

**SCN2B FUNCTIONS AS A CELL ADHESION
MOLECULE IN PROSTATE CANCER METASTASIS**

by

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A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Bachelor of Arts in Biological Sciences with Distinction.

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MOLECULE IN PROSTATE CANCER METASTASIS**

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ABSTRACT

Prostate cancer (PCa) is a silent disease treatable during its early stages but hard to diagnose due to the absence of symptoms and reliable disease markers. The five year survival rates of men with advanced stage and grade PCa are less than 30% and virtually all of these men have metastases to bone, predominantly in the lumbar and sacral regions of the spine. In over 85% of these cases, perineural invasion (PNI) is also observed. PNI is characterized by the close association of cancer cells along, around, and through nerve tissue. The currently accepted pathway of PCa metastasis is through the bloodstream or lymphatic system. However, these mechanisms do not account for the specific localization of PCa cells to the lumbar and sacral spine. This observation, combined with the frequency of PNI found in PCa cases and the abundance of nerves connecting the prostate and the spine, suggests that the cancer cells could be taking a more direct route of metastasis to these destinations by migrating along nerve cells. A protein complex believed to be involved in the migration of PCa cells is SCN2 β , a subunit of voltage-gated sodium channels (VGSC). These channels are made up of one pore-forming α -subunit and two auxiliary β -subunits. The β -subunits have extracellular V-set Ig-loop domains that are highly conserved when compared to neural cell adhesion molecules like Myelin Protein Zero Like protein 2 (MPZL2). The subunit SCN2 β , which functions both to traffic α -subunits to the cell surface and as a cellular adhesion molecule capable of forming both homotypic and heterotypic associations with other V-set Ig-loop domain containing proteins, is of particular interest. The expression of SCN2 β has been correlated to an increased metastatic phenotype in PCa cells. I hypothesize that SCN2 β is

required for PCa cell adhesion to and migration along peripheral nerve cells. To test this hypothesis, I had two aims. For my first aim, I cloned various proteins and protein domains associated with SCN2 β (both binding partners and repulsive domains). These proteins, when overexpressed in human PCa cancer cells, could be used in migration assays to determine their functions and interactions as well as purified to assay their binding strength directly. For my second aim, I performed several migration assays on cells overexpressing SCN2 β , with various conditions to inhibit or modify its function, to compare their migration with that of normal PCa cells. I have several protein cDNA sequences successfully ligated into mammalian expression vectors and ready for transfection into cancer cells. In my migration assays, I observed that overexpression of SCN2 β does increase migration in all cell types, and that inhibition of its normal function decreases migration.

Chapter 1

INTRODUCTION

1.1 Prostate structure and function

The prostate is a small, chestnut-sized organ surrounding the urethra and located beneath the bladder (Figure 1.1) [1]. This male accessory sex organ contains a ductal system of secretory epithelial cells [2]. Part muscle and part gland, its main function is to augment the seminal fluid with its own slightly alkaline secretions. These secretions enhance male fertility by promoting sperm survival, increasing sperm motility, and providing pH buffering in the vaginal tract [1].

The prostate is the most innervated organ in the male body, and such innervation is essential for its normal growth and secretory function [3, 4]. The prostate is sympathetically stimulated via the hypogastric nerve and parasympathetically innervated by the pelvic nerve [3]. Along with afferent nerves, these nerves connect to the thoracic, lumbar, and sacral regions of the spine; coincidentally these nerves colocalize to the same regions of the spine where prostate cancer metastasizes.

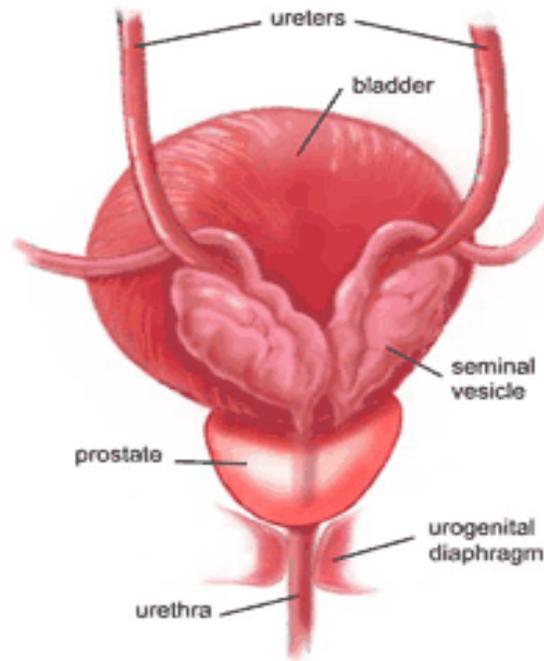


Figure 1.1 The prostate is located ventral-posterior to the bladder, surrounding the urethra. It releases its secretions into the seminal fluid, enhancing sperm survival and motility (source unknown).

1.2 Prostate cancer

With an estimated 192,000 new cases and over 27,000 deaths a year, prostate cancer (PCa) is the most commonly diagnosed cancer and the second leading cause of cancer-related deaths in U.S. male [5]. One in six men is predicted to develop PCa in their lifetime [5]. PCa originates predominantly from the epithelial cells lining the peripheral zone of the prostate. If PCa is diagnosed in its early stages while it remains localized, the disease can be treated and cured 95% of the time with surgery or radiation [1, 5]. However, the five-year survival rate drops drastically to only about 34% in those

diagnosed with advanced disease, which is characterized by the dissemination and metastasis of the PCa out of the prostatic capsule [6, 7]. Unfortunately, early PCa is a virtually silent disease marked by the absence of reliable symptoms, and is often misdiagnosed or missed entirely [6]. Therefore, it is most often diagnosed only after metastatic spread. PCa cells may migrate a small degree outward from the prostate, known as regional spread, or all throughout the body in the most aggressive cancer cases. Most often, PCa metastases are found in bone and the spine, particularly the lumbar and sacral regions of the spine (Figure 1.2) [6].

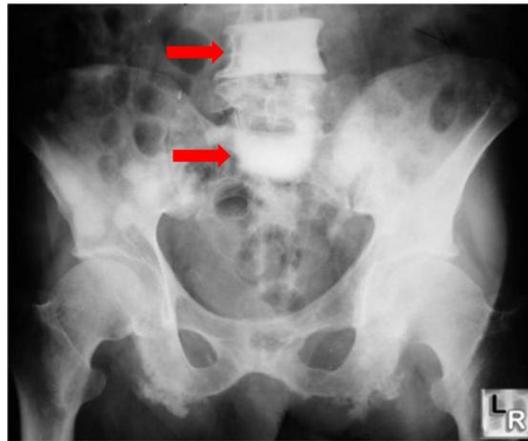


Figure 1.2 The red arrows on the x-ray of an advanced case patient indicate secondary PCa tumors at the lumbar and sacral segments of the spine.

1.3 Perineural invasion

Perineural invasion is a phenomenon that is frequently observed and remains poorly understood. It is defined broadly in cancer as the presence of metastases in and through nerve and nerve-associated fibers (Figure 1.3). In 85% of metastatic PCa cases, not only are secondary tumors found in the spine, but PCa cells are found closely

associated with the nerves bundles in the prostate gland itself [8] . This intimate association of PCa cells with nerve and propensity to metastasize to the lower spine suggest that the cancer is using the nerves to spread to the spine.

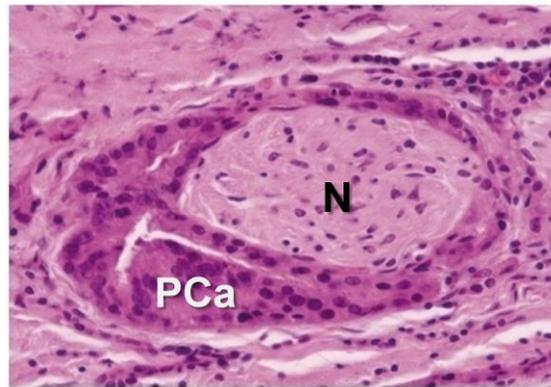


Figure 1.3 In the phenomenon of perineural invasion, PCa cells (the dark region labeled PCa) are found closely associated and often wrapped around nerve fibers (the lighter region labeled N). Interestingly, the PCa cells appear to be inside the perineurium [9].

1.4 Voltage-gated sodium channels and SCN2 β

In PCa cells, an increasingly metastatic phenotype has been correlated with the expression of voltage gated sodium channels (VGSCs) [10, 11]. VGSCs consist of one pore-forming α -subunit and one or two closely associated, dissimilar β -subunits (Figure 1.4). These channels most commonly function as pores for the propagation of action potentials in excitable cells. Their role in non-excitable cells is unknown. While the alpha subunit is homologous to the pore-forming units of other ion channels, the β -subunits are distinct from other channel-associated subunits [12].

