

**THE ROLE OF NEU1 SIALIDASE IN EPIDERMAL GROWTH
FACTOR RECEPTOR ACTIVATION**

by

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A thesis submitted to the Department of Microbiology & Immunology
In conformity with the requirements for the degree of
Master of Science

Queen's University
Kingston, Ontario, Canada
June 2011

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Abstract

The epidermal growth factor receptor (EGFR) exists as a single, highly glycosylated subunit receptor on the plasma membrane of a cell. Upon ligand binding to its extracellular domain, the EGFR dimerizes with an adjacent receptor. This results in activation of the EGFR's intracellular tyrosine kinase domain, and consequently, autophosphorylation of specific tyrosine residues on the receptor's cytoplasmic tail. Adaptor proteins bind to these phosphorylated tyrosine residues and transduce the message internally, initiating a multitude of signalling cascades which stimulate cell growth, division, and movement. Despite all that has been elucidated regarding the activation and signalling pathways of the EGFR, the parameters controlling dimerization and activation remain unknown.

Recently, Neu1 sialidase, an enzyme which cleaves α -2,3-linked sialic acids from glycosylated substrates, has been implicated as a critical mediator of TrkA receptor activation. Upon activation, the sialidase desialylates the external receptor glycosylation, removing a physical barrier which was formerly hindering receptor dimerization, and thus, receptor activation. Based on the known sialylation of EGFR glycosylation, as well as the demonstrated importance of receptor glycosylation in EGFR activation, we hypothesized that the EGFR may be activated by a similar mechanism.

Here, we report an identical membrane signalling paradigm initiated by epidermal growth factor (EGF) binding to EGFR to rapidly induce Neu1 sialidase activity in live NIH3T3-EGFR cells but not in live Neu1-deficient human fibroblast cells. Furthermore,

we report that Neu1 sialidase activity is required for EGFR activation, supported by the finding that tyrosine phosphorylation is inhibited in EGF-stimulated NIH3T3-hEGFR cells which have been pretreated with both broad-range (oseltamivir phosphate) and specific (anti-Neu1 neutralizing antibody) sialidase inhibitors. MMP-9 plays a role in the initiation of Neu1 sialidase post-ligand binding, as pre-treatment of NIH3T3-hEGFR cells with specific MMP-9 inhibitor prior to EGF stimulation blocks membrane sialidase activity as well as tyrosine phosphorylation. Of critical importance to this schematic is the finding that both Neu1 and MMP-9 co-immunoprecipitate with EGFR on the plasma membrane of both naïve and EGF-stimulated NIH3T3-hEGFR cells. Together, these findings reveal a novel EGFR activation mechanism in which cross-talk between Neu1 and MMP-9 plays a vital role in EGF-induced receptor activation.

Acknowledgements

Obtaining this degree would not have been possible without the support of many important people in my life during the past few years.

First of all, I would like to thank my supervisor, Dr. Myron Szewczuk. Thank you for taking a chance on me three years ago as an undergraduate thesis student, thank you for your guidance and mentorship throughout the ups and downs of research, for the time you've devoted to my education, and for believing in me and my capabilities every step of the way. I would also like to thank my committee members, Dr. Andrew Craig and Dr. Bruce Elliott. Your expertise, knowledge, and suggestions have been invaluable to me during this process. Additionally, thank you to Dr. Katrina Gee, Dr. Leda Raptis, and Dr. Ken Jarrell for your involvement as members of my thesis examination committee.

Thank you to the other members of the Szewczuk Lab, both past & present. It's been a pleasure working beside you, collaborating with you, and learning from you. I wish you all success in your current and future endeavours. Thank you to the rest of the Dept. of Microbiology & Immunology. To the grad students – thank you for two years of great camaraderie (and cooking tips). Thank you to the department's support and technical staff, especially Jackie, Chris, and Jerry – all that you do does not go unnoticed. A huge thank you to Julia Kim – for your friendship and for enabling my coffee addiction on a daily basis.

