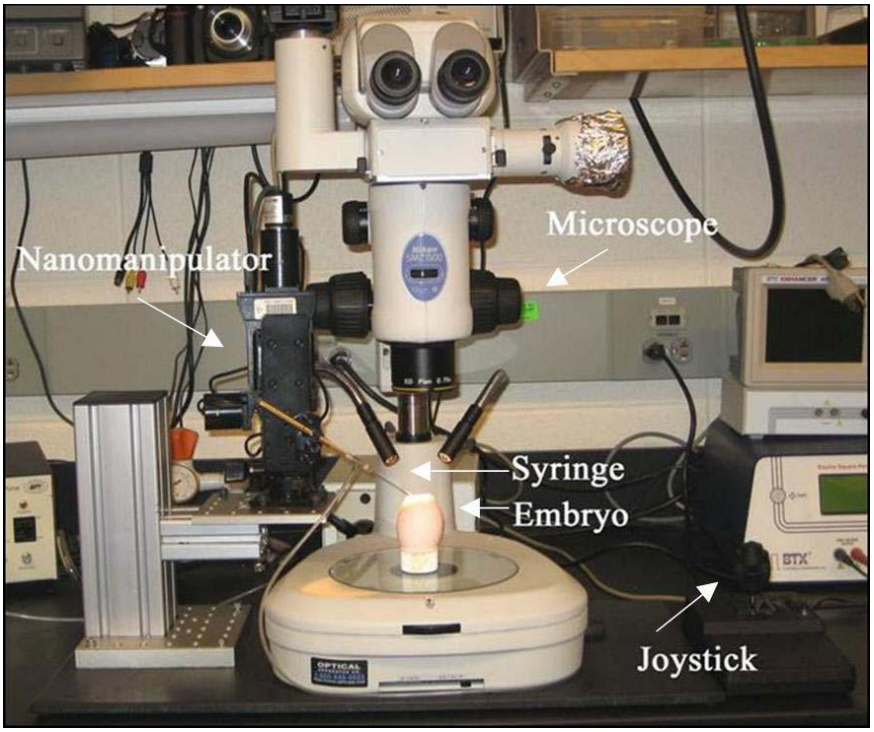

Figure 14 Microinjection Station.

An E5 chick embryo being injected with tumor cells colored with fast green. The embryo and blood vessels sit on top of the yolk at E5, allowing for injection into the extra-embryonic blood vessels (Hansen et al., 2006).

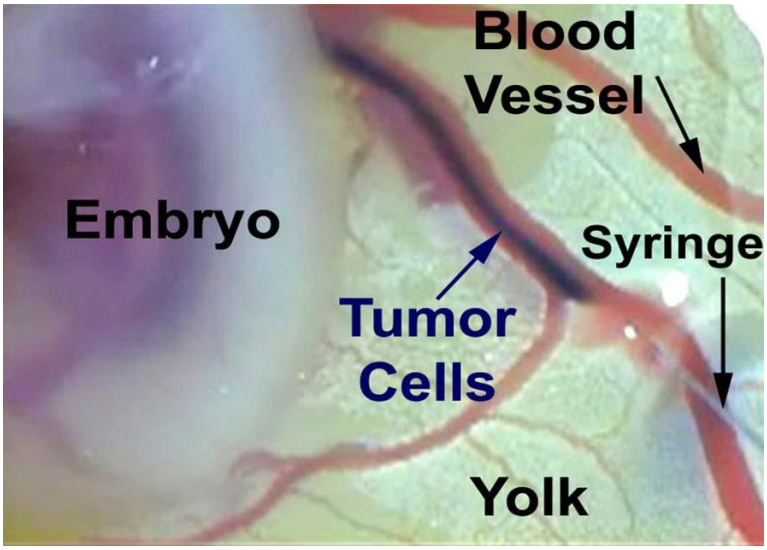

Figure 15 Injection of Cells into Chick Embryo Vasculature.

After injection of the cells, a drop of the antibiotic ampicillin (stock concentration: 50mg/ml) was added on the top of the vasculature to prevent any bacterial growth or contamination. Transparent tape was used to cover the opening to prevent drying up of the embryo in the egg. The embryos were placed back into the incubator for maturation until Day E9. Some of the embryos might bleed excessively while injecting the cells. They were taken note of, and they might have died few hours after injection. About 10-15 eggs were injected per experiment; the survival rate was about 30-50% of the embryos injected.

2.11. Recovery of Cancer Cells from Chick Brain

Embryos were sacrificed on E9 and brains were dissected to check the number of cancer colonies formed from the cells that have travelled through the vasculature to the brain. During brain dissection, the pia mater, the innermost layer of the meninges surrounding the brain was left intact.

Layers of the skin and the parts of the eyes were removed. Brains were dissected in 1x calcium and magnesium free Tyrode's saline solution (CMF) and then minced in fresh CMF using tiny serrated scissors.

The minced brain tissue was placed on ice immediately following dissection of all the embryos surviving from the experiment. They were centrifuged at 800 rpm for 5 minutes and the supernatant was removed. The tissue was further digested in 0.25% trypsin at 37^{o} C. After incubation in trypsin for 30 minutes at 37^{o}C, the digested tissue was kept on ice for 1 to 2 minutes. 2 ml of cold soybean trypsin inhibitor/DNase I (SBTI/DNase) was added to stop the trypsinization and also to digest the DNA that was released from lysed cells. It was then centrifuged again and the supernatant was removed. 2 ml of cold SBTI/DNase was again added, the tissue was triturated about 20 – 25 times using a pipette bulb, to release single cells from the suspension. The cell solution was then plated on a 10cm culture dish to allow the cells to grow and adhere for one day.

The selection marker in the cells is puromycin due to infection and expression from the viral vector. Puromycin was added to the cells to achieve a final concentration of 1 μg/ml concentration. While the chick brain cells died in 4 to 5 days, the breast cancer cells would survive the selection procedure.

CHAPTER 3

RESULTS

3.1. L1 expression in breast cancer cells

L1 (L1CAM, CD171) is present in several cancers including glioma (Yang et al., 2009) colon cancer (Gavert et al., 2005), ovarian cancer (Euer et al., 2005), lung cancer (Katayama et al., 1997), breast cancer (Shtutman et al., 2006), melanoma (Fogel et al., 2003), endometrial cancer and several others. Breast cancer metastasis to the brain usually causes death in breast cancer patients in few months to less than a year of the beginning of brain metastasis. It is important to study the molecular markers which could prove to be of therapeutic use against the advent of brain metastasis. L1 is not yet completely characterized in breast cancer and the mechanisms underlying L1 function in breast cancer have not yet been unraveled. The first step is to check the expression of L1 in several breast cancer cell lines.

The three different breast cancer cell lines we have access to in our lab are MDA-MB-231 (referred to as 231), MDA-MB-435 (referred to as 435) and MDA-MB-468 (referred to as 468) cells. Western blotting was done to probe for the presence of L1 in the cell extracts of each of these breast cancer cell lines using Method described in **Section 2.5**.

UJ127 was the monoclonal antibody used that binds to L1 on the ectodomain region and gives of a band of ~ 200kDa on the western blot. β-tubulin was used as the internal loading control, which is a housekeeping

protein present in presumably equal amounts in all the cells. The antibody against β-tubulin produces a band of about 50kDa (**Figure 16**).

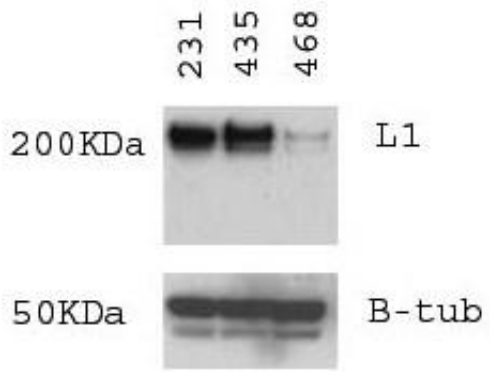

Figure 16 L1 expression in breast cancer cells.

Cell extracts of MDA-MB-231 (231), MDA-MB-435 (435), MDA-MB-468 (468) were probed against UJ127 antibody which recognizes the ectodomain region of L1CAM. β-tubulin is the housekeeping protein used as internal loading control.

231 and 435 cells clearly expressed larger amounts of the L1 protein compared to the 468 cell line. Interestingly, it is also known that both the 231 and the 435 cell lines are highly metastatic compared to the 468 breast cancer cell line. The high expression of L1 corresponds with the high metastatic potential of the breast cancer cell lines (231, 435), whereas L1 expression is very low in the less metastatic 468 cell line.

3.2. Lentiviral vector design to overexpress L1 ectodomain

The 435 cell line has an underlying controversy that it could be a melanoma cell line (Rae et al., 2007). Although there is recent evidence that MDA-MB-435 and M14 melanoma cell lines are similar in the expression of

certain genes but they are different cell lines of different origin (Chambers, 2009), I have decided to perform further experiments using the 231 cell line which was used as the model for the highly metastatic breast cancer cell line and 468 was the less metastatic breast cancer cell line. The L1LE vector is used to overexpress the L1 ectodomain in both the cell lines. Increasing the amount of L1 in 468 cells (which express very little L1) might alter the behavior of the cell line by increasing motility. Though 231 cells already express a substantial amount of L1, it might not be at the saturating level and, therefore, increased expression might result in increased motility. Hence both cell lines were stably infected with the L1LE vector or the control vector with the lentiviral backbone without the L1 ectodomain but having the puromycin resistance marker.

The L1LE vector was first tested by infecting 293T cells and the positive cells were selected using puromycin to create the 293T-LE cell line overexpressing L1 ectodomain (**Figure 17**).

Figure 17 LE vector validated by infecting 293T cells and probed with UJ antibody to detect L1 ectodomain.

Another potential modification is to knockdown the expression of L1 in the 231 cells. To achieve this, the short hairpin lentiviral vector L1shRNA from Open Biosystems was used. The vector has puromycin resistance

marker. The notation used for all the different cell lines made by infecting the 231 and 468 cells infected with the LE / shL1 vectors is shown in **Figure 18**.

Cell line	Description
468LE	468 cells infected with L1LE vector
468Ctrl	468 cells infected with 1879 vector
231LE	231 cells infected with L1LE vector
231Ctrl	231 cells infected with 1879 vector
231shL1	231 cells infected with L1shRNA vector

Figure 18 Different cell lines made for the experiments

3.2.1. Western blot showing L1 expression in the stable cell lines

Figure 19 shows the L1 expression in the 231shL1 cells, which was clearly much less than the normal 231 cells and 231Ctrl cells. Simliarly, 231LE cells expressed great amounts of the L1 ectodomain. The figure also shows tubulin internal loading controls.

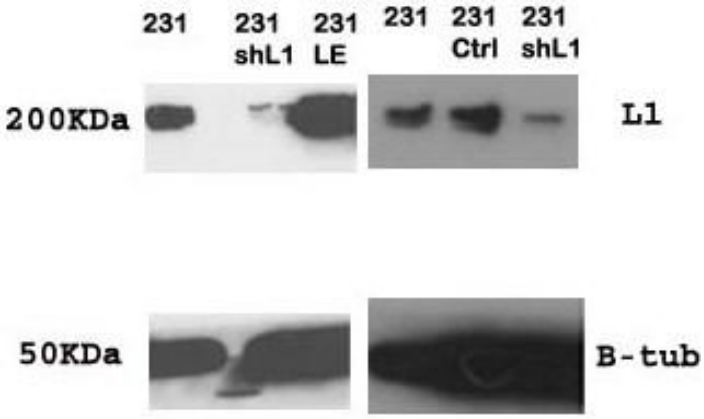

Figure 19 WB showing L1 expression in 231shL1, 231LE, 231Ctrl compared to the regular 231 cells.