Four different types of β -subunits have been identified, SCN1-4 β [13, 14]. They are type I single-pass transmembrane proteins with multiple functions that include

the regulation of the channel activity and its expression level at the cell surface [13]. They also contain a V-set Ig-loop in their ectodomain, which has more homology with neural cellular adhesion molecules than with ion channel components [13]. Due to this feature (Figure 1.5), the β -subunits have been recognized as potentially important molecules in cellular adhesion and migration. Of particular interest is the β -subunit SCN2 β , which is both necessary in the trafficking of α -subunits to the cell surface [15] and as a cellular adhesion molecule capable of forming both homotypic and heterotypic associations with other V-set Ig domain containing proteins [16-18].

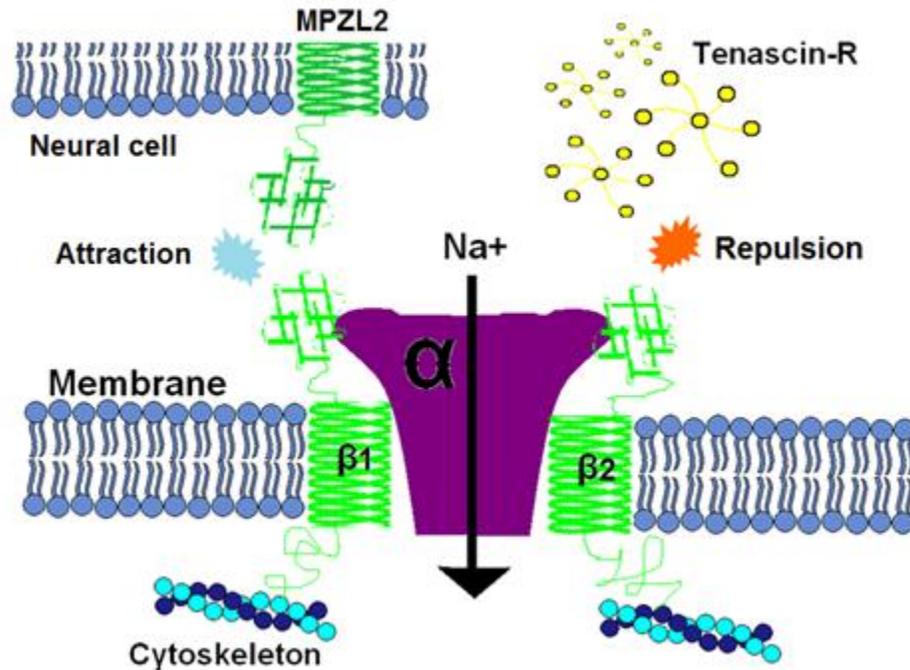


Figure 1.4 Voltage-gated sodium channels are comprised of a pore-forming alpha subunit (shown in purple) and one or two β -subunits (shown in green). The cytoplasmic domain of the β -subunits interacts with the cytoskeleton while their ectodomains interact with the extracellular matrix, presumably with other V-set Ig-loop proteins. Also depicted is tenascin-R (shown in yellow), which has some domains repulsive to SCN2 β , used in axon guidance, and MPZL2, a neural CAM binding partner of SCN2 β .

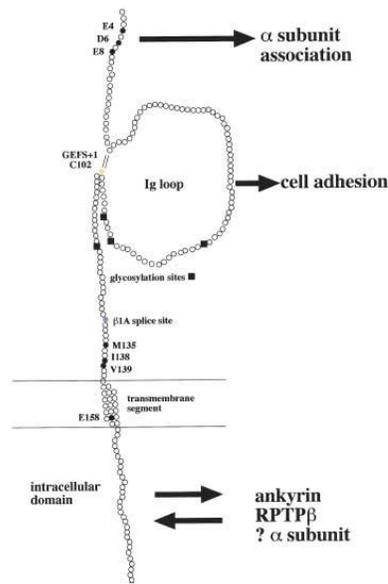


Figure 1.5 The structure of SCN2 β contains a V-set type Ig loop in the extracellular or “ecto” domain that is highly conserved when compared to several neural cellular adhesion molecules [19].

1.5 Project outline and Rationale/Impact

Prostate cancer treatments today are relatively effective for localized cancer, but once the disease spreads beyond the prostate, the average five year survival rate drop drastically to below 30%. The development of a successful treatment strategy for metastatic, advanced stage and grade PCa depends on a fuller understanding of how and when the cancer metastasizes. The currently accepted view on PCa dissemination invokes passage through the bloodstream and/or the lymphatic system. However, these mechanisms do not account for the specific preferential migration of PCa cells to the lower thoracic, lumbar, and sacral vertebrae (Figure 1.2). This, in combination with the high frequency of PNI observed, lead me to hypothesize that PCa cells migrate out of the prostate along the abundant nerves connecting the prostate to the base of the spine. Furthermore, as VGSCs (particularly SCN2 β) increase the metastatic behavior of cancer

cells, they most likely mediate PCa cell migration along nerve tracks. I hypothesize that SCN2 β promotes PNI association and migration.

My project includes several different approaches to studying the activities of cellular adhesion molecules potentially associated with prostate cancer metastasis. I have participated in many of the steps along the way to testing these molecules, from generating stable lines of LNCaP cells overexpressing desired proteins to performing a few functional assays to test the role of those proteins on cell behavior. My first aim was to clone the cDNAs for several cellular adhesion molecules (CAMs) and their ectodomains that are predicted to or have been shown to either adhere (positive controls) or repel (negative controls) SCN2 β . These included the full length and ectodomain of MPZL2, the EGF-L and EGF-S domains of tenascin-R (TnR), and the C-terminal domain of SCN2 β itself. MPZL2 is a neural CAM expressed by nerve-associated cells, called Schwann cells [20]. TnR is an extracellular matrix molecule that can interact with SCN2B, but its EGF-L and EGF-S domains have been shown to be repulsive to SCN2 β [19]. My second aim was to perform migration assays on cells overexpressing SCN2 β under a variety of different conditions. These included assaying cell migration on differential cellular matrices, treatments with certain protease inhibitors, and treatments with several conditioned media.

Chapter 2

MATERIALS AND METHODS

2.1 Cell lines and culture

Cells from the isogenic LNCaP lineage [21] were cultured in complete T-media (Invitrogen, Carlsbad CA), supplemented with 5% fetal bovine serum (FBS) and 1% penicillin streptomycin, and incubated at 37°C in 95% humidity. The cells were grown on 100mm dishes in about 10 ml of media. Their media was replaced every 2 to 3 days and passaged 1:8 about once a week between 90% and 100% confluence with trypsin (Invitrogen, Carlsbad CA, R001100). The LNCaP cell lineage mimics clinical PCa by progressing from a weakly metastatic, androgen sensitive phenotype representative of early PCa to a castrate resistant and strongly metastatic phenotype characteristic of advanced PCa.

2.2 Cloning for overexpression of desired CAMs in the LNCaP lineage

The cDNA sequence encoding the full length open reading frame and ectodomain of MPZL2, the C-terminal domain of SCN2 β , and the EGF-S domain of Tenascin-R (TnR), were subcloned into mammalian expression vectors for downstream transfection into the LNCaP cell line (Figure 2.1). Primers were designed to amplify the desired insert with PCR. Primers also contained a peptide tag (myc-His) and specific restriction sites on either side of the sequences for easy insertion into cloning vectors. Primers used and their sequences are shown in Table 1. The PCR products were extracted from 0.8% agarose gels and ligated into the TA cloning vector pCR2.1-TOPO (Invitrogen,

Carlsbad, CA, KNM4500-01) via TOPO isomerase following the manufacturer's recommendations. Clones were confirmed by direct sequencing of the plasmid DNA acquired after bacterial transformation and amplification with TOP10 E. coli (Invitrogen, Carlsbad, CA, C404010) in LB broth supplemented with kanamycin (50µg/ml). The domain sequences were then digested from the vector using the restriction enzymes *BamHI* and *AgeI* (*NheI* and *XhoI* for TnR) overnight at 37°C. The products were gel purified and then ligated into the mammalian expression vector pcDNA3.1 for 3 hours at room temperature. Following the ligation, TOP10 E. coli cells were transformed, and the clonal DNA digested for confirmation of insert. Upon the presentation of a proper sized insert, the clones were sequence again. DOTAP liposomal transfection reagent (Roche, Germany, 1181117 001) and Lipofectamine LTX with PLUS reagent (Invitrogen, Carlsbad, CA, 15338-100) were used to transfect recombinant DNA constructs into Chinese Hamster Ovary (CHO) for protein expression. Selection for stable clones was done using 800 µg/mL G418 Sulfate.