I have been blessed with great friends who must be thanked for helping me maintain balance in my life during my time as a graduate student. A special thanks to Jill

Martens and Rachel Merino, as well as those I have lived with during my six years here at Queen's. I am so thankful for you all.

Finally, I'd like to thank my family, without whom none of this would be possible. Thank you to my grandparents, Ian and Marie Brown, and my aunt Sheila for supporting me in everything I do. Thank you for celebrating my victories with me and for encouraging me during my trials. Thank you to my Dad for inspiring me to keep challenging myself. To my sister, Stephanie – thank you for the phone calls, for all of the advice (yes, even the unsolicited!), for your “science facts”, and for your ways of finding the humour in our day-to-day lives. Finally, thank you to my Mom - you have always been my #1 fan and no words that I write here could ever sufficiently thank you for that. You are a daily example of strength and perseverance – attributes which I have tried to emulate myself while completing this degree. I love you and I dedicate this thesis to you.

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List of Abbreviations

4-MUNANA	2'-(4-methylumbelliferyl)- α -D-N-acetylneuraminic acid
EBP	Elastin binding protein
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
FBS	Fetal bovine serum
JAK	Janus Kinase
GPCR	G-protein coupled receptor
MAPK	Mitogen-activated protein kinase
MMP	Matrix metalloproteinase
NGF	Nerve growth factor
NF κ B	Nuclear transcription factor kappa-B
PBS	Phosphate-buffered saline
PI3K	Phosphoinositide 3-kinase
PIPZ	Piperazine
PPCA	Protective protein cathepsin A
pEGFR	Phosphorylated epidermal growth factor receptor
pTyr	Phosphorylated tyrosine
RTK	Receptor tyrosine kinase
STAT	Signal transducer and activator of transcription
Tami	Oseltamivir phosphate
TBS	Tris-buffered saline
T.cruzi	Trypanasoma cruzi
TLR	Toll-like receptor
TrkA	Trk tyrosine kinase receptor A
TS	Trans-sialidase

Chapter 1: Literature Review

This body of work represents a novel intersection between two established fields of study: sialidase biology and epidermal growth factor receptor (EGFR) activation. Therefore, in order to fully appreciate the specific scientific niche which this work aims to address, it is important to first gain a deeper understanding of the past and present research occurring in both of these disciplines. In addition, it is necessary to explore previous work pertaining to Neu1-mediated TrkA and Toll-like receptor activation, as it represents the foundation upon which this project is built.

1.1 Receptor Tyrosine Kinase (RTK) Family of Receptors

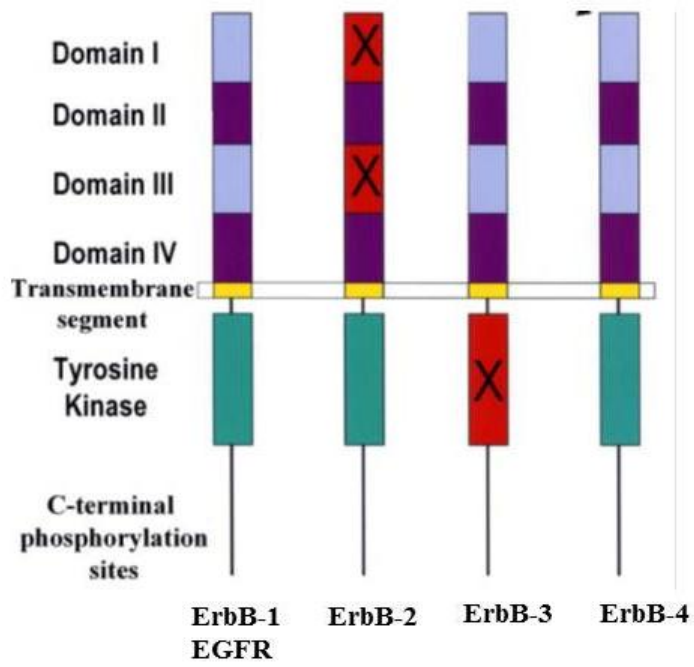
The receptor tyrosine kinase (RTK) family is a group of receptors characterized by their subcellular localization on the plasma membrane, their trans-membranal orientation, and the presence of a tyrosine kinase domain on the cytoplasmic portion of the receptor. The RTK family is an extensive collection of receptors, with humans currently known to possess 58 different RTKs present in a diverse array of cell types throughout the body [1]. In order to survive and thrive, a cell must be able to evaluate its extracellular environment and respond to it both efficiently and effectively. RTK receptors are key mediators in this process, specializing in receiving external signals via a binding peptide ligand and transmitting these signals to the cell's intracellular environment [2], ultimately altering the cell's gene expression patterns in order to suit its