Similarly, 468LE cells expressed very large amounts of L1 compared to the normal 468 cells. Whereas, 468Ctrl cells expressed similar amounts of L1 as the 468 cells (**Figure 20**).

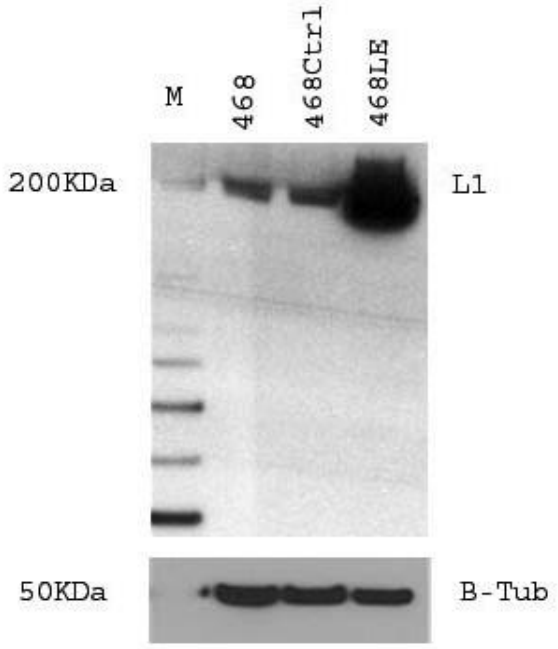

Figure 20 L1 expression in 468 breast cancer cells.
Cell extracts of 468, 468Ctrl, 468LE were probed against UJ127 antibody. β-tubulin is the housekeeping protein used as loading control. (M-Marker)

3.2.2. FACS analysis of the cell lines

FACS analysis using the UJ127 antibody was done for 468 cells, 468Ctrl cells and 468LE cells. The samples were either fixed plain cells (referred to as 'cells'), probed with the secondary antibody only without the addition of the primary antibody UJ127 (referred to as 'sec only'), both of which served as controls for the fixed cells probed with UJ127 followed by the secondary antibody (referred to as "UJ") (**Figure 21**). The 468LE cells clearly expressed a larger amount of L1 compared to 468 and 468Ctrl cells.

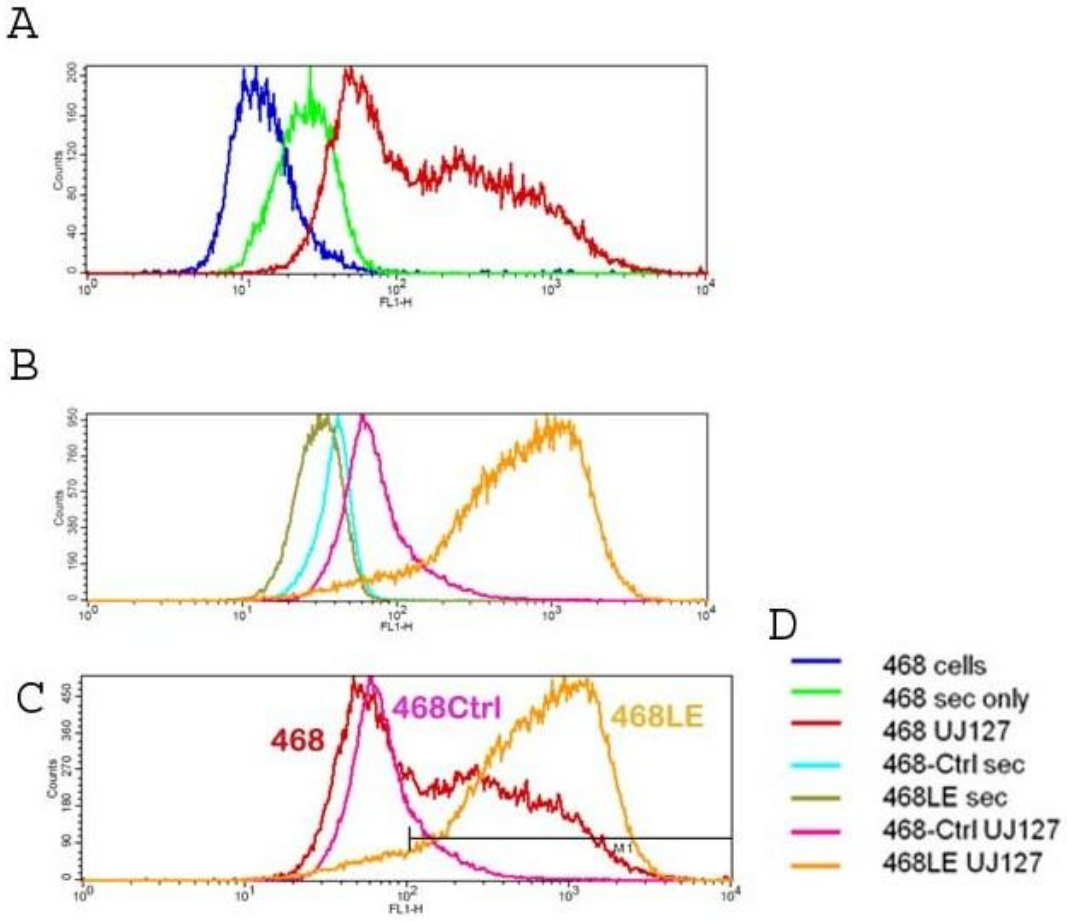

Figure 21 FACS analysis with UJ shows different amounts of L1 on 468, 468Ctrl and 468LE cells.

A. The top panel shows a positive shift of the fluorescence of 468 cells with UJ compared to the plain cells and the secondary only controls. **B.** 468Ctrl and 468LE show a positive shift and indicate the presence of L1 compared to their no primary (secondary only) controls. **C**. Collage of the 468, 468Ctrl and 468LE cells incubated with UJ. The 468LE cells clearly express a large amount of L1 compared to 468 and 468Ctrl cells. **D**. Legend.

FACS analysis using the UJ127 antibody was done for 231 cells, 231shL1 and 231LE cells. The same notation is used as mentioned above for the samples referred to as 'cells', 'sec only' and 'UJ'.

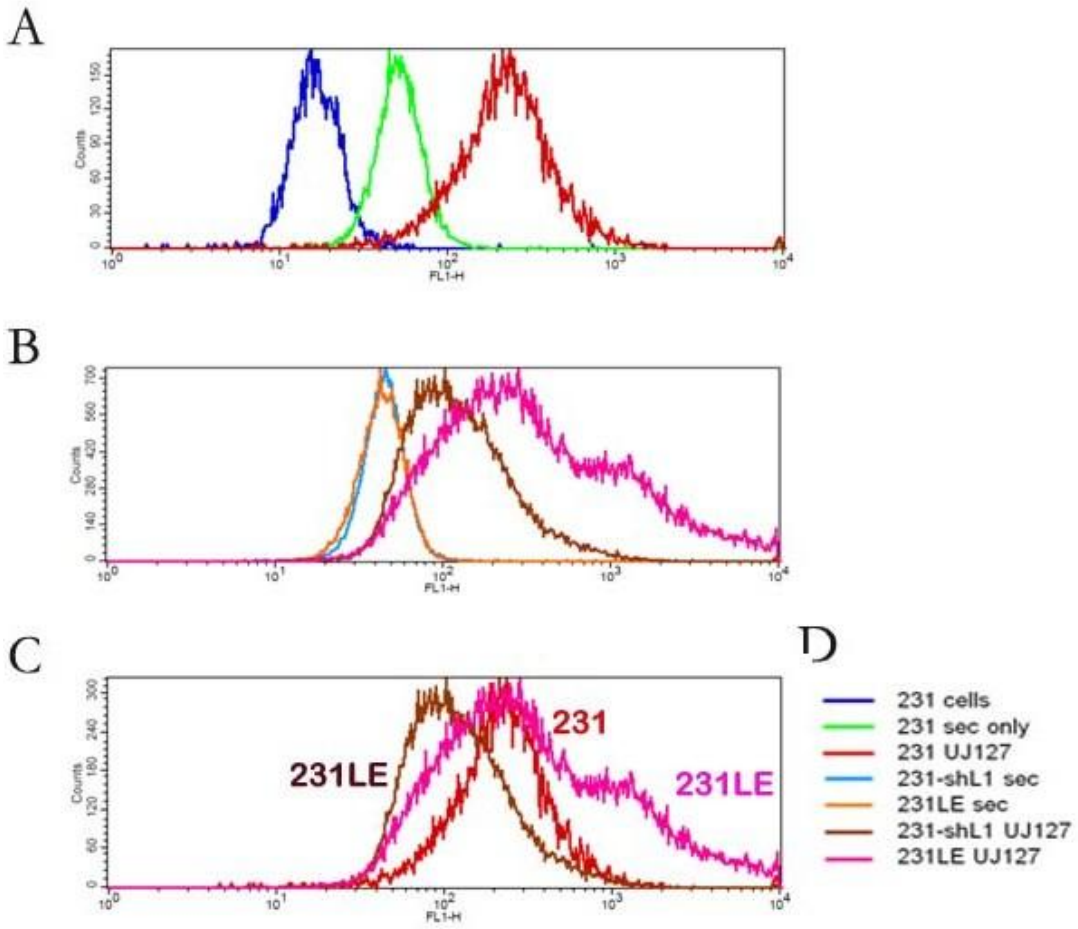

Figure 22 FACS analysis with UJ shows different amounts of L1 on 231, 231shL1 and 231LE cells.

A. Shows a positive shift of the fluorescence of 231 cells with UJ compared to the plain cells and the secondary only controls. **B.** 231shL1 and 231LE cells show a positive shift in fluorescence and indicate the presence of L1 compared to their no primary (secondary only) controls. **C.** Collage of the 231, 231shL1 and 231LE cells incubated with UJ. **D.** Legend.

The 231shL1 cells UJ express lesser L1 than 231cells and the peak of 231shL1 is shifted to the left of the peak of the 231 cells (**Figure 22B**). And 231LE express a larger amount of L1 compared to 231 cells . Hence, the 231LE, 231shL1 cells show significant changes in L1 expression compared to the

normal 231 cells and can be used for further motility studies (**Figure 22C**). FACS data for the 231Ctrl cells compared to the regular 231 cells along with their secondary only (no primary antibody) controls is shown in **Figure 23.** Expression of L1 in 231Ctrl cells is similar to that of 231 cells.

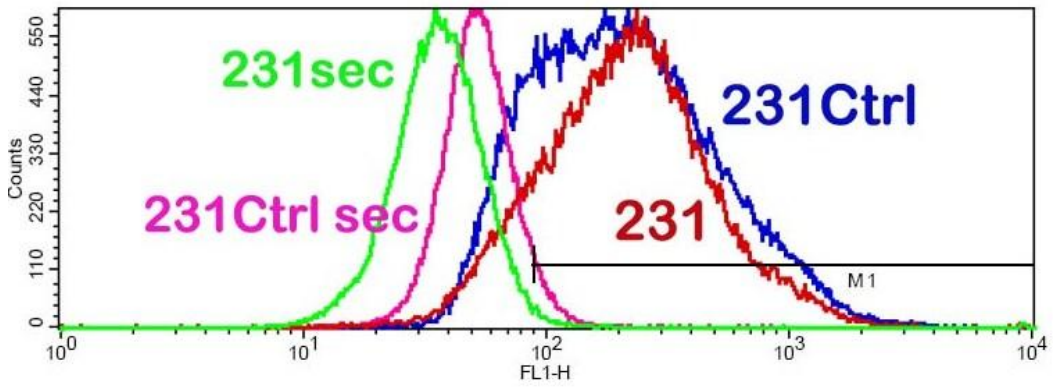

Figure 23 FACS analysis with UJ shows L1 on 231 and 231Ctrl 231LE cells along with the secondary only controls.