Table 1. Primers used to amplify protein domains from corresponding mRNA with RT-PCR. Forward (F) and reverse (R) primers were designed to include restriction sites on either side of the insert, as well as peptide tags (myc-His) and Kozak sequences when necessary.

Gene	Primer sequence (5'→3')	Size (bps)
MPZL2 full length (F) MPZL2 full length (R)	-CGGATCCTTATGTATGGCAAGAGCTCTACT -TCTCTGTTTATTTAGAAGACACAGACGGTAAGCCTATCC CTAACCCCTCTCCTCGGTCTCGATTCTACGACCGGT	560
MPZL2 ectodomain (F) MPZL2 ectodomain (R)	- CGGATCCTTATGTATGGCAAGAGCTCTACT -ACCGGTCGTAGAATCGAGACCGAGGAGAGGGTTAGGGAT AGGCTTACCCAGGAAGTGGATCTCAGAGAAGCCT	463
SCN2β C-terminal (F) SCN2β C-terminal (R)	- GGATCCCCAGCCATGGNNTGTGTGAGG -TTCGGACTACTTGCGCCATCATCCGG	103
TnR EGF-S (F) TnR EGF-S (R)	-GCCTAGGAATAGACGTTGCTTCCGACC -ACCGGTCGTAGAATCGAGACCGAGGAGAGGGTTAGGGAT AGGCTTACCGCAGTCAGGGCCCTGGTA	408
TnR EGF-L (F) TnR EGF-L (R)	-TTGCGGTTGACGACGGTTAAGGAGCCG -ACCGGTCGTAGAATCGAGACCGAGGAGAGGGTTAGGGAT AGGCTTACCGCAGTCAGGGCCCTGGTA	504

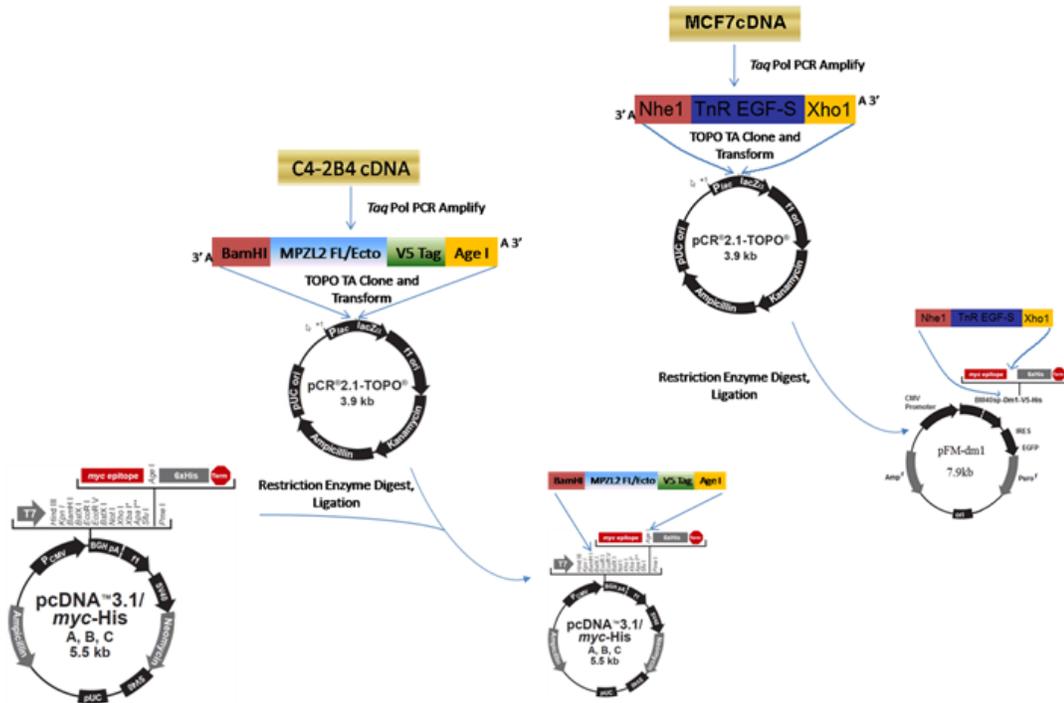


Figure 2.1. The strategy used to subclone MPZL2 (full length and ecto, on the left) and TnR EGF-S (right) into mammalian expression vectors. The C-terminal domain of SCN2 β was later cloned into the pcDNA 3.1 myc-His vector using the strategy on the left.

2.3 Migration Assays

Cells were seeded on 60mm plates at approximately 3.0×10^6 cells per plate and allowed to pack tightly overnight. The following day, after a media change and/or the appropriate treatment, the plates were scratched with a 1000 μ L silicone coated pipette tip. The scratches were photographed at 4 zones every 24 hours until they closed completely. Migration assays were quantified using Adobe Photoshop by measuring wounds in pixels, converting pixels to μ m by direct comparison to a micrometer photographed at the same magnification. Width was measured at multiple points along each photographed scratch,

in the same location on each day, to determine the average μm migrated per day. The migration rate was determined by the average microns migrated each day in all the zones. Assays were repeated a minimum of 3 times with different cell passage numbers. Statistical significance was determined using a student's t-test and a p-value <0.05 was considered significant.

Chapter 3

RESULTS

3.1 Cloning results

3.1.1 MPZL2 full length and ectodomain

I began cloning the full length and ectodomain of the myelin associated protein, MPZL2, using bacterial colonies isolated by Brian Grindel, who had already subcloned MPZL2 full length and ectodomains successfully into the pCR 2.1-TOPO vector. These were digested out of the TOPO vector (Figure 3.1) and then ligated into the mammalian expression vector pcDNA 3.1 myc-His. After digestion to confirm the presence of inserts of the correct size, 650 bps for full length protein and 463 bps for ectodomain (Figure 3.2), the inserts were sequenced before considering the full length of MPZL2 ready for transfection into LNCaP cells. During this process, the ectodomain found to have mutations found at three different locations and cloning was started over.

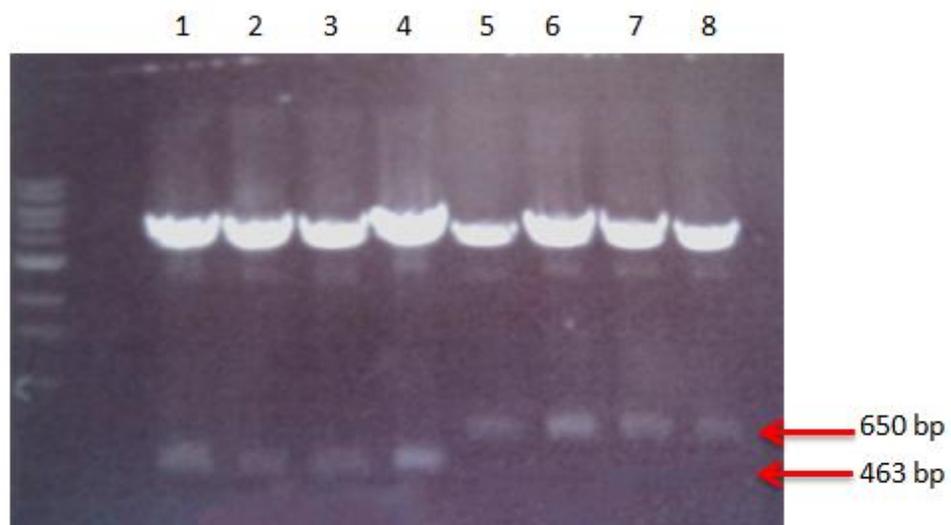


Figure 3.1 The full length and ectodomains of MPZL2 were digested out of the pCR 2.1 TOPO cloning vector using the restriction enzymes *BamHI* and *AgeI*. The fragments indicated were gel purified for use in successive subcloning steps.