particular needs at the time [3]. While RTKs exhibit a fair amount of redundancy within their signalling pathways, often relying on the same major signalling networks to fulfill their missions within the cell, their downstream effects within different cell types are varied and complex, ranging from embryogenesis and cell growth to neurite extension and apoptosis [3, 4].

1.2 The ErbB Family of Receptors

The ErbB family of receptors, an RTK sub-family, is composed of four different receptors: ErbB-1, -2, -3, and -4. While significant homology does exist between the receptors, they also display several key differences in receptor structure which impacts their eventual function. For example, the ErbB-2 receptor is unable to bind ligand due to a truncated extracellular domain and the ErbB-3 receptor lacks a functional intracellular tyrosine kinase domain [5]. Each receptor, aside from ErbB-2, is able to bind a multitude of ligands, and redundancy exists within their ligand specificities. ErbB-1 binds epidermal growth factor (EGF), transforming growth factor- α , heparin binding-EGF, amphiregulin, epiregulin, epigen, and betacellulin. ErbB-3 binds neuregulin-1 and -2. ErbB-4 binds neuregulin-1,-2,-3,-4, betacellulin, epiregulin, and heparin binding-EGF [6-8] (Figure 1.1).

Figure 1.1. The ErbB family of receptors. The ErbB family of receptors consists of ErbB-1 (EGFR), -2, -3, and -4. These trans-membrane receptors are characterized by their location on the plasma membrane, as well as the presence of a tyrosine kinase domain on their intracellular domain. Despite their similarities in structure, they also have differences: ErbB-2 does not have a functional ligand-binding domain on the extracellular portion of the receptor, and ErbB-3 does not have a functional tyrosine kinase domain. Figure adapted from Roskoski, 2004.



1.3 ErbB-1, The Epidermal Growth Factor Receptor

1.3.1 Overview

ErbB-1, hereafter referred to as the epidermal growth factor receptor (EGFR), is expressed in high levels on epithelial, smooth muscle, neural, and embryonic mesenchymal cells [9]. EGFRs play a crucial role in a vast array of cellular and physiological processes, having been implicated in everything from cell homeostasis and growth to cell movement. In recent years, research in this field has focused on EGFR activation and signalling as it relates to the current hot topics of oncogenesis and embryogenesis. EGFR overexpression has become a well-established oncological biomarker (particularly in breast, pancreatic, non-small cell lung, and prostate cancer), providing important information to clinicians about the pathological state of a patient. Additionally, EGFR inhibition is now considered a desired outcome when developing novel chemotherapeutics [10-12]. The EGFR is also required for embryogenesis. EGFR knockdown/out mouse models have been used extensively in this field to highlight the importance of proper EGFR function in the prevention of glandular, neural, and cardiac developmental defects [9, 13]. It is important to note that the EGFR is not only required for embryonic development, but also in the mature organism, a prime example being the EGFRs role in the growth, differentiation and re-modelling of pre- and post-natal mammary gland and tissues [14].