3.3. Effect of L1 overexpression on 468 cell motility

468LE cells had a distinctive more spread-out morphology compared to the normal 468 cells and appeared to be flattened out. Qualitatively, the cells seemed to develop long processes or extensions. As hypothesized earlier, L1 could play a role in cell motility. Hence, the cell motility of 468LE and the 468Ctrl cells was analyzed for 24 hrs using the random motility assay.

3.3.1. Random motility of 468LE compared to 468Ctrl cells

Fifteen cells were tracked for random motility analysis over 24 hours. All the cells in a particular dish did not have the same motility in any particular experiment. Some cells were more rounded through the entire

course of the experiment, while some of the cells developed long cytoplasmic extensions, helping them to move about longer distances and more quickly. Hence, the cells, which were inactive during the course of the experiment were avoided from the analysis. The 468Ctrl cells and the 468LE cells, which were tracked are shown in **Figure 24** (their tracks in red).

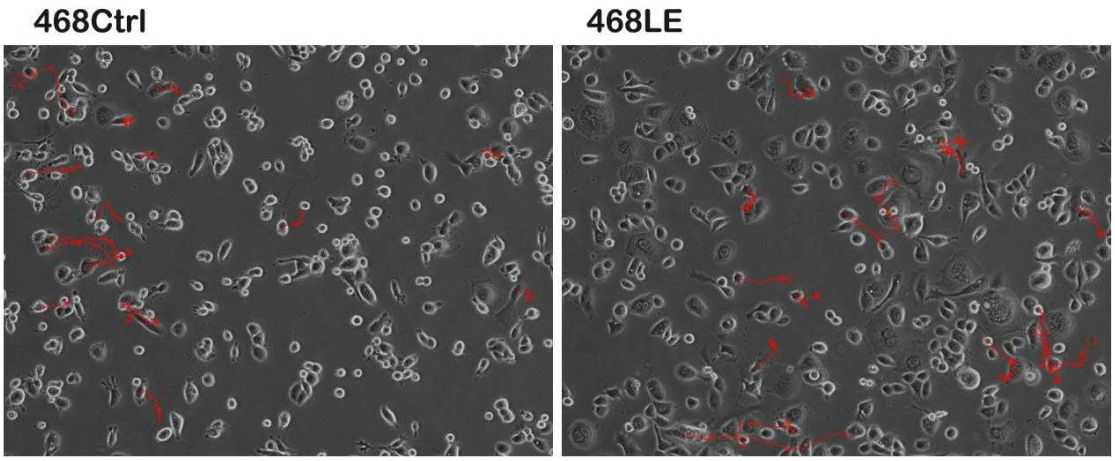

Figure 24 **Time lapse images of 468Ctrl cells and 468LE cells (Tracks of the cells are shown in red).**

The average velocity of fifteen cells at every timepoint was calculated and plotted to show the trend of average cell velocity with time (**Figure 25**). A third order polynomial best fit curve was drawn by fitting the data points to highlight the trend of the cell velocity in each case. The polynomial curves show that the 468LE cells (with greater L1) showed a consistently greater average velocity compared to 468Ctrl cells (less L1).
The overall average velocity of all the cells tracked was plotted (

Figure 26). The overall average velocity of 468LE cells (with more L1) is about **45%** greater compared to the overall average velocity of 468Ctrl

cells. Statistical analysis shows that the results are significant ($p<0.002$). This indicates that increased expression of L1 can further increase 468 cell motility.

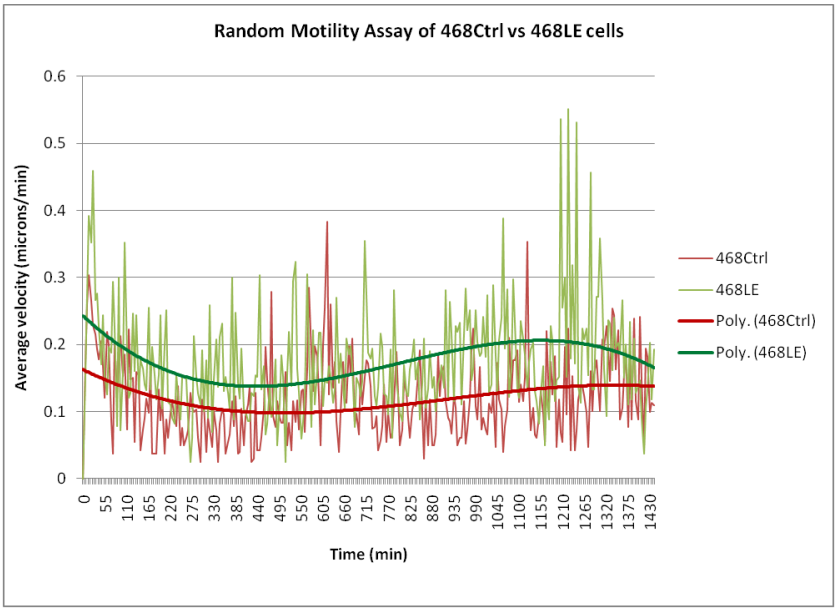

Figure 25 Random motility assay: Average velocity of 468Ctrl cells vs 468shL1 cells plotted over time.

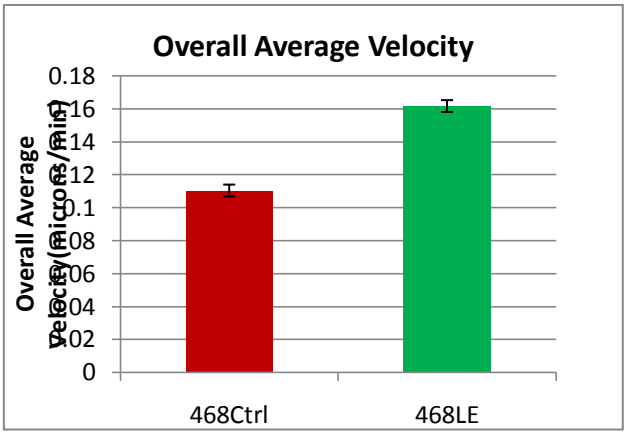

Figure 26 Overall Average Velocity of 468LE cells (green) was 45% greater than the overall avg. velocity of 468Ctrl cells (red). (p= 2.5196E-19).

3.3.2. *Super Scratch* assay of 468LE vs. 468Ctrl cells

A *Super Scratch* assay was done in order to compare the motility cells along the leading edge for 48 hours. While most of the 468LE cells were normal, some of the cells were highly active in moving across the scratch. The images of the cells tracked along the scratch are shown in **Figure 27**.

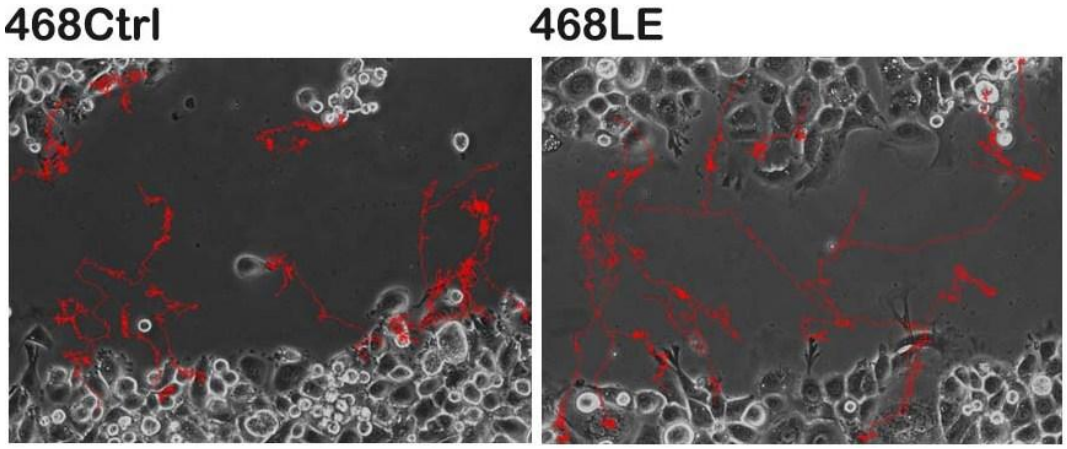

Figure 27 ***Super Scratch*** **Assay of 468Ctrl cells and 468LE cells (bottom).(Tracks of the cells shown in red)**

Ten cells were tracked for 48 hours and the average cell velocity was plotted. There was no particular trend which can be identified from the graph except for the fact that the velocity of 468LE cells peaked to a maximum of 1.2microns/min while the maximum value observed in the case of 468Ctrl cells was a much lesser value of 0.4 microns/min (**Figure 28**). This was observed at multiple timepoints. This gave an indication that probably some of the cells are highly active compared to others at specific timepoints. When the overall average velocity of the cells was calculated, 468LE cells along the scratch showed a significantly higher velocity by about **10%** compared to the 468Ctrl cells ($p<0.002$), shown in **Figure 29**.

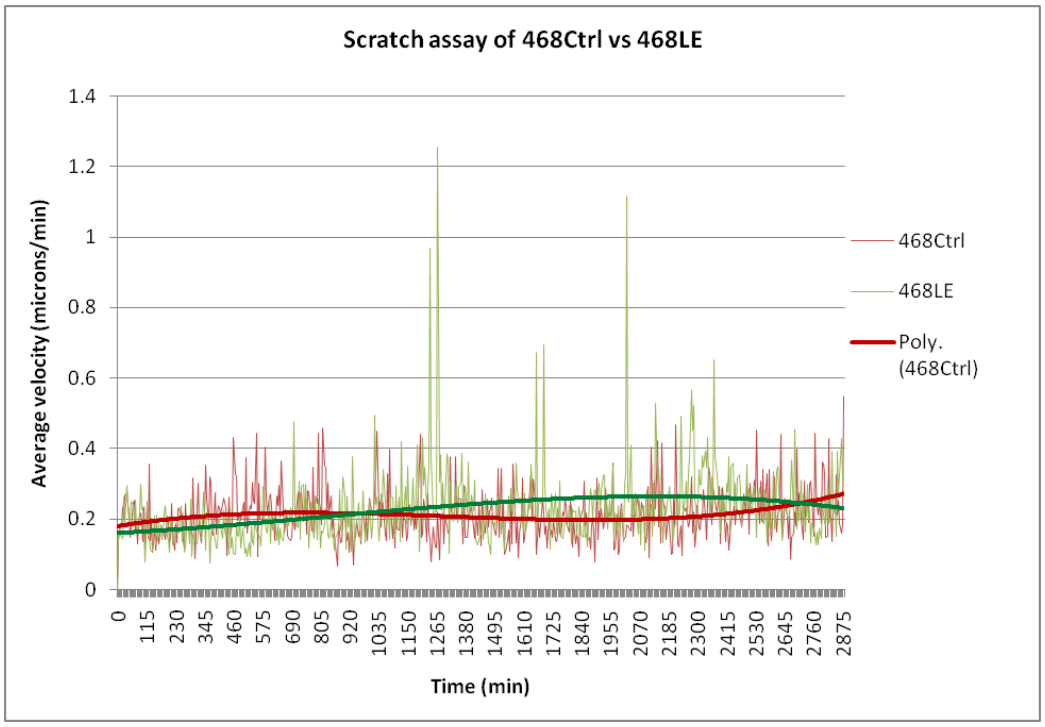

Figure 28 *Super Scratch* **assay: Average velocity of 468Ctrl (red)cells vs 468shL1 (green) cells plotted over 48 hours.**