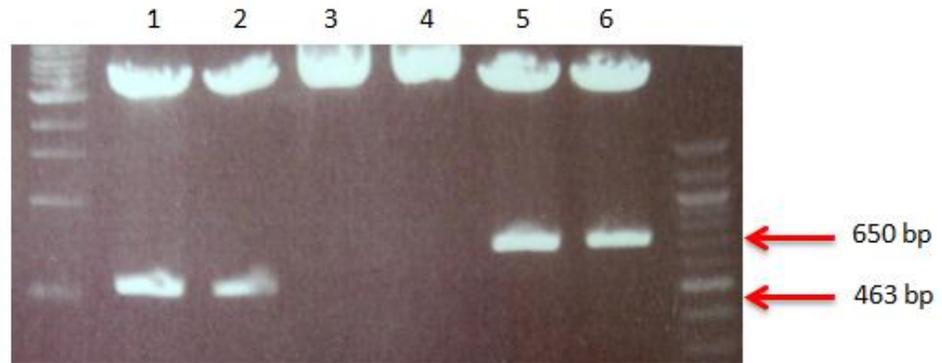


Figure 3.2 The full length of MPZL2 (right side) cDNA clone was digested from the pcDNA 3.1 myc-His vector with the restriction enzymes *BamHI* and *AgeI*. It was 100% sequence confirmed. The cDNA clones of the ectodomain fragment (left side) were found to be mutated at three nucleotides. The full length was used to transfect into CHO and LNCaP cells.

Because of the potential for all other bacterial colonies containing the ectodomain of MPZL2 in the pCR 2.1 vector to be mutated, cloning of MPZL2 ectodomain was restarted with fresh PCR. After a new forward primer for the ectodomain was designed, I amplified the domain using LNCaP cDNA as the template (Figure 3.3) and I subcloned this product into the pCR 2.1 TOPO vector (Figure 3.4) and then into mammalian expression vector pcDNA 3.1 myc-His (Figure 3.5).

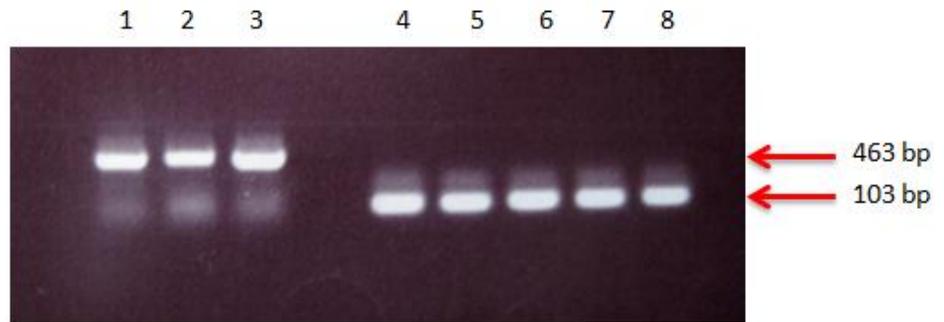


Figure 3.3 Reverse transcription PCR was used to amplify the ectodomain sequence of MPZL2 (lanes 1-3) and the C-terminal domain of SCN2 β (lanes 4-8) using LNCaP cDNA as the template.



Figure 3.4 The RT-PCR products from Figure 3.3 were subcloned into the pCR 2.1 TOPO vector and then digested out with restriction enzymes *Bam*HI and *Age*I for MPZL2 ectodomain (lanes 1-8), and with *Bam*HI and *Csp*451 for the SCN2 β C-terminal fragment (lanes 10-15).

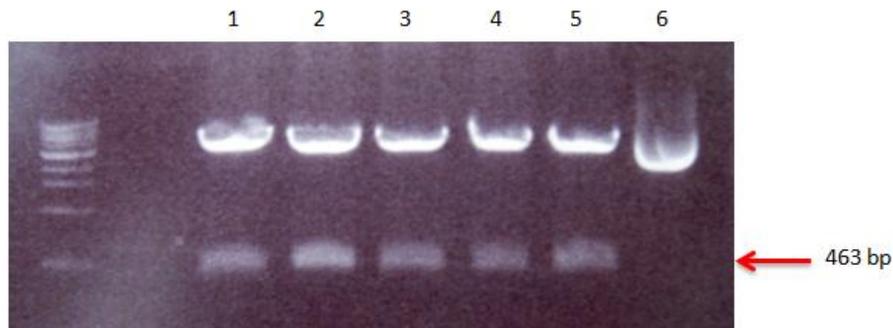


Figure 3.5 The successful ligation of the 100% sequence-confirmed ectodomain of MPZL2 into the mammalian expression vector pcDNA 3.1 myc-His was verified by digestion of the insert out of the vector using restriction enzymes *BamHI* and *AgeI* (lanes 1-5). In lane 6, the uncut, supercoiled vector containing the MPZL2 ectodomain insert is shown for comparison.

3.1.2 Tenascin R, EGF-S and EGF-L

Primers were ordered for two domain sequences of tenascin-R (Tn-R) that are repulsive to the ectodomain of SCN2 β . The primers included restriction sites on either end for the enzymes *BamHI* and *Csp451* along with an inserted stop codon. Similar to the domains of MPZL2, the PCR product was amplified using MCF-7 cDNA as the template that (Figure 3.6) was subcloned into the pCR 2.1 TOPO vector, digested with restriction enzymes (Figure 3.7), and ligated into the pCR 2.1 TOPO cloning vector. As seen in Figure 3.6, the MCF-7 cDNA was a good source from which to amplify the EGF-S domain, but not for the EGF-L domain of TnR. The EGF-S domain was transferred successfully into the TOPO cloning vector and could be used for further downstream cloning.

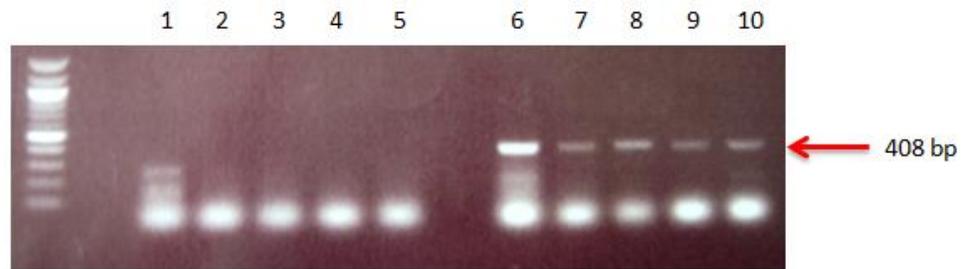


Figure 3.6 The EGF-L (lanes 1-5) and EGF-S (lanes 6-10) domains were amplified with PCR using MCF-7 cDNA as the template at a range of temperatures from 50-60°C. This cDNA was effective as a template for the EGF-S domain (lanes 6-10), particularly at 50°C, but not very effective for amplifying the EGF-L domain (lanes 1-5). While several other templates were attempted, a useful product has yet to be amplified.

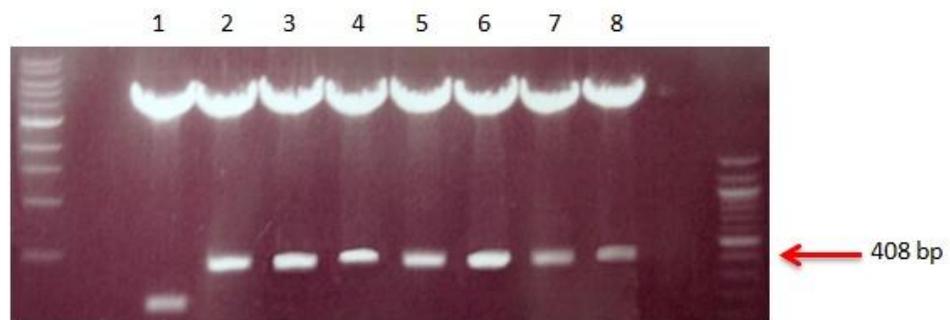


Figure 3.7 After transformation of TOP10 *E. coli* with TnR EGF-S in the pCR 2.1 TOPO cloning vector, digestion with restriction enzymes *Bam*HI and *Csp*45I revealed several bands of the correct size (lanes 2-8). Samples in lanes 3,4,5, and 8 were 100% sequence-confirmed.

3.1.3 C-terminal of SCN2 β

The cDNA sequence corresponding to the C-terminal domain of SCN2 β was cloned next to study its potential role in cell migration. Primers were ordered to amplify

the domain starting from the transmembrane segment on the inner surface to the C-terminal end. Restriction sites were added on each end, and a Kozak sequence was added after the restriction site in the forward primer. Lanes 4-8 of Figure 3.3 show the PCR product of the SCN2 β C-terminal segment using LNCaP cDNA as a template. The purified product was ligated into the pCR 2.1 TOPO cloning vector, as described previously with other inserts. After bacterial transformation and restriction digest of their DNA, three of the clones were found to contain a 100% sequence-confirmed insert for the C-terminal, cytosolic domain of SCN2 β (Figure 3.4, lanes 10-15).