The EGFR also plays a major role in epithelial wound repair. Epidermal growth factor (EGF), a ligand of the EGFR, becomes up-regulated due to increased mRNA expression of EGF by macrophages and epithelial fibroblasts present at the wound

location [15]. During the re-epithelialization stage of wound healing, EGFR signalling plays a major role not only in stimulating keratinocyte growth and multiplication, resulting in an increase in epithelial thickness at the wound site, but also in promoting cell migration to the injured area [16].

1.3.2 Structure of the EGFR

The EGFR is composed of three distinct domains: an extracellular domain, a trans-membrane domain, and an intracellular domain [11, 17]. Each domain has its own purpose and function in EGFR signalling. The extracellular domain is further sub-divided into four domains: 1, 2, 3, and 4 [18]. Domains 1 and 3 are composed primarily of leucine-rich β -helical barrels and together form the ligand-binding site [19, 20]. Domains 2 and 4 are cysteine-rich and regulate ligand-binding affinity by altering the conformational arrangement of domains 1 and 3 [21]. The single trans-membrane portion of the EGFR is α -helical in structure and ensures that the EGFR remains stably anchored in the plasma membrane. The intracellular domain of the EGFR consists of an internal tyrosine kinase enzyme as well as specific tyrosine residues on its C-terminal tail that become phosphorylated during EGFR activation and serve as docking sites for secondary signal transducers [11]. The EGFR has an average half-life of 20hrs on the plasma membrane before it becomes internalized. This time period is reduced to 8hrs in the presence of EGF stimulation.

1.4 Epidermal Growth Factor

As its name suggests, the natural ligand of the EGFR is epidermal growth factor (EGF) [22]. Epidermal growth factor is a 53 amino acid long polypeptide, containing three disulphide linkages which segment the polypeptide into a single α -helix and two β -sheets [20]. EGF naturally exists as a trans-membrane protein, requiring cleavage before it is able to associate with the extracellular ligand-binding domain of the EGFR and induce activation [6].

1.5 EGFR Activation

1.5.1 Overview

The EGFR activation mechanism has been studied extensively with great interest over the past three decades. In this section, the elucidated steps of the EGFR activation mechanism will be outlined, followed by a discussion of the finer details of EGFR activation which still remain a mystery to the scientific community.

1.5.2 Ligand Binding

The activation of the EGFR begins with the ligand-binding step. Protein crystallography studies have provided us with valuable structural information, conclusively showing that two EGF molecules bind to two separate EGFRs in the dimer pair prior to dimerization [23, 24]. The EGFR has been found to exist in two affinity

states, with ~3% displaying high-affinity for ligand, and ~97% possessing low-affinity for ligand [21]. In order for a successful ligand-receptor interaction to occur, the Arg41 residue of the EGF must associate with the Asp 355 residue of the EGFR, followed by the EGF's Leu47 residue nestling within a hydrophobic pocket on the extracellular domain of the EGFR [25, 26].

1.5.3 Receptor Dimerization

When EGF binds to the EGFR, the receptor's extracellular domain undergoes a conformational change, resulting in the protrusion of a dimerization arm from each receptor to aid in the orchestration of receptor dimerization [27, 28]. When the dimerization arm region was deleted in a mutant EGFR, the result was the complete inhibition of activation [20], suggesting that EGFR dimerization is a required step in the receptor activation mechanism. The EGFR is capable of both homodimerization and heterodimerization with similar receptor structures, such as other members of the ErbB family or the insulin receptor. Interestingly, increased tyrosine kinase activity is observed in the case of an EGFR:ErbB-2 heterodimer in comparison to an EGFR:EGFR homodimer. Plausible explanations for this phenomenon include the idea that the heterodimer has a higher affinity for EGF [11], or that this specific heterodimer complex enjoys increased stability at the plasma membrane and by extension, decreased internalization [29]. The formation of heterodimers represents a way in which the ErbB family of receptors can manipulate and expand the specificity and diversity of the signal transduction pathways it activates upon ligand binding [13, 30, 31].