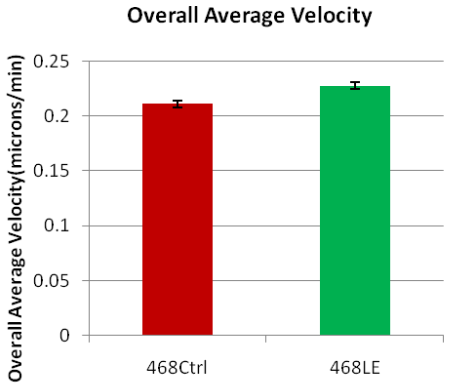

Figure 29 **Overall average velocity of 468LE (green) cells was 10% greater than the 468Ctrl (green) cells. (p=0.0019)**

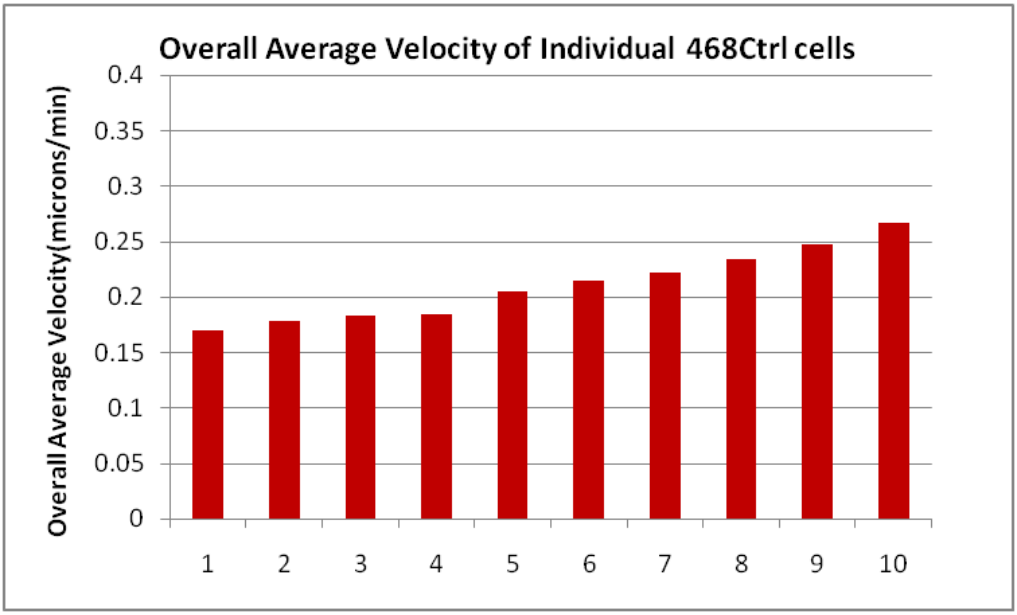

Figure 30 **Average velocity of each of the ten 468Ctrl cells tracked along the scratch.**

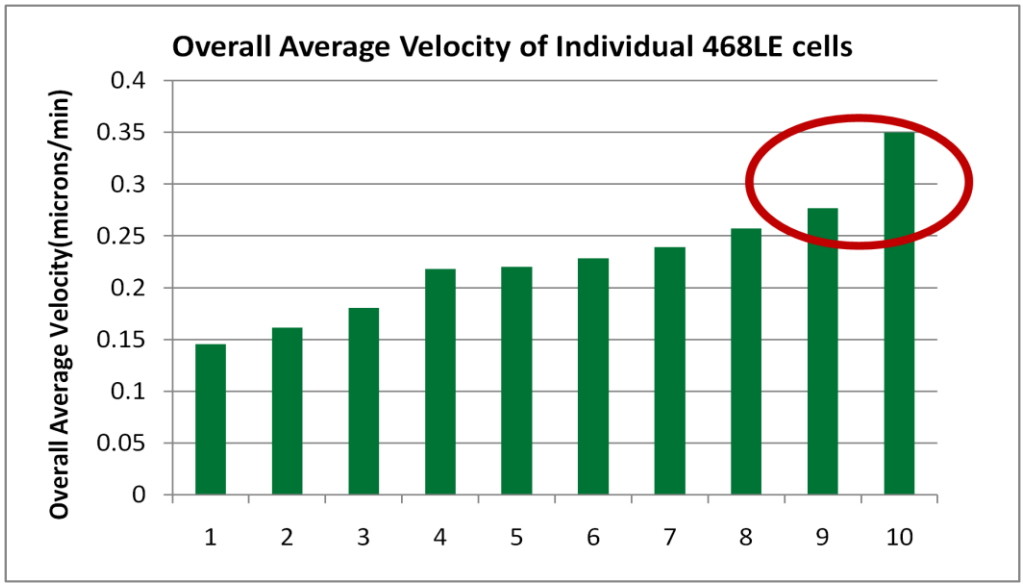

Figure 31 **Average velocity of each of the ten 468LE cells tracked along the scratch. (Red oval indicates the cells which are extremely active)**

Though the 468LE cells show a higher average velocity, it cannot be generalized for all the cells. Therefore, we wanted to extract more information by plotting the overall average velocity for each cell separately for the 468Ctrl cells (**Figure 30**) and 468LE cells (

Figure 31). Thus, it appears that the extreme motility of some 468LE cells was largely masked when grouped with the rest of the cells.

By observing the individual cells from 468LE and 468Ctrl, there is more variation in the 468LE cells. Few cells had much higher average velocity compared to the rest of the cells (indicated by the red oval in **Figure 31**). This suggests that the cells along the scratch are not all active in the case of 468LE cells. There could be a subpopulation of cells that for some reason are more susceptible to stimulation by L1ED and, thus, have increased cell motility. Alternatively, it could be that culture conditions or an individual cell's juxtaposition to neighboring cells that causes differences in cell velocities.

3.4. Effect of L1 overexpression on the cell motility of the more metastatic 231 cells

231 cells already express easily detectable L1 (**Figure 16**). Nevertheless, overexpressing L1 caused the cells to flatten out more and send out longer extensions and processes. Random motility analysis was performed by tracking the images of cells for 24 hours in serum-free media. Images of the tracked cells from both 231Ctrl and 231LE cells are shown below (**Figure 32**).

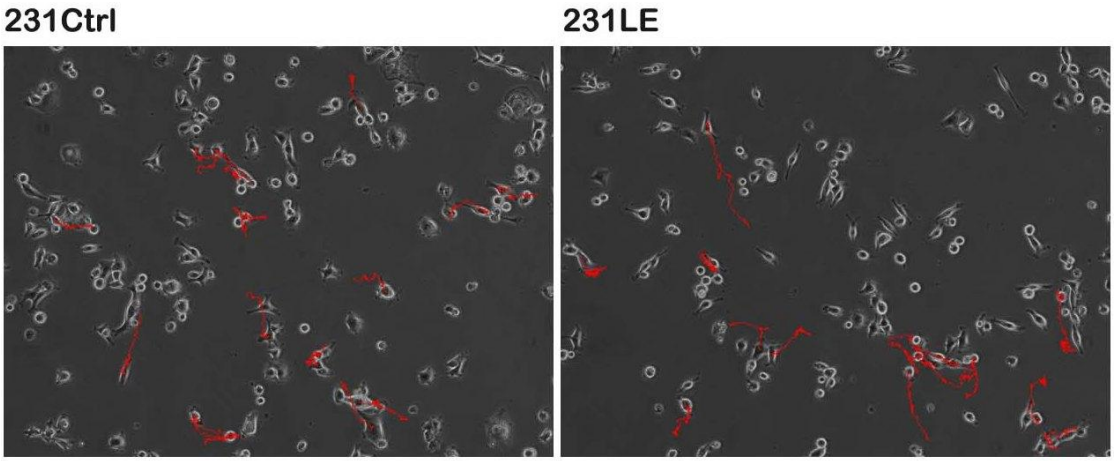

Figure 32 Time lapse images of 231Ctrl cells and 231LE cells (Tracks of the cells are shown in red).

3.4.1. Random motility of 231LE compared to 231Ctrl cells

Images of 231Ctrl and 231LE cells, sparsely distributed in the cell culture dish, were taken every 5 minutes for 24 hours. The average velocity of 15 cells at every timepoint was calculated and plotted to show the trend of average cell velocity with time (**Figure 33**). A third order polynomial best fit curve was drawn by fitting the data points to highlight the trend of the cell velocity in each case. The polynomial curves show that the 231LE cells (with greater L1) showed a consistently greater average velocity compared to the 231ctrl cells.

The overall average velocity of all the cells tracked was plotted (**Figure 34**). The overall average velocity of 231LE cells (green; with more L1) was about **18%** greater compared to the overall average velocity of 231Ctrl (red) cells ($p<0.001$). This shows that increased L1 expression can further increase the motility of 231 cells.

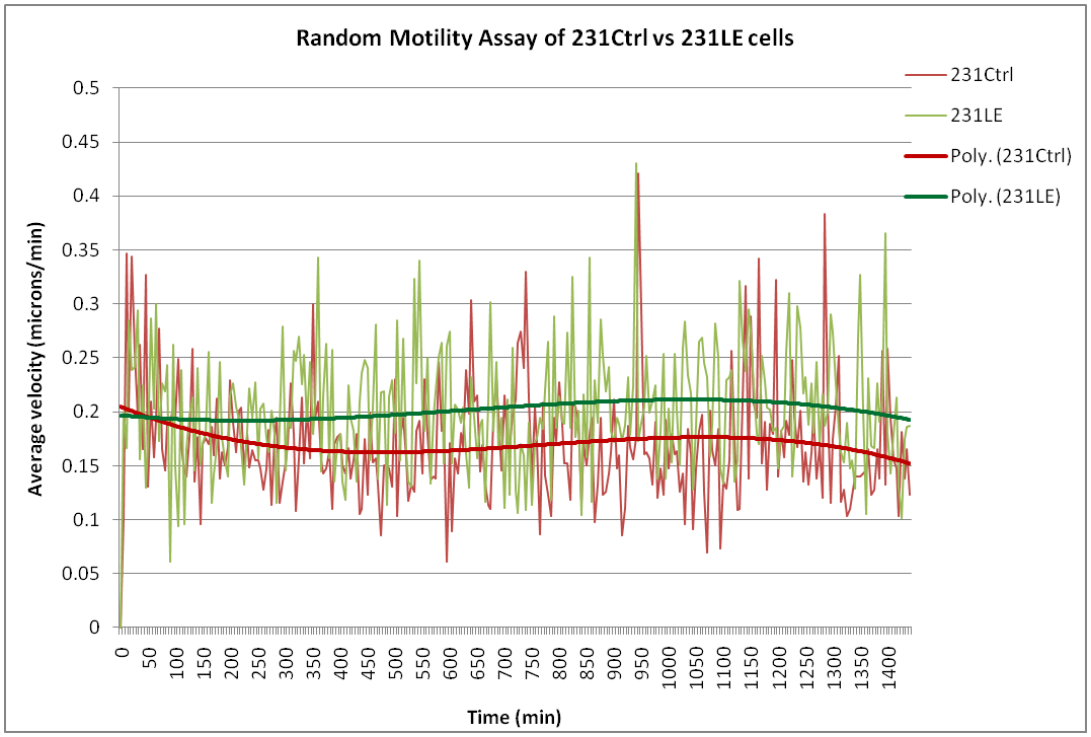

Figure 33 **Random motility assay: Average velocity of 231Ctrl cells vs 231LE cells plotted over time.**

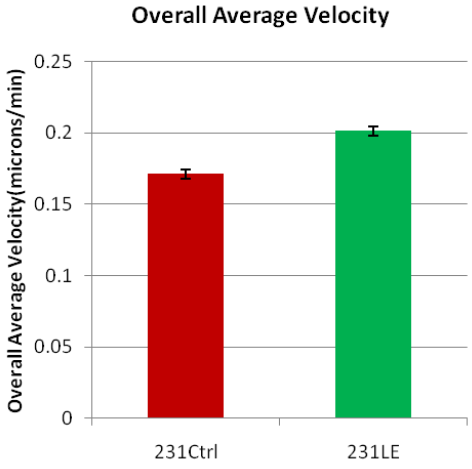

Figure 34 **Average Velocity of 231LE cells (green) was 18% greater than the average velocity of 231Ctrl cells (red). (p= 7.99E-11).**

3.5. Effect of L1 knockdown in 231 cells

231 cells showed a high level of expression of L1. In order to investigate the role of their endogenous L1 in breast cancer cell motility, L1 was attenuated in 231 cells to create 231shL1 cells. A random motility analysis was done by tracking the images of 231Ctrl and 231shL1 cells for 24 hours in serum-free media. The images of the tracked cells from both the cell types are shown below (**Figure 35**).