3.2 Migration Assay Results

Migration assays were started with SCN2 β before the subcloning of the other proteins was complete and mainly used cells transfected and confirmed to overexpress the full length SCN2 β as a fusion protein with enhanced cyan fluorescent protein (CFP) affixed to its C-terminus.

3.2.1 Matrix assays

The first assays were done on various cell matrices to compare migration rates of the cells on different substrata. The rates of migration of LN2 β CFP cells were measured on plastic versus dorsal root ganglia (DRG) derived cell matrix to emulate a nerve environment. Cells were also prepared on CHO matrix plates to be used as a negative matrix control, but there was so little initial adhesion that the all the cells lifted off the matrix within 24 hours after the scratch. Figure 3.8 shows the results of the LN2 β CFP migration; the LN2 β CFP cells migrated nearly twice as far on a plain plastic surface than on the DRG matrix.

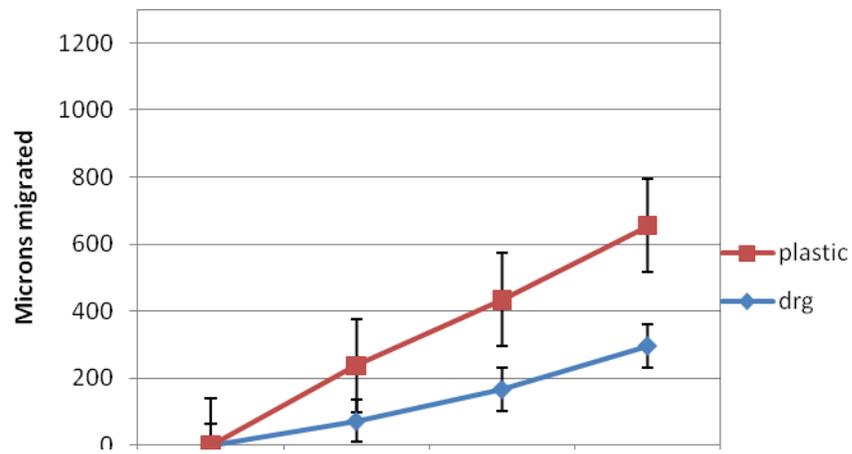


Figure 3.8 LN2 β CFP cells were seeded on DRG matrix, CHO cell matrix, and on plastic, scratched, and the average microns migrated was calculated each day. The cells on DRG matrix migrated consistently about half as far as the cells on plastic. CHO cell matrix had very little LNCaP cell adhesion and produced no data.

3.2.2 Gamma secretase inhibitor assays

SCN2 β is a gamma secretase substrate. If the cleavage of SCN2 β 's intracellular domain is critical to its function in cell migration, inhibiting this action would result in decreased migration. This result is seen in Figure 3.9, C and D. In these assays, both the LNCaPs and the LN2 β CFPs treated daily with gamma secretase inhibitor in DMSO migrated half as far as the control group treated daily with just DMSO. In Figure 3.9 A and B, the media on the control plates was not changed daily, and they were not given the control treatment of just DMSO. In these trials, no difference was seen in the migration of the treated versus zero treatment groups. These data indicate that gamma secretase is required for cell migration.

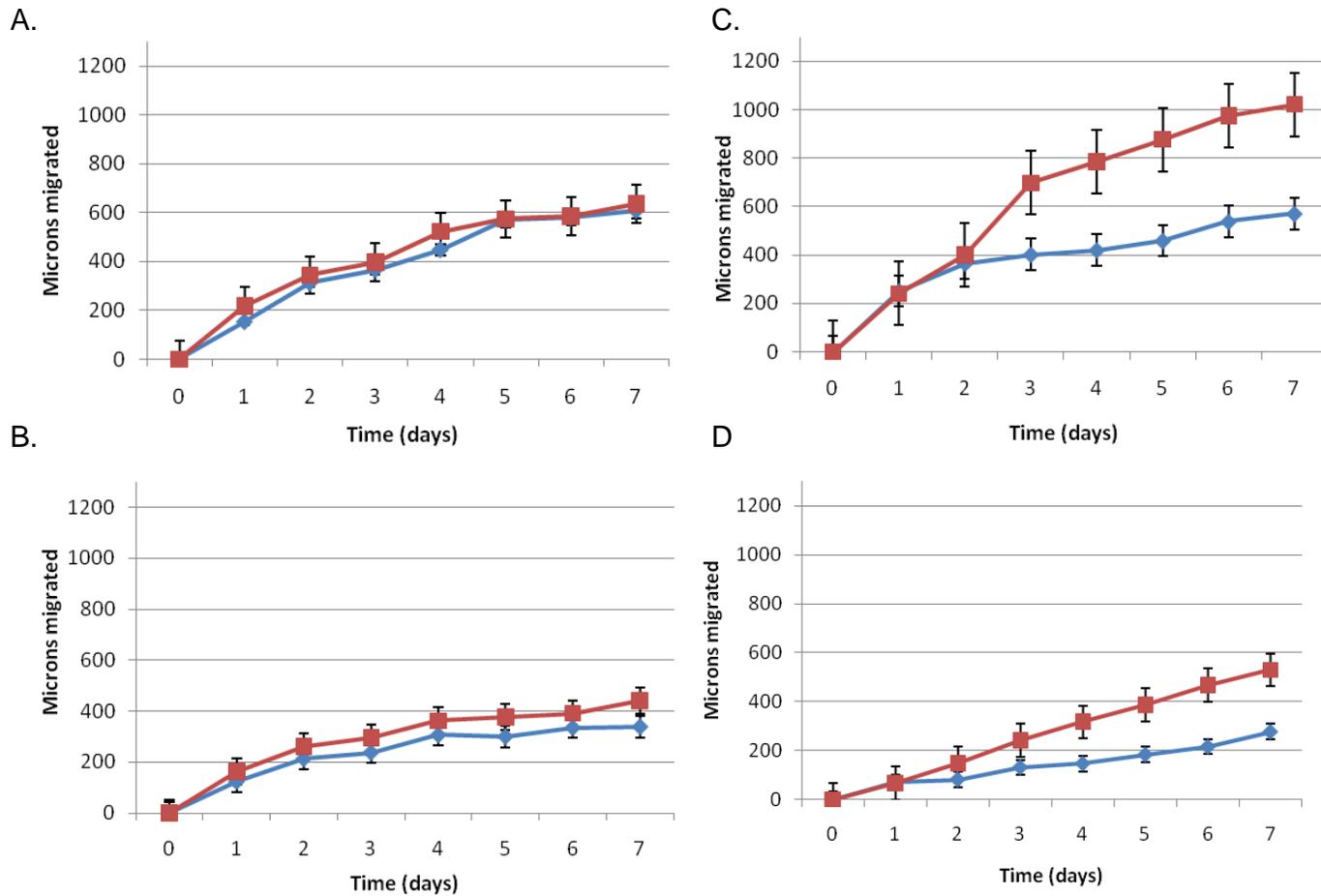


Figure 3.9. Graphs A and C are data obtained with migrations done with LN2 β CFPs; graphs B and D represent assays with LNCFP control cells. Graphs A and B represent the initial assays in which the “control” plate (red squares on both graphs) was not given any treatment at all; in graphs C and D, the control plate was given an equivalent dose of DMSO (vehicle) and their media was changed at the same time as the plates treated with gamma secretase inhibitor. In each assay, the gamma secretase inhibitor plate data is shown in blue circles.

3.2.3 Combination of treatments-matrix and protease inhibitor

Another assay was set up to compare the effects of gamma secretase inhibitor on LN2 β CFP cells plated on plastic versus DRG derived matrix. Cells were seeded on plastic or DRG-derived matrix and each plate then was treated with 4 μ l of either gamma secretase inhibitor or DMSO as control. Unfortunately the gamma secretase inhibitor treatment plate on DRG matrix failed after 24 hours (all cells lifted from plate). Even without that data, though, the cells on DRG-matrix seemed to migrate less than those on plastic, and the gamma secretase inhibitor also seemed to moderately decrease migration.