1.5.4 Tyrosine Kinase Activity

Once a dimer has formed, the tyrosine kinase domain of the EGFR becomes activated. This kinase enzyme catalyzes the removal of γ -phosphate from an ATP molecule and facilitates its addition to a tyrosine residue on the intracellular domain of its partner receptor [32, 33]. The major sites of tyrosine phosphorylation are the Tyr 1068, 1148, and 1173 residues on the intracellular domain of the EGFR [34].

1.5.5 Receptor Internalization

Post-phosphorylation, the receptor becomes internalized via several different mechanisms depending on the situation at hand. By internalizing the receptor into the cell, the EGFR is no longer becoming activated by extracellular ligand, allowing the cell to control and regulate the degree and transience of EGFR signalling depending on its specific needs at the time [35]. There are currently known to be two major mechanisms by which the EGFR dimer becomes internalized in the cell: clathrin-mediated endocytosis and caveolae-mediated endocytosis. Clathrin-mediated endocytosis is the dominant form of internalization at low levels of EGF stimulation, in direct contrast to caveolae-mediated endocytosis which predominates at high levels of EGF [36, 37]. Clathrin-mediated endocytosis is characterized by the internalization of the receptor dimer into clathrin-coated pits and the subsequent containment of these receptors within an endosome. The future of the receptor is based on the type of ligand bound to the receptor. When an EGF:EGFR complex is present in an endosome, the complex is ubiquitinated by Cbl ligase, marking it for degradation by the cell via lysosomal action [17]. Caveolin-mediated endocytosis involves the internalization of receptor along caveolin-rich

invaginations along the plasma membranal surface. Like the clathrin-mediated process, the receptor will be internalized into an endosome and tagged for eventual degradation [36-38].

1.5.6 Signal Transduction

Once the intracellular tyrosine kinase domain of the receptor autophosphorylates its tyrosine residues, the receptor has done its part in transducing the signal and now it is the duty of intracellular molecules to continue to propagate the signal internally. Adaptor molecules containing the Src-homology-2 domain or the phosphotyrosine-binding domain mediate this process by binding onto the newly phosphorylated tyrosine residues on the EGFR's intracellular domain [39], acting as the starting point for several major signalling pathways. The phosphoinositide 3-kinase (PI3K) pathway functions to activate Akt kinase, which can go on to mediate dissociation of Bcl2-associated-death-promoter (BAD) from its cellular complex resulting in a loss of BAD's pro-apoptotic agenda in the cell. Additionally, Akt signalling results in the increased expression of NFκB and a corresponding boost in the transcription of pro-survival genes [40]. The key player in the Ras/MAPK pathway is activated MAP kinase, which is able to phosphorylate, and thus modify, a variety of nuclear-destined transcription factors, resulting in altered cell cycle regulation [23]. Additionally, the Jak/STAT pathway accomplishes its agenda via STAT phosphorylation, dimerization, and nuclear localization, resulting in increased transcription of cell division and differentiation genes [40].

1.5.7 The Unknown

Despite all that has been discovered about the EGFR, several elements of its activation mechanism still remain unclear. While it is accepted that the ligand-binding step and the dimerization step are both critical in proper EGFR function, elucidating the molecular mechanism linking these two events together continues to be a major focus of EGFR-related research [40]. Various groups have suggested that receptor glycosylation modification may in fact be the link connecting ligand-binding and dimerization [41-44].