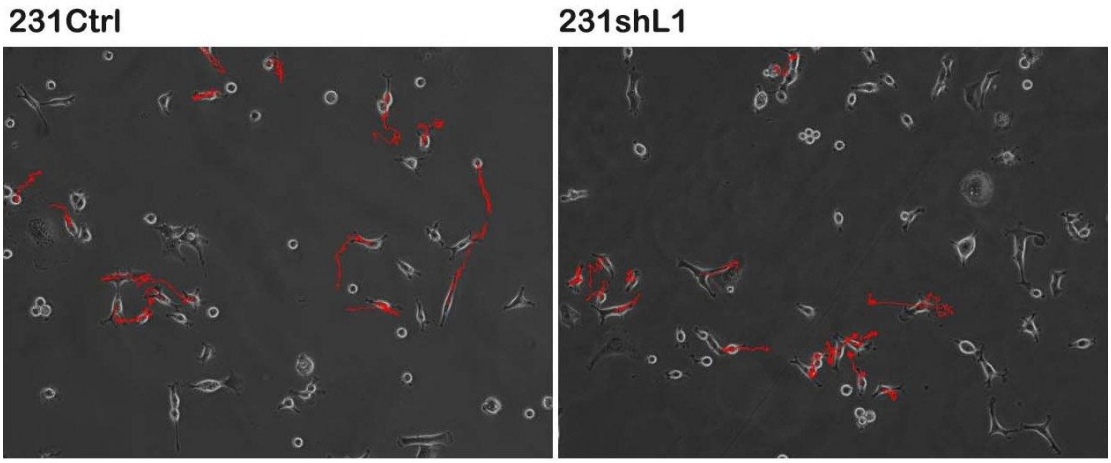

Figure 35 Time lapse images of A. 231Ctrl cells B. 231shL1 cells (Tracks of the cells are shown in red).

3.5.1. Random motility of 231shL1 compared to 231Ctrl cells

Images of 231Ctrl and 231shL1 cells, sparsely distributed in the cell culture dish, were taken every 5 minutes for 24 hours. The average velocity of fifteen cells at every timepoint is calculated and plotted to show the trend of average cell velocity with time (**Figure 36**). A third order polynomial best fit curve was drawn by fitting the data points to highlight the trend of the

cell velocity. The polynomial curves show that 231Ctrl cell showed a greater average velocity at most timepoints than 231shL1 cells.

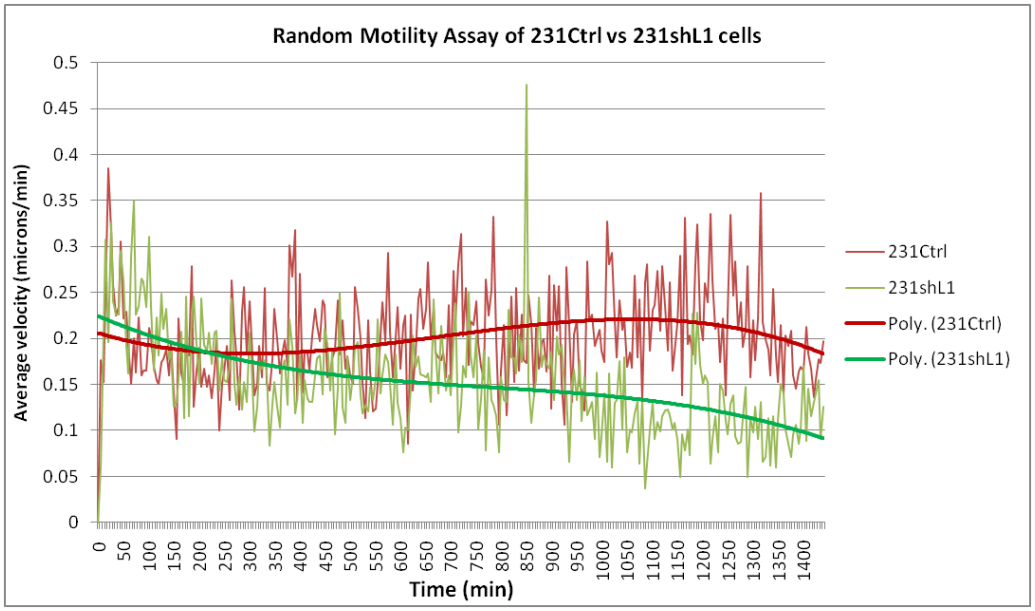

Figure 36 Random motility assay: Average velocity of 231Ctrl cells vs 231shL1 cells plotted over time.

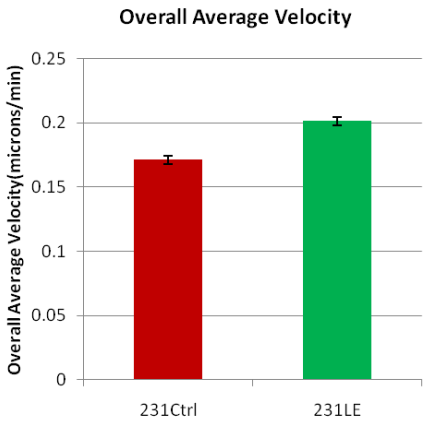

Figure 37 Overall Average Velcocity of 231shL1 cells (green) was 25% lower than the overall average velocity of 231Ctrl cells (red) (p= 2.57925E-25)

The overall average velocity of all the cells tracked was plotted (**Figure 37**). The overall average velocity of 231shL1 cells was about **25**% lower compared to the overall average velocity of 231Ctrl cells ($p<0.001$). This shows that attenuation of L1 expression in 231 cells reduced the motility of 231 breast cancer cells.

3.6. L1 is present in exosomes released by breast cancer cells

Exosomes are used as a potential means of communication by several kinds of mammalian cells and was first discovered in reticulocytes (Keller et al., 2006). Exosomes are membranous vesicles which pack and send out specific proteins that serve as extracellular messages (Raposo et al., 2004). Several cancer cells also release exosomes. L1 was found to be released in the exosomes of 435 breast cancer cells in unpublished data from the thesis of a previous lab member (Adla, 2007). However, the presence of exosomes has not been confirmed in different breast cancer cells.

Exosomes were isolated from the media of 231, 435 and 468 cells by incubating them in serum-free media overnight. The cell extracts were prepared. And the supernatant was subject to a series of differential centrifugation steps of 1000 x g, 10000 x g and the last being ultracentrifugation at 100,000 x g for 20 hours (as described in the Methods **Section 2.7.**). Exosomal extracts were dissolved in PBS + Protease Inhibitor. The supernatant from the last centrifugation and the exosomal pellet, along with the cell extracts are run in an SDS gel and the presence of L1 is checked using western blot analysis (**Figure 38**). As expected, more L1 was found in the supernatants and exosomes of 231 and 435 compared to 468 cells.

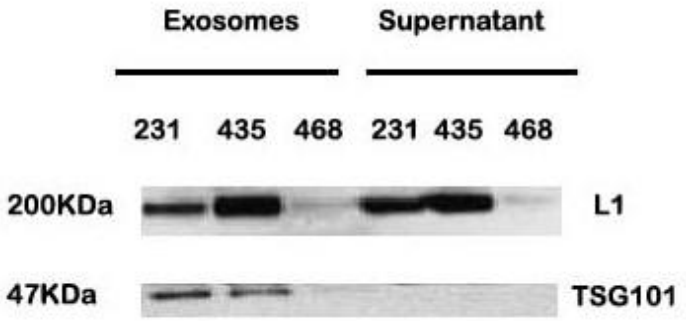

Figure 38 Western blot showing the expression of L1 (using antibody UJ)and the exosomal marker TSG101 in the exosomes and cell supernantants of 231, 435 and 468 cells.

TSG101 was one of the several exosomal markers used. Here, the presence of TSG101 was confirmed in the exosomes of 231 and 435 cell lines while absent in their respective supernatants. Exosomes from the 468 cell line, however showed a very insignificant band of TSG101, either because the cell line did not produce enough exosomes or TSG101 was absent in these exosomes.

3.7. Visualizing exosomes using TEM

Transmission Electron Microscopy was used to visualize the exosomes. The serum free media from 435 breast cancer cell line was centrifuged and the resulting pellet was resuspended in PBS, transferred to the grids, centrifuged again for 2 hours, then stained with PTA and imaged by the transmission electron microscope after drying the grids according to the method described in **Section 2.8.** The images are shown in **Figure 39** below for both the exosomes and the control without the exosomes. The dried excess

stain forms blobs on the grids, while the exosomes are the tiny round particles as indicated in the **Figure 39B**.

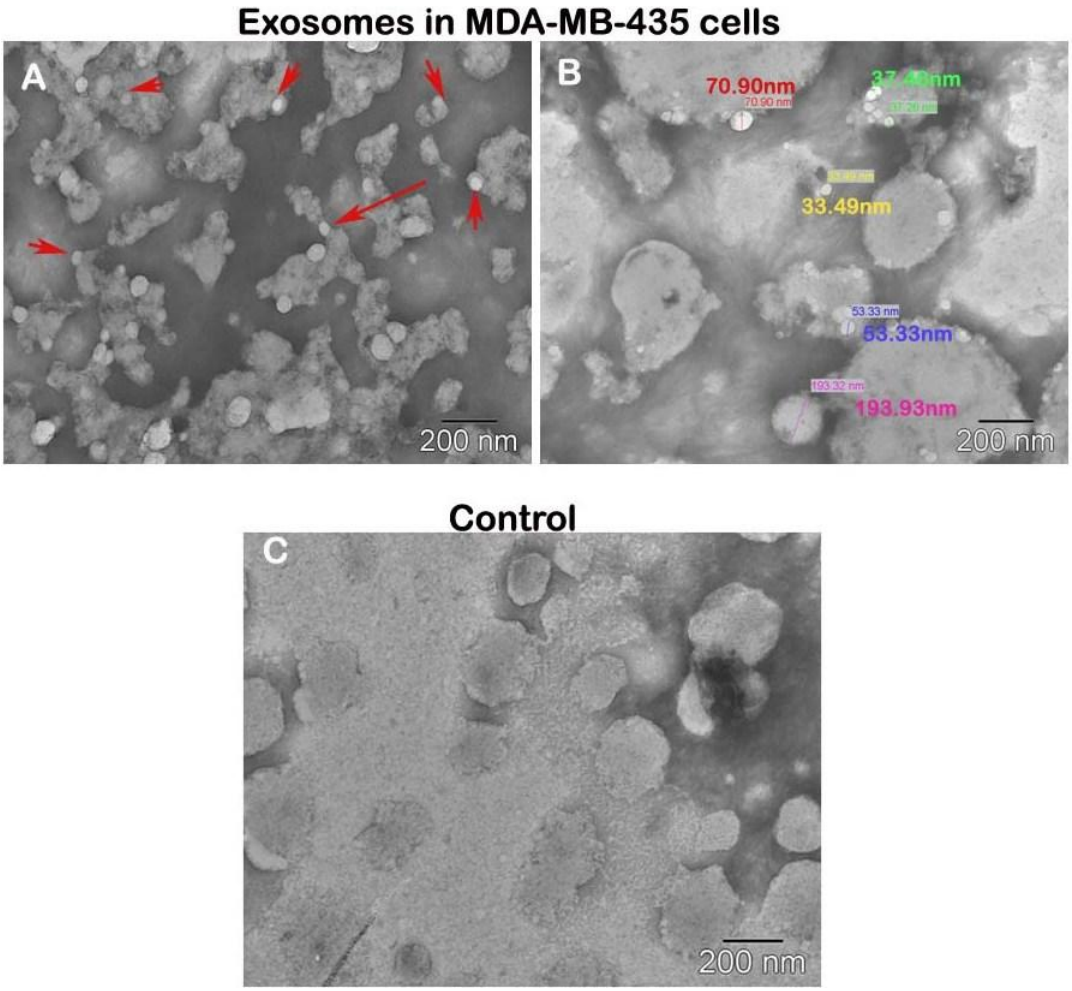

Figure 39 Transmission Electron Microscopy revealed exosomes released MDA-MB-435 cells.