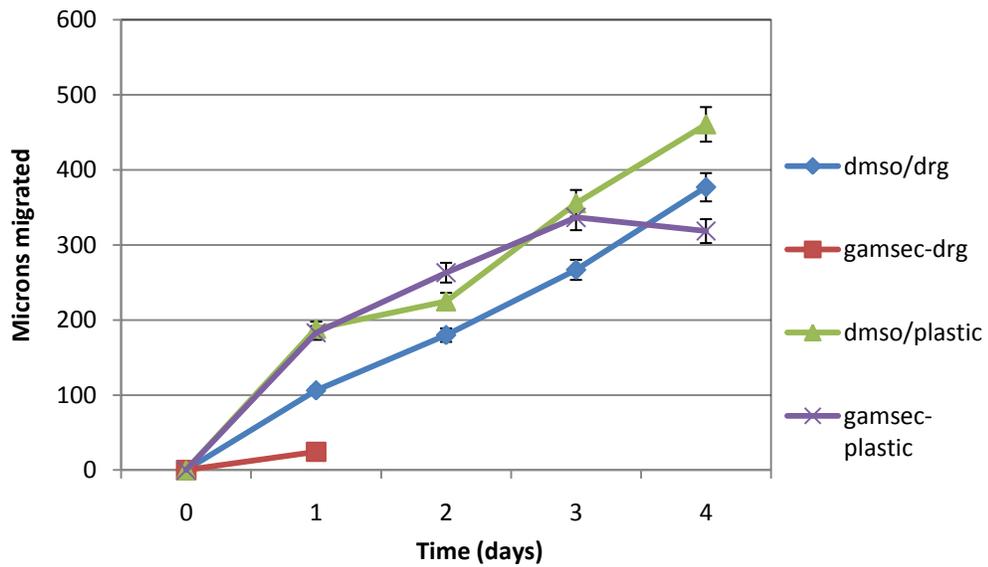


Figure 3.10 This assay comparing the effects of a combination of treatments. LN2 β CFP cells were placed on both plastic and DRG-derived matrix, then treated with gamma secretase inhibitor or DMSO.

3.2.4 Conditioned media assays

The next set of assays involved treating LNCFP cells and LN2 β CFP cells, as well as some using C4-2B cells, with various conditioned media and observing their migration rates. The conditioned media came from regular CHO cells and from CHO cells transfected with SCN2 β 's ectodomain (2 β CHO).

3.2.4.1 LNCFPs and LN2 β CFPs with both CHO media and 2 β CHO media

LNCFP cells and LN2 β CFP cells were treated with the conditioned media collected from CHO cells and CHO cells transfected to overexpress SCN2 β (henceforth 2 β CHO cells). The culture media was changed every other day, with 1.5 ml of complete-T media and 1.5 ml of the appropriate conditioned media treatment. As seen in Figure 3.11, the migration rate of cells treated with CHO media versus 2 β CHO media was nearly identical over the course of the assay. The migration was even similar between LNCFP cells and LN2 β CFP cells.

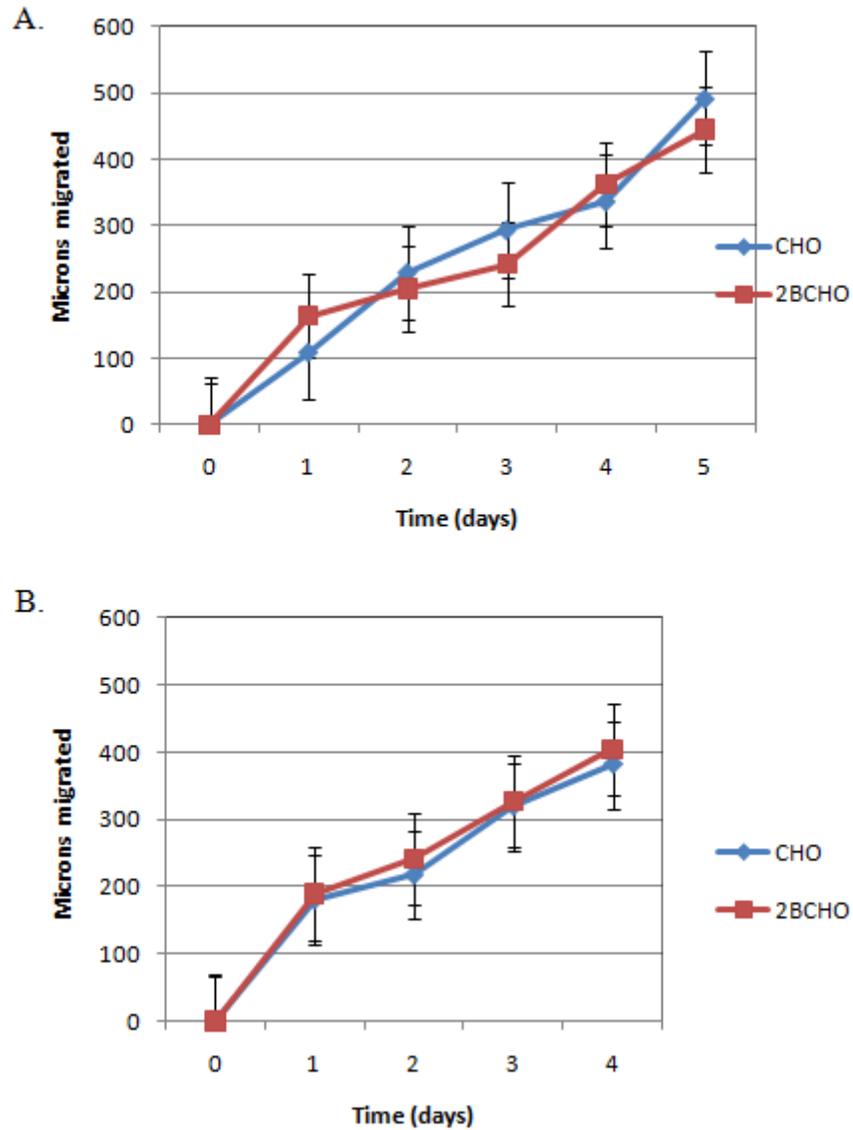


Figure 3.11 In A, LN2 β CFP cells were treated with conditioned CHO media and 2 β CHO media. In B, LNCFP cells were used. The LN2 β CFP cells, regardless of treatment, migrated slightly farther than the LNCFP cells, but within each cell type, the conditioned media treatment did not appear to affect migration.

3.2.4.2 LNCaP with both CHO and 2 β CHO complete and serum free media

The next assay tested whether the effects of growth factors in the complete media were masking quantifiable differences in migration. Untransfected LNCaP cells were used for this assay, and complete and serum-free CHO and 2 β CHO conditioned medias were prepared as treatments. The cells were cleaned and fed every other day with 1.5 ml of complete media, then given their different treatments of 1.5 ml complete CHO media, complete 2 β CHO media, serum-free CHO media, or serum-free 2 β CHO media. The results of this assay are shown in Figure 3.12. Although for the most part, when comparing CHO versus 2 β CHO media treatments, the 2 β CHO media treated cells seemed to migrate somewhat farther, there was very little difference from treatment to treatment overall.

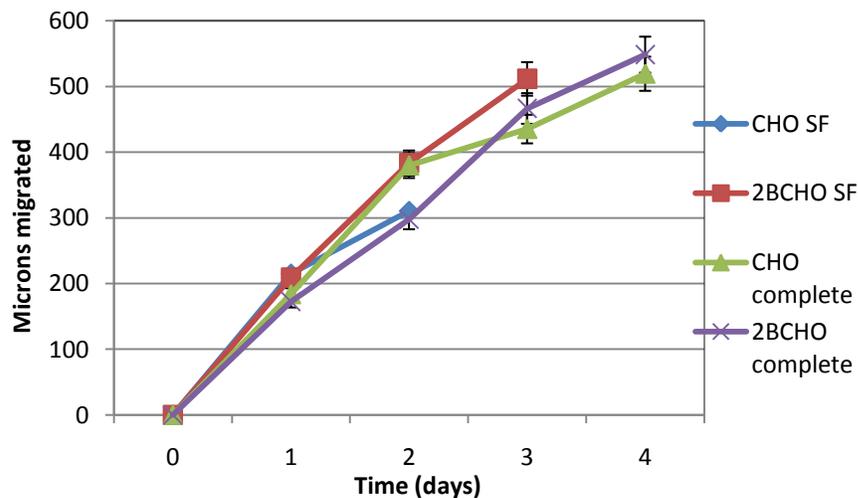


Figure 3.12 The effects of serum vs. serum-free conditions was assayed in LNCaP cells. Complete CHO/2 β CHO conditioned media was given to two plates, and serum-free CHO/2 β CHO media was given to the other two plates.

3.2.4.3 LNCaPs in 2%TCM and with serum-free CHO/2 β CHO media

One more assay was done with LNCaP cells and CHO versus 2 β CHO media, this time in 2% TCM was used to ensure serum-free conditions under optimal nutrient levels from the 1.5 ml of non-treatment media. As seen in Figure 3.13, there was no significant difference between the migration of LN cells treated with CHO media and LN cells treated with 2 β CHO media in totally serum-free conditions.