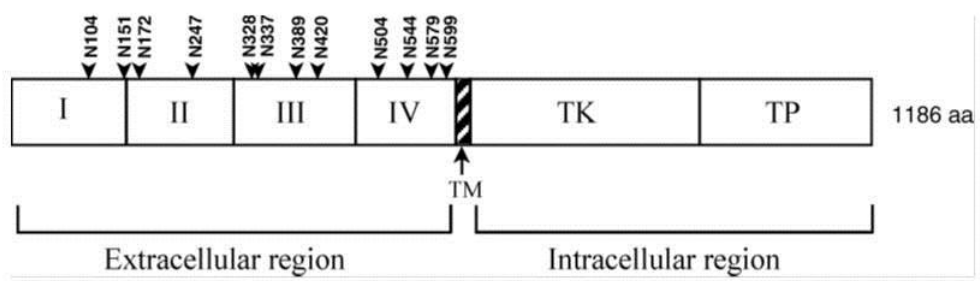
1.6 Receptor Glycosylation

The EGFR undergoes extensive post-translational modifications within the Golgi apparatus before it is considered a mature receptor and is transported to the cell membrane. The extracellular domain of the EGFR contains 12 potential glycosylation sites (Figure 1.2). One of the major post-translational modifications that a wild-type EGFR will undergo is glycosylation at 9 of these sites [32]. The glycosylation present on the mature receptor is composed of two molecules of N-acetylglucosamine, nine molecules of mannose, and three molecules of glucose [42].

1.7 Sialic Acids

After the glycosylated EGFR has been sorted to the cell membrane, it undergoes even more modifications. Approximately 40-60% of receptors will have 1-2 sialic acid molecules transferred onto their N-glycosylation sites [42, 45]. ‘Sialic acid’ is a broad term used to describe over 50 monosaccharides which each share a 9- α -keto acid

Figure 1.2. N-glycosylation on the extracellular domain of the EGFR. The extracellular domain of the EGFR consists of 12 potential N-glycosylation sites. In the Golgi apparatus, a wildtype EGFR will become glycosylated at 9 of these sites. This glycosylation consists of 2 molecules of N-acetylglucosamine, 9 molecules of mannose, and 3 molecules of glucose. Once transported to the plasma membrane, this glycosylation undergoes extensive modification, including the addition of 1-2 sialic acids to the terminal, non-reducing ends of the glycosylation. EGFR glycosylation is thought to play an important role in receptor function, cellular localization, and activation. Figure adapted from Takahashi, 2004.



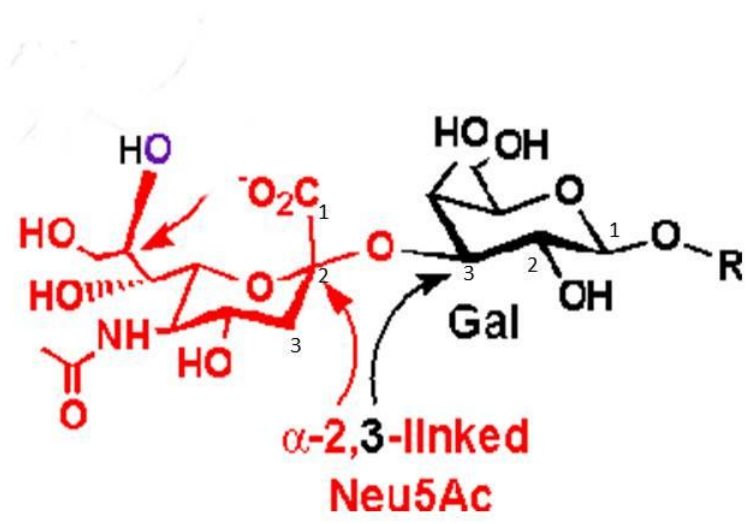
structure. The 5th position carbon exists as a common site of functional group modification, with hydroxylation, methylation, and acylation being popular modification options. Sialic acids may float freely in the cell, although the majority secure themselves to a galactose residue at the terminal, non-reducing ends of glycoproteins or glycolipids via an α -2,3 or α -2,6-linkage [46] (Figure 1.3). Their differences in function and purpose stem from the types of molecules to which they are anchored, as well as their functional group modifications [47, 48].