The vesicles pelleted from the serum-free media of the 435 breast cancer cells. 200 nm scale bar is shown. **A:** Exosomes are pointed out using red arrows. **B**: Several exosomes within 30-100 nm are marked (33nm, 37nm, 53nm, 70nm) along with a blob of accumulated stain (193nm in pink colour) to show the difference. **C**. Control-media without exosomal pellet.

3.8. Exosomes increased cell motility

Though exosomes have been detected to be produced by in several types of cancer cells, the function of exosomes is not characterized completely. Exosomes are found in the breast cancer cells 231 and 435 and L1 is present in the exosomes (as shown in **Results 3.8.**). L1 in the cells was found to increase the cancer cell motility. To extend these results, I have done experiments with exosomes to check if the L1 in the exosomes could also stimulate and increase cell motility.

3.8.1. Characterizing L1 expression in exosomes released by 231shL1 and 231Ctrl cells

In order to find the effect of the exosomal L1, exosomes were isolated from 231shL1 where L1 is attenuated and the 231Ctrl cells. The exosomal proteins were analyzed by western blot and probed with UJ for the presence of L1 in both the cases (**Figure 40**).

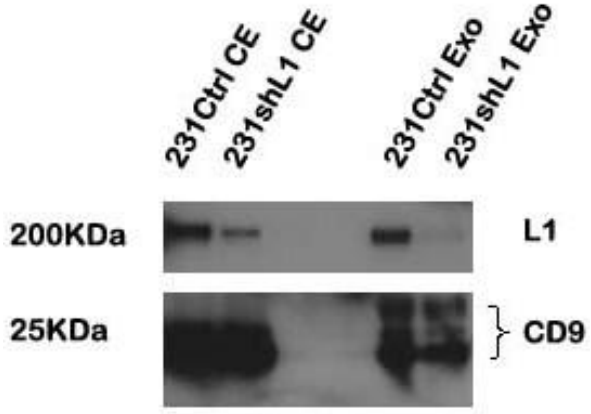

Figure 40 **Western blot showing the presence of the L1 and the exosomal marker CD9 in 231Ctrl and 231shL1 Cell Extracts (CE) and exosomal extracts (Exo).**

As seen from the western blot, the cell extract of 231Ctrl has more L1 than the 231shL1 cell line. Quantitation of exosomes was done using BCA

assay and eual amounts of total exosomal protein was loaded in each well. As expected, the exosomal extract from 231Ctrl cell medium contained a substantially greater amount of L1 compared to the exosomes of 231shL1 cell medium. Since there was such a great difference in the L1 in exosomes of 231Ctrl and 231shL1 cells, these exosomes were further used to explore the function of exosomal L1.

3.8.2. Effects of exosomes on motility of 231shL1 cells

231shL1 cells were incubated either with PBS + Protease Inhibitor with no exosomes (served as Control) or with the exosomes from 231Ctrl cells or the exosomes released by the 231shL1 cells. Exosomes were highly concentrated by ultracentrifugation. The concentrated exosomes released by 231Ctrl or 231shL1 cells from a 10 cm cell culture dish (10 ml) were finally dissolved in 50 µl PBS + Protease Inhibitor. After saving some of the exosomal extract (about 10 µl) for western blot analysis and for quantitation of exosomes, equal amounts of total protein in the exosomal extracts (or just PBS for the control) was made up to a total volume of 200 µl using serum-free media. This was then added to 231shL1 cells in an 8-well Lab-Tek II Chamber Slide. About 40,000 cells of 231shL1 were grown overnight in each well of the chamber slide. Once the 200 µl media with or without exosomes was added to each well, the cells were incubated with or without exosomes for 4 hours before setting up the time lapse experiment for measuring the random motility of the cells. Cells were tracked for 560 minutes (~9 hours), which is less than for other experiments. Evaporation of the media covering the cells takes place over time when the hot air chamber is used to maintain the

temperature of the cells. Only 200 µl media was used in each well to maximize the concentration of the exosomes. Hence, to prevent evaporation of the media during the course of the experiment, the edges of the chamber slide were sealed with petroleum jelly. Due to some evaporation despite the above precaution, cells were tracked only for 9.5 hours.

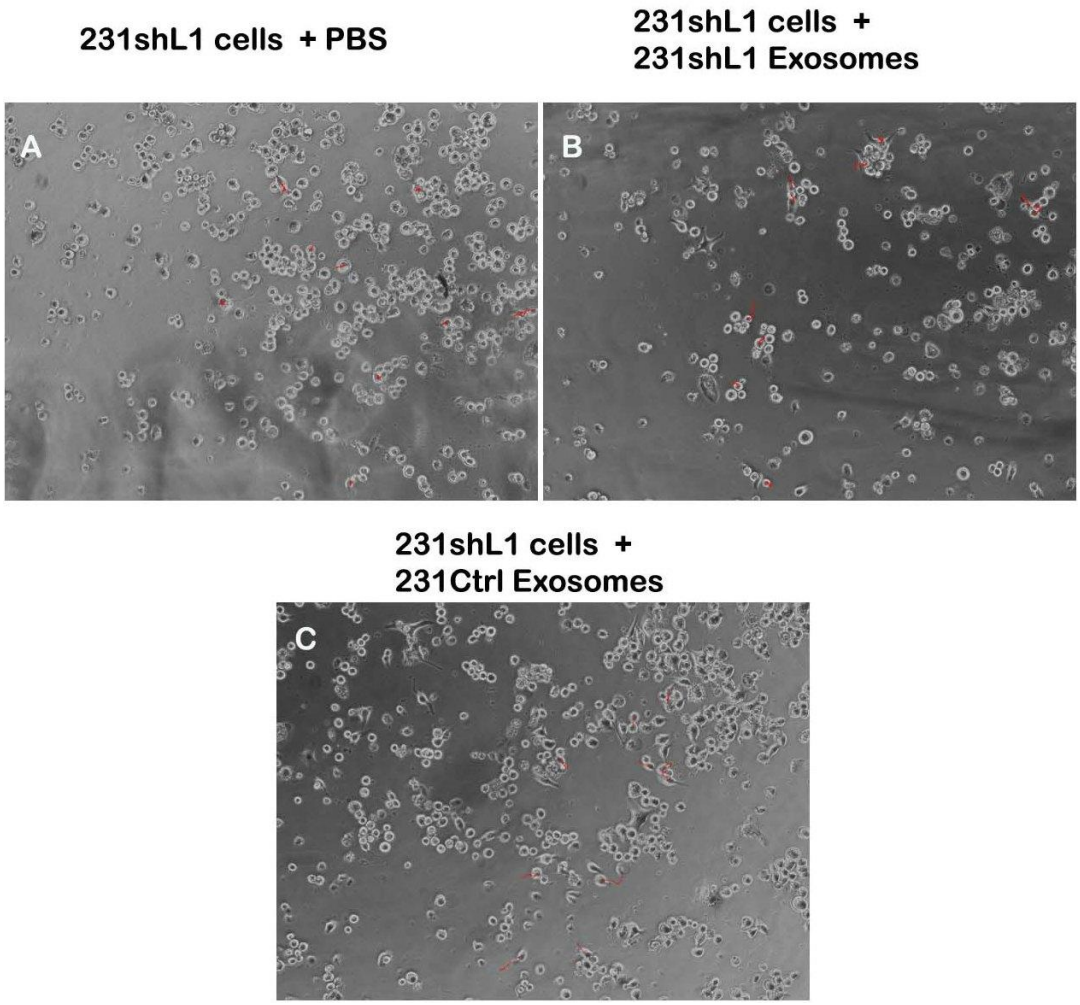

Figure 41 **Images of 231shL1 cells incubated with A.PBS(Control, without exosomes), B. Exosomes from 231shL1 cells, C. Exosomes from 231Ctrl cells.**

Images of the cells tracked (in red) are shown in

Figure 41. The striking observation was that most of the 231shL1 cells were rounded when they were incubated in media with PBS alone. On the other hand, when the cells were incubated with exosomes, more cells flattened out in the well. Maximum numbers of cells flattened out and appeared to be more motile in the case of 231Ctrl exosomes compared to the exosomes from 231shL1 cells.

The average velocity of 10 cells was calculated every 5 minutes for 9.5 hours and plotted to show the trend of average cell velocity with time (**Figure 42**). A third order polynomial best fit curve was drawn by fitting the data points to highlight the trend of the cell velocity in each treatment. The polynomial curves show that the cells incubated with PBS showed the least average velocity at every timepoint and the cells incubated with the exosomes of 231Ctrl cells (with more L1) showed a greater average velocity curve compared to the 231shL1 cells (with lesser L1).

The overall average velocity of all the cells tracked in each treatment was plotted (**Figure 43**). The overall average velocity of cells incubated with exosomes from 231Ctrl cells (with more L1) was almost double that of the overall average velocity of cells incubated with the exosomes from 231shL1 cells (about **100%** greater). Multiple trials of the experiment achieved a statistically significant increase in cell motility of about **80-100%** in the case of 231Ctrl exosomes compared to 231shL1 exosomes ($p<0.001$).

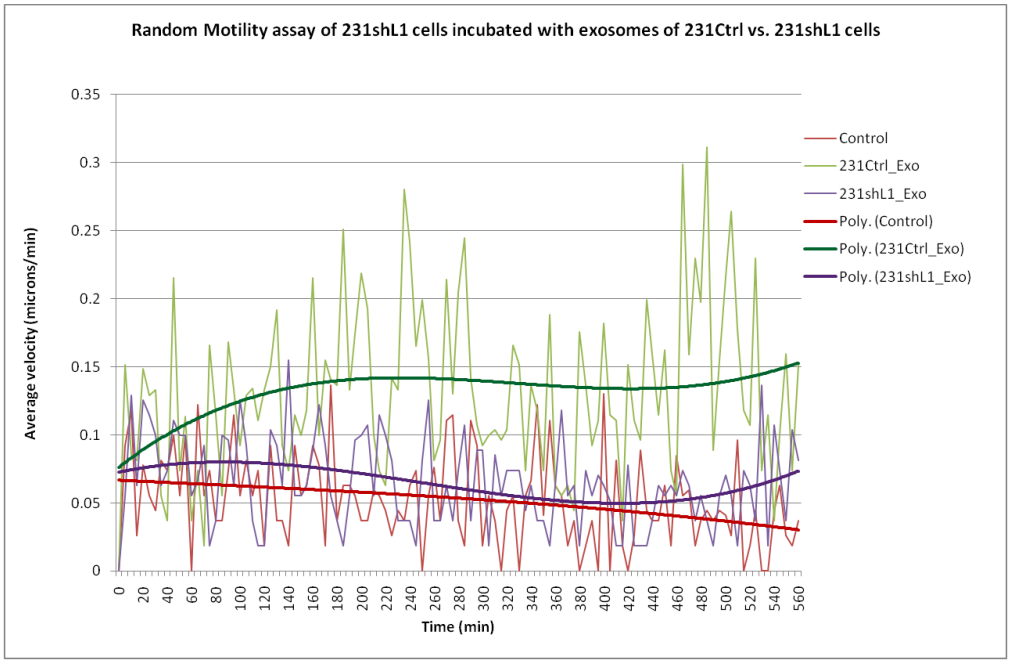

Figure 42 **Average velocity of 231shL1 cells after incubation with exosomes from 231Ctrl and 231shL1 cells, and PBS (control).**