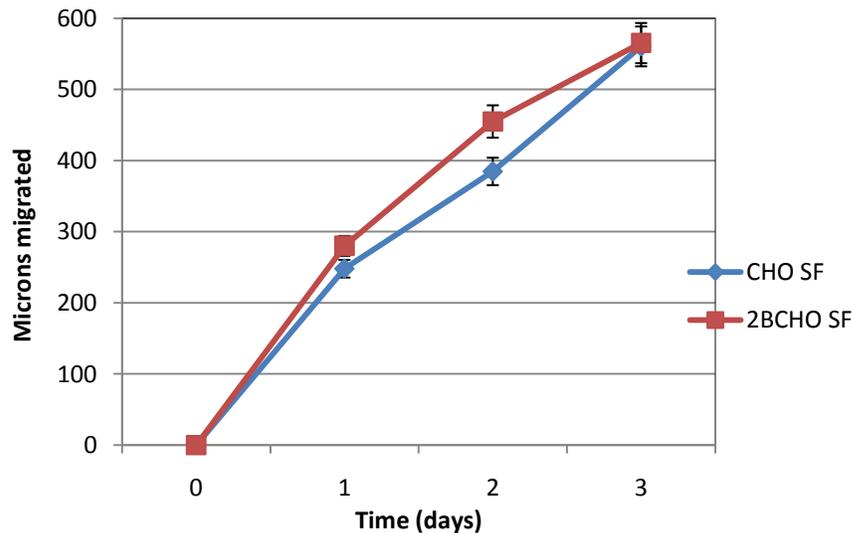


Figure 3.13 LNCaP cells adapted to 2% TCM media overnight were treated every other day with serum-free CHO or 2 β CHO media. Their migration over three days, shown here, is nearly identical.

3.2.4.4 C4-2Bs with CHO media and 2 β CHO media

The first conditioned media experiment was also planned on C4-2B cells to see if the results would be different on the more metastatic cells. From the one short

preliminary assay shown in Figure 3.14, it looks like C4-2B cells treated with 2β CHO conditioned media (complete media) respond and migrate much more than C4-2B cells treated with just CHO conditioned media. By 48 hours the cells treated with 2β CHO media had migrated almost twice as far as untreated cells.

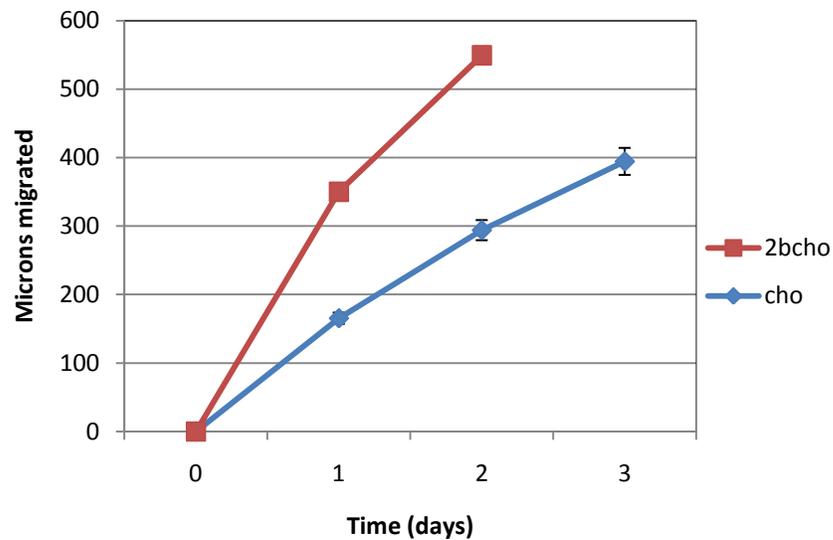


Figure 3.14 B4 cells were cleaned and fed every other day with 1.5 ml of complete media and 1.5 ml of either CHO conditioned media or 2β CHO conditioned media. The 2β CHO media-fed cells migrated almost twice as far as the CHO media-fed cells in just the first 48 hours.

Chapter 4

DISCUSSION

4.1 Cloning

Thus far, I have generated several populations of stable LNCaP transfectants containing the gene for overexpression of the full length protein of MPZL2. Additionally, the ectodomain of MPLZ2, the C-terminal domain of SCN2 β , and the EGF-S domain of TnR have all been cloned into mammalian expression vectors and are ready to be transfected into the LNCaP cell line. These last three protein segments were not transfected due to time constraints and technical issues relating to cloning.

The ectodomain of MPZL2 for example, which had been PCR amplified with the full length, was subject to various setbacks early on during cloning. First, the initial subcloning process was carried out with a mutated DNA segment, which was not identified till the sequencing step after the mutant was cloned into and digested out of the pCR 2.1 TOPO cloning vector. After some troubleshooting as to what may have happened, I found out that the bacterial source from which I took the DNA was already mutated. I decided the best idea would be to begin again with fresh product from PCR. This was done successfully once I learned how to run PCR, but around this time I also started having problems with my gel purifications. With the same protocols and materials, I was getting extremely low yields, which prevented me from performing successful ligations. As a result, my cloning progress stalled. I tried using different agarose, freshly made TAE buffer, and different gel purification kits. Finally I began having more success, most likely as a result of fresh buffer and/or gel agarose because nothing else about my protocols was changed in terms of procedure. The cDNAs of the ectodomain of

MPZL2 and the other proteins could again be isolated following successful ligation, amplification, and restriction enzyme confirmation.

A similar problem occurred with the ligation step, once both digested and purified mammalian expression vector and insert(s) were obtained. Troubleshooting this step also took an unnecessarily long time and included trying several ligation reaction conditions. In the end I determined that the ligase enzyme and my reaction volumes were the problem. When the ligation was done with fresh ligase and ligase buffer in a small volume (10 μ l), it worked. After this, it was easy to ligate all the inserts into their designated expression vectors.

The only insert that was not followed through beyond primer design is the EGF-L domain of Tn-R. I tried several different cDNAs sources as template, including LNCaP, C4-2, C4-2B (prostate), MCF-7 (breast), HEK (kidney), but none yielded a clear product. Instead of wasting time trying to find one while I had the EGF-S domain PCR product, I went ahead with subcloning this domain into the cloning vector. Unfortunately, I did not have time to go back to the EGF-L domain primers.

4.2 Migration Assay Discussion

4.2.1 Matrix assays discussion

The most consistent result in these assays is that migration for both kinds of cells is reduced on DRG matrix when compared to tissue culture plastic. There seems to be something inhibiting the cells from migrating, especially within the first 24 hours after making the wound. In LNCaP cells, the rates are similar at first, but then the cells on plastic continue to migrate rapidly, while the rate of migration of cells on DRG tapers off. In the LN2 β CFP cells, the cells on plastic are clearly the more motile, traveling over twice

the distance of the cells on DRG matrix with cells in each plate moving at what appeared to be a constant rate over the period of the assay. A possible explanation for this observation is that on DRG matrix, the cells adhere more tightly to the matrix through more interactions of SCN2 β . The cells on plastic may just slide along with more ease as well due to charge, as it is a smooth surface unlike almost any material found in the body.

The results of this assay were originally expected to be reversed, and were intended to show greater migration on DRG-derived matrix than anything else. However, as mentioned above, plastic was perhaps not the best control for a “matrix” that PCa cells would normally be exposed to. Instead of showing increased migration on DRG-derived matrix, this assay showed increased adhesion to DRG-derived matrix. Also, three plates per experiment were initially seeded with cells, including one of CHO cell derived matrix. This plate was intended to be the negative control for the assay, as LNCaP cells do not adhere well to matrix derived from these cells. However, the scratch made on the plate caused a most of the cells to shear off immediately, and within 24 hours after there were no cells remaining on the plate even though great care was taken when handling the plates to prevent peeling of the cell monolayers. Therefore, another control matrix is required, either from a new cell type or purified matrix component, like fibronectin or Matrigel.

4.2.2 Gamma secretase inhibitor assays

This next set of assays sought to show that migration would decrease if normal activity of SCN2 β in the cell was inhibited. SCN2 β is a substrate of gamma secretase, an integral membrane protein on the cytosolic leaflet of the plasma membrane that functions as a protease. By treating cells with an inhibitor of this protease’s activity, migration dependent on this activity should have decreased.

The good thing about these assays is the consistent response from all the cells treated with gamma secretase inhibitor. As long as the cells treated with the inhibitor were fed every day and fresh drug (inhibitor) replaced, then the cell behavior was very reproducible. It is also interesting to note that the LN2 β CFPs always migrated more than the LNCFPs in each set of assays. In both assays involving LNCFPs, the cells in the treatment plates migrated between 300 and 400 microns in 7 days. The LN2 β CFPs migrated a bit more, but in both assays, consistently reached 600 microns in 7 days. This is good, but the most interesting information comes from comparing the migration of the “control” plates from each assay.