Sialic acids have unique characteristics. At physiological pH, the carboxylate group of the acid exists in a deprotonated form, possessing hydrophilic and acidic properties. These properties are optimized by both the sialic acid in question, as well as the molecule to which it is attached [47, 49, 50]. For example, negatively charged sialic acids may aid in the transport of positively charged molecules, as well as the repulsion of negatively charged molecules. In general terms, sialic acids function to fulfill two important, yet opposite, tasks: the first is to physically conceal a recognition site, and the second is to act as the recognition site for a particular receptor [51-53].

1.8 Sialidases

Based on the two general functions of sialic acids described above, it is evident why the addition, removal, or modification of sialic acids can have a significant effect on many cellular processes. One family of the enzymes responsible for decreasing sialic acid expression levels are sialidases, which hydrolytically cleave the α -2,3 or α -2,6-linkages connecting sialic acids to their glycosylated molecules [49, 54]. The more externally

Figure 1.3. Structure of sialic acids. Sialic acids (red) have a common 9-carbon core and the 5th carbon in this structure is a common modification site. In this diagram, a sialic acid (red) is α -2,3-linked to a galactose residue (black) at the terminal, non-reducing end of glycosylation. Sialidases are able to cleave these α -2,3-linkages, removing the sialic acid from the glycosylation. Figure adapted from Jakubowski, *Cell & Society*.



located the sialic acid is on the glycosylated molecule, the greater the rate of sialidase activity due to the sialic acid's increased accessibility and decreased steric hindrance [51].

Sialidases have many different roles in the cell. They have the ability to affect cellular function by altering and manipulating the expression of sialic acids, and can regulate the expression of sialic acids depending on the cell's specific needs during its current stage of development or differentiation [46]. For example, sialidase activity is important in the process of erythrocyte recycling. Sialidase will cleave sialic acids from the surface of a dying or damaged erythrocyte, exposing galactose residues. Phagocytic cells are now able to recognize and bind onto these residues through their galactose receptors and engulf the unwanted erythrocyte [51].

In a mammalian system, there are four types of sialidases: Neu1, -2, -3, and -4. Structurally, these four enzymes are quite similar, each possessing a β -propeller structure, a F.YRIP motif (X-Pro-Arg-Pro) in the enzyme active site, and repeats of the Asp-box motif (Ser-X-Asp-X-Gly-X-Thr-Trp) [55, 56]. However, despite their similar structures and common goal of cleaving sialic acids from glycosylated molecules, they each possess a unique substrate and cellular localization profile. Neu1 is characterized as a lysosomal sialidase which cleaves sialic acids from oligosaccharides and glycopeptides. Its activity is critical in lysosomal particle degradation, exocytosis, and elastin fiber formation [49, 57, 58]. Lysosomal exocytosis is mediated by the binding of lysosomal-associated membrane protein-1, -2 (LAMP-1, -2) to the plasma membrane. Within the lysosome, Neu1 functions to control the rate of lysosomal exocytosis by desialylating LAMP, thereby removing the ability of LAMP to bind to its receptor on the membrane [59].

Sialidosis, an autosomal disorder characterized by a Neu1 deficiency, is a debilitating condition marked by neurological and neuromuscular problems [46]. Neu2 is a cytosolic sialidase and its principal substrates include oligosaccharides, glycoproteins, and gangliosides. Neu2 has been shown to be important in neural and smooth muscle cell differentiation [57]. Neu3 is found on the plasma membrane of the cell and cleaves sialic acids from gangliosides. It has been thought to play a role in neuron differentiation, cell apoptosis and cell movement [60]. Neu4 is the most recently discovered sialidase and has been found to be located in numerous locations in the cell, including the lysosome, mitochondria and intracellular membranes, where it acts on oligosaccharides, glycoproteins, and gangliosides. It is believed to play an important role in apoptosis and is expressed at its highest levels in the liver [60, 61].

1.9 Role of Receptor Glycosylation in EGFR Activation

There exists a considerable amount of speculation as to the exact role of glycosylation in EGFR activation and signalling. Over the past three decades, receptor glycosylation has been heralded by various researchers as being crucial to a number of cellular processes, including the proper transport of the premature receptor to the plasma membrane, the maintenance of a stable pre-activation structure, direct ligand binding, and receptor dimerization.