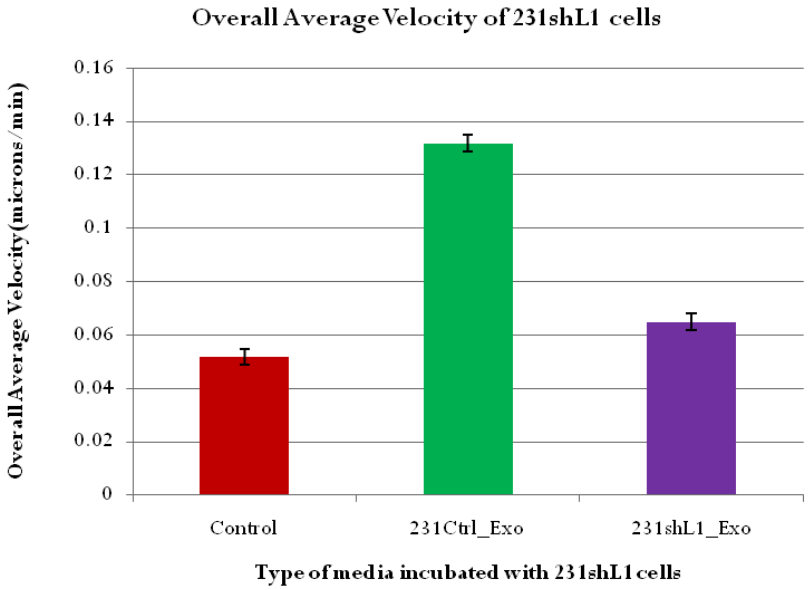

Figure 43 **Overall average velocity of the cells when incubated with Control (PBS; red), exosomes from 231Ctrl cells (green) and exosomes from 231shL1 cells (purple) (p-value= 1.59092E-20).**

3.9. *In vivo* model to study metastasis

The results from the time lapse experiments above show that overexpression of L1 can increase cell motility of the breast cancer cells *in vitro*. However, *in vivo* studies would help us understand the exact mechanisms by which L1 might influence the metastasis of the breast cancer cells to the brain.

In vivo studies on breast cancer metastasis to the brain were done by injecting 231 cells into nude mice (Kim et al., 2004). Chick embryos have been used in our lab to study the metastatic potential of breast cancer cells. It was shown previously in our lab that 435 and 231 breast cancer cell lines extravasate into brain tissue after injection into the extra-embryonic vasculature of chick embryos (Hansen, 2006; Teixeira, 2009).

3.9.1. Injection of 231shL1 and 231Ctrl cells into the chick embryo vasculature

In order to study the metastatic potential of L1, the 231shL1 and the 231Ctrl cells were injected into the vasculature of E5 chick embryos. These cancer cells had the puromycin resistance marker. The eggs were incubated for 4 additional days and were then sacrificed on E9, the brains were dissociated and the single cells were selected for puromycin resistance. The resultant 231 cell colonies were supposed to be counted to quantitate the metastatic potential of the control cells and the cells in which L1 is knocked down. Though there were a few cells surviving the puromycin selection, they did not live long enough to make visible colonies and eventually all the cells died out. Hence, nothing could be concluded about the change in the metastatic potential due to L1 knockdown.

3.9.2. Injection of 468LE and 468Ctrl cells into the chick embryo vascuature

In order to verify if increased expression of L1 could increase the metastatic ability of 468 breast cancer cells, 468Ctrl cells and 468LE cells both having the puromycin resistance marker were injected into the vasulature of E5 embryos. As explained above, brains were dissociated on E9 and were selected for puromycin resistance to quantitate the number of cancer cells which have metastasized into the brain from the blood vessels.

The same problem was encountered here and there were no cells surviving the puromycin resistance.

3.9.3. Identifying the problem with *in vivo* experiments

In the earlier chick embryo studies in the Galileo Lab, G418 resistance was used to select the cancer cells which have metastasized to the brain. With the neo^r marker, the cells would go for about 10 days before starting to die out. The cancer cells would develop visible colonies in about two weeks. They were also equipped with the lacZ marker to be able to visualize the blue color in the positive colonies with X-Gal. However, in the case of puromycin selection, most of the cells died out in the first couple of days. As there were very few cancer cells compared to the enormous amounts of the brain tissue, it was speculated that probably there was not enough time for the cancer cells to attach to the dish and they could grow to multiply more.

In order to verify that the puromycin selection was the problem, 468-GFP cells (with the GFP marker and the puromycin marker) were injected into the chick embryos and the cells were checked under the fluorescence

microscope for the presence of the cancer cells at the end of every crucial step in the microinjection and the brain dissociation process (**Figure 44**).

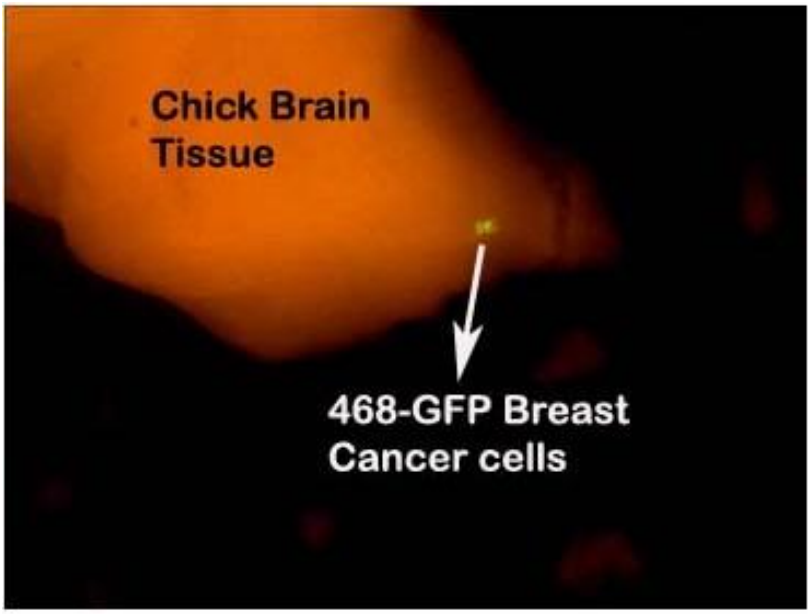

Figure 44 **Dissociated brain tissue piece with a cluster of 468-GFP cells (fluorescing in green) metastasized into the brain tissue.**

(Image taken at 5X magnification using a camera attached to the microinjection stereomicroscope).

Hence, it was shown that 468 cells could actually metastasize into the brain by visualizing the GFP + cells under the fluorescent light. But the number of cells were very small (about 30 cells in the entire brain tissue) and some of them were buried under the layers of the brain tissue and the fluorescence could not be detected. The cells were then plated and allowed to grow for 2 days before adding puromycin. The culture dish was again checked under the fluorescent microscope to confirm the presence of GFP cells adhering to the dish. After the puromycin was added, there was massive cell

death and none of the cancer cells seemed to survive after a week of addition of puromycin. So, the problem with the metastasis experiments could be the usage of the puromycin marker to select the cancer cells which have metastasized to the brain. Having the same set of cell lines (231Ctrl and 231shL1; 468ctrl and 468LE) with neo^r marker could help us investigate the metastatic abilities of the cell lines either overexpressing L1 or where L1 is attenuated.

CHAPTER 4

DISCUSSION

L1CAM is a neural cell adhesion protein which was shown to be primarily important in neural development (Hortsch, 1996). The normal expression of L1 is limited to the CNS and PNS during development and certain other specific cell types like the intestinal epithelial cells (Probstmeier et al., 1990) and cells of the urogenital tract (Kujat et al., 1995). However, recently L1 is found to be overexpressed in several cancers including melanoma (Thies et al., 2002), lung cancer (Katayama et al., 1997), glioma (Yang et al., 2009), colon cancer (Gavert et al., 2005), ovarian cancer (Euer et al., 2005), lung cancer (Katayama et al., 1997) and breast cancer (Shtutman et al., 2006). The aggressiveness of the cancer, bad prognosis and poor outcome is correlated to the presence of L1 in several of these cancers. Though L1 was detected in some of the breast cancer cell lines like MCF7 (Shtutman et al., 2006), the molecular mechanisms of L1 in breast cancer are yet to be explored.

In normal human brain development, shedding plays an important role in releasing L1 on the axonal surface to facilitate more L1-L1 interactions and promote cell adhesion and cell motility. But, 'Shedding' of L1 was found to be one of the major mechanisms by which soluble L1 is released by cancer cells and increase the cell motility and migration ability of the cancer cells and therefore increase the aggressive of the cancer cells (Gutwein et al., 2003). L1 is found to increase the cell motility of cancer cells. Previous work shows that L1 can cause transformation of cells and is expressed at the

invasive front of colon cancer cells (Gavert, 2005). L1 was also found to correlate with metastasis in renal cell carcinomas (Allory, 2005).

Another form of soluble L1 is the L1 present in the exosomes in the cancer cells. Exosomes are widely used by different cell types to promote intercellular communication. Exosomes are tiny membrane vesicles which are formed by invaginations of the membrane of late endosomes (Niel et al., 2006). Certain proteins are packaged into the exosomes with the help of the chaperone proteins and the proteins which are the constituents of the ESCRT (Endosomal Sorting Complex). Recently, exosomes have been widely detected in several cancers and the mechanisms by which the proteins packaged in the exosomes influence cell-cell behavior are not completely understood (Marsh & van Meer, 2008).

Normally, L1 is not expressed in breast tissue, neither in the epithelium nor the duct cells (Huszar et al., 2006). But L1 is overexpressed in certain breast cancer cells (e.g. MCF7) and was found to cause cell scattering and increased cell motility in its membrane bound full-length form and not in the soluble ectodomain form (Shtutman et al., 2006), but this effect appears to be unusual compared to many other cancer cell types. The exact functions of the two soluble forms of L1 (shed ectodomain and the exosomal L1) have not yet been elucidated for breast cancer cells. It is important to answer these unknown questions, which might provide a clue to finding the therapeutics to prevent breast cancer aggressiveness and metastasis to the brain. L1 is an important molecule in the extracellular environment of the brain. The possible interactions between L1 on the cancer cell surface or the soluble L1 released by

the breast cancer cells might influence their ability to interact with the brain extracellular environment. A similar mechanism found in other cancers could be found in breast cancer as well. L1 could influence the motility and the metastatic ability of the breast cancer cells to migrate and disseminate in the brain.

In order to address the unknown mechanisms of L1 in breast cancer, I have investigated the role of L1 primarily in breast cancer cell motility. The effects of both the soluble forms of L1: shed ectodomain and the L1 present in the exosomal membrane, on the breast cancer cells were targeted. Experiments were also done to investigate the ability of L1 ectodomain to stimulate motility and metastasis in breast cancer cells. Furthermore, because of its correlation with metastasis of other cancer cell types (Raveh et al., 2009), L1 may also operate to facilitate metastasis of breast cancer in general.

4.1. L1 is present in breast cancer cells

First of all, the presence of L1 was characterized in three breast cancer cell lines MDA-MB-231 (231), MDA-MB-435 (435) and MDA-MB-468 (468). Cell extracts were prepared, western blot analysis was done and the protein was probed using the antibody UJ127 (a mouse monoclonal antibody which binds to ectodomain segment of human L1 protein). Results in **Figure 16** show that substantially greater amount of L1 is present in 231 and 435 cells compared to the 468 cells. According to previous breast cancer studies, 231 and 435 cells are found to be more metastatic whereas 468 is considered to be

less metastatic (Pollack et al., 2002). Here, the western blot analysis shows that there is a correlation between the presence of L1 and the metastatic ability.