In the first two assays (Figure 3.9, Graphs A and B), the media was changed every other day and no DMSO (vehicle for the inhibitor) was added to non-treated cells, while in the second assays (Graphs C and D), a vehicle control was performed and the cells were fed and treated daily with DMSO or gamma secretase inhibitor in DMSO. In the first two assays, the no treatment cells migrated almost exactly as much as the treated cells. In the second two assays, however, both the LNCFPs and the LN2 β CFPs cells migrated nearly twice as much as the treated cells.

Though at first, the data from the first pair of assays proved disappointing in supporting the role of SCN2 β in enhancing cell migration, this data is useful to illustrate the general migration of cells on plastic under my treatment conditions. When cells are provided with a constantly renewed source of nutrients, they migrate at the higher rates seen in the control plates of the second two assays. If they are only fed every 2-3 days, their migration is slower. However, as they migrate faster under the same conditions as cells treated with the inhibitor, the conclusion can still be made that the inhibitor could be responsible for this observation. In the first two, poorly controlled assays, the gamma

secretase inhibitor treatment was not affecting cell migration, rather, the lack of constant care treatment was slowing untreated cells' migration, incidentally, to the same rate as gamma secretase inhibited cells. The vehicle control, in this case DMSO, may also have had an effect on the cells' migration, as it differed between cells treated with DMSO and cells receiving no treatment whatsoever. Nonetheless, as DMSO was the inhibitor, the best control for this experiment is a DMSO treated control.

4.2.3 Combination of treatments: matrix and protease assays

Based on the results from the previous sets of assays, this assay was set up to test the effect of several treatments in combination. The cells were plated on both plastic and DRG-derived matrix as well as treated with gamma secretase inhibitor (or a vehicle control treatment). Unfortunately, the gamma secretase inhibitor treatment plate of cells on DRG matrix failed after 24 hours; this may be due to the plate being handled too roughly at times. This also may have been the reason for all cells in this experiment performing poorly, because the cells on other plates also were lifting off the plate in several areas including some of the zones that were photographed. Data points could still be collected from these cultures, however, and are shown in Figure 3.10.

Other than that one data set, the cells with the shortest/slowest migration were those on DRG matrix. These were given just a DMSO treatment, so the only real factor was the matrix. The cells on this plate migrated less than cells on plastic with this treatment, and also less than cells on plastic with gamma secretase inhibitor. This is consistent with the results from previous DRG migration assays, although a few more runs would be helpful to replace the data from the lost plate. There is not much difference between the migration of cells treated with the protease and untreated cells on plastic.

This could be due to the fact that the cells were disturbed, and the zones where the migrated distance was quantified may have not represented actual migration.

4.2.4 Conditioned media assays

These assays were meant to test the effect of a lot of soluble SCN2 β floating around outside the cell. Presumably, if abundant SCN2 β , even just a part of it with the important Ig-loop present, was swirling around the LNCaP cells, there would be many homotypic interactions with the cellular SCN2 β . Decreased migration was expected across all cells by virtue of occupying the SCN2 β with soluble ectodomain or saturating the plate with ectodomain. This was not the case and virtually no effect could be observed with LNCaP cells. However, the SCN2 β ectodomain cloned into the CHO cells lacked a Kozak sequence. The apparent lack of effect could be due to low expression and secretion of the protein by the CHO cells into the media.

4.2.4.1 LNCFPs and LN2 β CFPs with CHO or 2 β CHO media

In this first conditioned media assay, the LNCFP and LN2BCFP cells were given a 50:50 media change, with half of the new media being complete-T media and the other half being conditioned media from either CHO or transfected 2 β CHO cells. This assay sought to test the effect, if any, of soluble SCN2 β available in the medium, and the cells exposed to SCN2 β in the media were expected to migrate less quickly. My results showed no difference in migration with or without SCN2 β added. However, it is possible that the concentrations of SCN2 β in the treatment media were too low to have any effect whatsoever.

4.2.4.2 LNCaP with both CHO and 2 β CHO complete and serum free media; LNCaP in 2%TCM and with serum-free CHO/2 β CHO media

This next assay was thought of to account for the serum in the conditioned media; perhaps the cells in regular CHO conditioned media were getting extra growth factors as well as the cells receiving 2 β CHO conditioned media. This time, the cells were fed 1.5 ml complete T-media and 1.5 ml of either CHO or 2 β CHO serum-free media. LNCaP cells were used this time since there had been no difference detected between the migration of LNCaP cells and LN2 β CaP cells; one type of untransfected cell was used to keep it simple. The plates were also less traumatized during data collection and media changes, so this data is more reliable. However, as seen in Figure 3.12, the results of these assays also showed no difference in LNCaP cell behavior when conditioned SCN2 β ectodomain was added.

Then, it was realized that the plentiful growth factors in the complete media might be having an effect in masking differential migration as well. To account for this, the next assay I set up was similar to the former, but the cells were adapted to 2% TCM for 24 hours before the assay was started. The cells were fed each day with 1.5 ml of 2% TCM and 1.5 ml of serum-free CHO or 2 β CHO media. Even with every possible source of interfering growth factors controlled, there was no difference in the migration of LNCaP cells treated with CHO and 2 β CHO conditioned media (Figure 3.13). Most probably, the problem was with the conditioned media not being concentrated enough, and therefore having no effect on the cell migration.

4.2.4.3 C4-2B with CHO media and 2 β CHO media

Interestingly, C4-2B cells, a castrate resistant and metastatic variant of LNCaP that expresses more SCN2 β mRNA migrate twice as fast in 2 β CHO conditioned medium than CHO control medium. In spite of the interference believed to be a result of

soluble SCN2 β , C4-2B4 cells appear to be stimulated to enhance motility by soluble SCN2 β ectodomain expressing CHO cell conditioned medium. This contrasts what I thought would happen at the beginning of the assay. In contrast to the LNCaP results, these data suggests that 2 β CHO are producing enough SCN2 β -ectodomain to cause an effect if the cells are capable of responding to soluble ectodomain, and unlike LNCaP cells, C4-2B cells are primed to respond to SCN2 β cues. Therefore, SCN2 β induced cell migration appears to correlate more with the migration of castrate resistant PCa cells as compared to androgen sensitive PCa cells.

4.3 Summary and future work

Prostate cancer is a progressive disease with no treatment or cure for its advanced and metastatic stages. 85% of these advanced cases exhibit perineural invasion and in 90% of cases, secondary tumors are found forming in the lumbar and sacral regions of the spine. These statistics are too great to be meaningless. Understanding the molecular mechanisms of prostate cancer progression and how the metastasis occurs is crucial to the development of novel treatments for advanced stage and grade PCa. If the general hypothesis that PCa cells migrate along nerve fibers to the spinal cord rather than through the bloodstream/lymphatics is correct, new treatments of metastatic PCa are required. It would also explain why current treatments of PCa once it has metastasized rarely succeed due to the lack of a proper therapeutic target.

As for SCN2 β , it is clearly an important molecule in PCa metastasis. Blocking its normal function in the cell with a protease inhibitor decreased migration of both cells overexpressing it and not. Cells overexpressing the protein migrated farther in the same time than normal control cells, most likely due to an excess of the protein performing its normal function in promoting cell migration. This also indicates that

gamma secretase inhibitors that are non-toxic could provide significant therapeutic effect. When the cells are plated on matrix derived from DRG cells, their migration is decreased; this is probably also due to the fact that the overexpressed SCN2 β proteins are interacting with the surface and are turning over more slowly. Plastic was probably not such a great control, because it has no structures the cells could adhere to that resemble anything in a human body. A different matrix (other than CHO) similar to perhaps the PCa cells' own matrix would have been a better choice. Nonetheless, that assay serves to illustrate the strength with which the PCa cells adhere to nerve-associated cell matrix, and the cells did not just stay still upon the matrix.

An interesting future experiment would be to test the binding strength with atomic force microscopy (AFM) of the cell on the surface of a DRG-derived matrix coated plate. It should be several times stronger than the adhesion force of the cells on plastic. The other protein domains I have in mammalian expression vectors need to be transfected into the LNCaP cell line, the cells selected and amplified, and then expression of the desired proteins confirmed by Western blot. Once this is done, these protein-expressing cells can be used in various assays to further discover SCN2 β 's interactions. AFM could be done to measure the strength of SCN2 β 's adhesion with MPZL2, and many different migration assays with cells overexpressing any combination of the proteins would yield enlightening results.

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