Previous results from the unpublished data of Shalini Adla's thesis show that the breast cancer cells shed L1 ectodomain. This was confirmed through the presence of the cytoplasmic segment (32kDa) in western blot analysis (Adla, 2007).

4.2. Breast cancer cells release exosomes containing L1

Several cancer cells release exosomes like melanomas (Riteau et al., 2003), (Mathivanan et al., 2010), nasopharyngeal carcinoma cells (Keryer-Bibens et al., 2006) to serve variuos functions and comprising different kinds of proteins. In order to investigate the presence of exosomes in breast cancer cells, exosomes were isolated from 231, 435 and 468 cell lines using a series of differential centrifugation steps as described in Methods **Section 2.7.** After the final ultracentrifugation step, the pellet contains the exosomes released by the cells. This was confirmed by the presence of TSG101, which is a widely used exosomal marker (shown in the Western blot in **Figure 38**) (Guescini et al., 2010; Sharples et al., 2008). Exosomes of about 40-100nm from 435 cells were also visualized by using transmission electron microscopy as show in

Figure 39. The cell extracts, the exosomal pellets and the final supernatants were run in a western blot to check the amounts of L1 in each case. As expected, the more metastatic cell lines 231 and 435 showed a substantially greater amount of L1 than the less metastatic 468 cells.

This shows that the amount of L1 in the exosomes also correlates with the metastatic ability of the cell lines. Tumor released exosomes are

known to mould the host environment either by altering the signaling pathways in the host through growth factor or receptor transfer, factors promoting angiogenesis or proteins suppressing the immune response in the host (Iero et al., 2008). Considering the general mechanisms of exosomes, in the case of L1 it is possible that exosomes released by breast cancer cells could be a mediator to alter the brain microenvironment around the disseminating tumor cells by promoting hemophilic L1-L1 interactions or L1 signaling through integrins and thereby contributing to increased cell adhesion and motility of the breast cancer cells.

4.3. L1 ectodomain can stimulate breast cancer cell motility

In order to study the functional significance of L1 ectodomain in breast cancer cells L1 ectodomain was overexpressed in 231 and 468 cells and the cell motility was analyzed using random motility assays.

L1 ectodomain was shown to increase cell motility and metastasis in other cancers like melanoma (Fogel et al., 2003), ovarian cancer (Mechtersheimer et al., 2001), uterine cancer (Fogel et al., 2003) and colon cancer (Gavert et al., 2007). Here, in the case of breast cancer, I have chosen one highly metastatic cell line 231 and the less metastatic 468 cell line to investigate the role of L1 in breast cancer cell motility and metastasis. Lentiviral vectors were used to overexpress L1 ectodomain (LE) in both these cell lines. The cells in which L1 ectodomain was overexpressed were more stretched out and seemed to develop longer cytoplasmic extensions compared to the control cells. Random motility analysis of 231LE cells demonstrates a statistically significant increase of **18%** in the overall average cell velocity

compared to the 231Ctrl cells (**Figure 34**). The average velocity of all the 231LE cells also followed a polynomial curve, which always showed a greater value than the 231Ctrl cells (**Figure 33**).

A similar trend was observed in the random motility assay of 468 cells (**Figure 25)**. However, the increase in cell motility of 468LE cells was far more than the 468 Ctrl cells. 468LE cells exhibited a much more significant increase in the overall average cell velocity of about **45**% compared to the 468Ctrl cells (

Figure 26).

231 cells already express a large amount of L1, which is shed to release the L1 ectodomain. Even though they already have L1, overexpression of L1 ectodomain in these cells further increased the cell motility of 231 cells. This shows that the L1 receptors are not saturated enough and further L1 ectodomain promotes further L1-L1 interactions and further L1 signaling to increase their cell motility. Whereas 468 cells have a low expression of L1 to begin with. So, overexpression of L1 ectodomain caused a greater effect on the cell motility of 468 cells (45% increase) compared to 231 cells (18% increase).

Scratch assays were performed on the 468LE and 468Ctrl cells to understand the cell behavior along the leading edge of the migrating cells in the scratch. The overall average velocity of 468LE cells in the scratch is greater than that of the 468Ctrl cells by 10%. But, this result is not as large as the difference of 45% achieved in the random motility assay. The cells in the scratch also exhibited a non-uniform behavior. Some of the 468LE cells were

extremely active and exhibited highly motile behavior compared to the others along the scratch.

This leads to a possible conclusion that 468LE cells are more active when they are sparser and have the ability to interact in random fashion, developing the cytoplasmic extensions from all possible directions. On the other hand, in a scratch assay in a directed motion, the 468LE cells are not as active as in the random motility analysis. Only few of the cells along the scratch are highly motile compared to the rest of them. It is not clear why the 468LE cells do not have a higher motility in the scratch assay. But one possible reason is the tighter cell junctions in 468 cells which reduces the cell motility. But overexpression of L1 might provide a selective advantage to few cells to overcome the attachment with the neighboring cells. This could be the case in the actual breast cancer tissue, where a few of them are highly motile, disseminate from the tumor mass and are able to become highly active and have the potential to migrate and metastasize away from the primary tumor mass and have a selective advantage in cancer cell motility in the brain.

4.4. L1 knockdown decreased the cell motility of breast cancer cells

L1 was attenuated in the highly metastatic 231 cells using short hairpin lentiviral vector to create a stable cell line 231shL1 expressing much less L1 compared to the 231Ctrl cells (**Figure 19**). These cells were tracked in a random motility assay for 24 hours and their cell velocities were analyzed. Random motility analysis of 231shL1 cells demonstrate a statistically significant decrease of **25**% in the overall average cell velocity compared to the 231Ctrl cells (**Figure 37**). The average velocity of all the 231shL1 cells at the

different timepoints also followed a polynomial curve, which always showed a lesser value than the 231Ctrl cells (**Figure 36**). This shows that L1 is essential for the motility of 231 cells and the attenuation of L1 leads to a reduction in their cell motility of breast cancer cells.

L1 is shown to interact with several binding partners in the extracellular matrix, especially the integrins. It was shown that L1 stimulates motility by autocrine/paracrine binding with the integrins (Mechtersheimer et al, 2001). 231 cells express the integrin subunits for $\alpha_V\beta_5$ and $\alpha_V\beta_3$, which are known L1 receptors (Adla, 2007). Thus, the attenuation of L1 could reduce the interaction with integrins present in 231 cells and also cause reduction in L1 signaling by which the cells tend to become less motile.

4.5. L1 in exosomes can stimulate cell motility of breast cancer cells

Though exosomes containing L1 have been found in ovarian cancer cells (Keller et al., 2009), glioma cells (Yang et al., 2009) and other cancerous cells, the functional significance of exosomes containing L1 is not known. I have used the exosomes released from 231shL1 cells and 231Ctrl cells. The presence of the exosomal marker CD9 confirmed these extracts as exosomes before using them for experiments (**Figure 40**). As expected, the amount of L1 in exosomes of 231Ctrl cells was much more than the L1 in attenuated 231shL1 cells.

231shL1 cells in which L1 was attenuated were used to see if the exosomal L1 could stimulate them to increase their cell motility. The interactions of L1 along with other proteins on the exosomes have shown an

effect on the number of cells being more stretched out in the chamber slide as shown in

Figure 41. There was no significant difference in motility for the cells incubated with the control PBS and they also had the least average velocity throughout the time course. On the other hand, the cells incubated with exosomes from 231Ctrl cells had more average velocity than the PBS control and also more number of cells stretched out on the slide. Among the exosomes, the exosomes from 231Ctrl had a great increase in the cell motility compared to the cells incubated with the exosomes from the 231shL1 (**Figure 42**). The results were very significant as the overall average velocity of the 231shL1 cells incubated with the exosomes from the 231Ctrl had about **100%** increase compared to the cells incubated with 231shL1 exosomes with less L1 as illustrated in

Figure 43. This result clearly demonstrates the L1 on the exosomes plays a significant role in breast cancer cell motility. Not only in breast cancer, it is possible that this mechanism of the role of exosomal L1 is very important for the cell motility of other types of cancers as well. Here, I clearly unwind a new role of the exosomes in influencing cancer cell motility. Exosomes released by cancer cells are known to modulate the microenvironment around the tumor by altering the signaling pathways through redistribution of receptors (Iero et al., 2008). Here, L1 is a transmembrane protein with a potential of interacting with other L1 binding partners and especially other L1 molecules present in the brain microenvironment and thus stimulate cell motility. L1 signaling could occur through MAPK pathway (Whittard et al.,

2006) or possibly through FAK activation as in glioma cells (data from unpublished data of Yang, manuscript submitted), which can regulate the cancer cell motility.

4.6. Role of L1 in metastasis in vivo

The chick embryo system has been demonstrated to be an excellent model to study metastasis of cancer cells (Cretu et al., 2005). Breast cancer cells 231 and 468 cells, with the puromycin resistance marker, with and without the overexpression of L1 ectodomain were injected into the vasculature of the chick embryo, and incubated in the chick system for 4 days before recovering the breast cancer cells which have migrated to the brain. Though the experimental setup was ideal, we could not recover enough cancer cells, and they all died before forming quantifiable colonies.

Previously, the lacZ marker and the neomycin resistance marker were used in the Galileo lab for selecting the recovered cancer cells. I have tried the puromycin resistance marker for the first time and this did not seem good enough to select the cancer cells over the brain tissue successfully. In order to confirm that it was the selection marker, which was the problem, the 468 breast cancer cells with GFP marker were injected and GFP fluorescent cells were seen at the end of every crucial step in the experiment. The 468-GFP cells were successfully recovered from the brain tissue. However, they did not survive the puromycin selection process for more than a week.

Very few (about 20-30) cancer cells were recovered from the brain compared to the enormous number of cells ($> 10^7$) from the dissociated brain tissue. As puromycin acts very rapidly, many cells started dying in 2 to 3 days

and the few cancer cells likely were not able to attach well to the culture dish amongst the dying brain tissue masses. In the case of neomycin resistance, the cells grew for about 10 days before starting to die out. This difference in the time course of cell death is thought to be a significant factor in my unsuccessful *in vivo* experiments.

Role of L1 in metastasis *in vivo* in breast cancer cells is an important function to be understood further. New breast cancer cell lines overexpressing L1 and equipped with neomycin selection marker would be needed to study metastasis in the chick embryo system.

4.7. Conclusions and Future Work

All my results so far suggest the importance of L1CAM in breast cancer cell motility *in vitro.* **The two soluble forms of L1, L1 ectodomain shed by the cancer cells and the L1 present in the exosomal membrane, contribute to the breast cancer cell motility *in vitro*.** The functional role of intact exosomes in contributing to breast cancer cell motility has been shown for the first time. This could be an important step in understanding the significance of exosomes released by cancer cells. L1 could also be an important biomarker for the breast cancer cells and further research could lead to discovering the therapeutic potential of L1 antibodies specific to the L1 ectodomain or the exosomes released by the breast cancer cells. Anti-L1 antibodies have been shown to have an effect on the growth or metastasis of other cancer cell types (Wolterink et al., 2010; Gast et al., 2008).

Further work needs to be done in analyzing the cytoskeletal changes associated with L1 ectodomain overexpression to pinpoint the exact

mechanisms by which the L1 overexpressing cells have a higher cell motility. For example, our lab has shown that FAK-containing focal complexes are altered (M. Yang, submitted for publication).

Exosomes can be further studied to find out the signaling mechanisms triggered by the L1 present in the exosomes. It would be very helpful to check if there are any additional ways of communication via exosomes in the cancer cells.

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