In 1984, Soderquist *et al.* performed ground-breaking research that, for the first time, examined EGFR glycosylation within the context of EGFR activation. They discovered that when N-linked glycosylation was inhibited, tyrosine phosphorylation could not be detected. They concluded that N-linked glycosylation was either directly

involved in binding ligand to the EGFR, directly involved in tyrosine phosphorylation, or was responsible for holding the EGFR in a specific conformation that was required for proper activation [42].

Takahashi *et al.* were determined to identify the specific N-binding sites involved in the glycosylation functions proposed by previous researchers. They employed a mutagenesis approach, altering each N-glycosylation site on the extracellular portion of EGFR and observing the behaviour of each individual mutant upon ligand stimulation. Interestingly, the Asn-420-Gln mutant that provided the most intriguing results: this mutant receptor was incapable of binding ligand, yet it spontaneously dimerized and underwent full activation. Based on these results, they concluded that the Asn-420 glycosylation site must function to inhibit spontaneous activation of the EGFR, perhaps through the maintenance of an inhibitory conformation. They further suggested that the conformational change present in the EGFR post-ligand binding must result in a direct conformational change to the Asn-420 glycosylation, therefore blocking its inhibitory effects on EGFR dimerization and activation [43].

1.10 Role of Neu1 Sialidase in Toll-like and Trk Receptor Activation

Recently, a novel mode of Toll-like and Trk receptor activation has been described involving desialylation of receptor glycosylation by Neu1 sialidase. Woronowicz *et al.* (2004) observed that when trans-sialidase (TS), an enzyme isolated from *T.cruzi* which transfers α -2,3-linked sialic acids between molecules, was incubated with TrkA receptors, TS was able to cleave sialic acids from the receptor, resulting in TrkA activation (tyrosine phosphorylation). They followed up on this work, this time

focusing on a membrane-bound sialidase which was induced upon stimulation of the TrkA receptor with nerve growth factor (NGF) and cleaved α -2,3-linked sialic acids anchored to β -galactosyl residues present on the TrkA receptor. When oseltamivir phosphate, a broad-range sialidase inhibitor, was used to treat the cells before stimulation with the ligand, the result was a block in TrkA phosphorylation. Together, these results suggest that membranal sialidase activity is in fact required for proper TrkA activation [62, 63].

Jayanth *et al.* (2010) further elucidated this sialidase-dependent mode of TrkA receptor activation. They reported that the type of sialidase activated upon ligand stimulation of the TrkA receptor was Neu1 sialidase. Additionally, their results suggest that Neu1 activity post-ligand stimulation is mediated by MMP-9, an enzyme found to exist in direct complex with the Trk receptor on the cell surface [64]. MMP-9 is a type IV collagenase enzyme whose major substrates include collagen, elastin, and gelatin.

Amith *et al.* (2009) examined a similar scenario in Toll-like receptors (TLRs). When Toll-like receptors (specifically TLR-2, -3, and -4) were stimulated with their ligand lipopolysaccharide (LPS), Neu1 sialidase became activated, cleaving an α -2,3-linked sialic acid attached to a β -galactosyl residue present on the TLR ectodomain. It was shown that NF κ B activation, the major downstream effect of TLR signalling, could be blocked when Neu1 sialidase was inhibited. Based on their results, Amith *et al.* hypothesized that the sialic acids on the TLR formed a barrier which sterically hindered receptor dimerization until the sialic acids were cleaved by Neu1 sialidase [65].

1.11 Project Rationale: Reasons to Explore a Neu1-mediated Mechanism of EGFR Activation

This project centers around the idea that EGFR activation may also be mediated by Neu1 sialidase activity. The reasons for considering this concept are summarized below:

1.11.1 Common Receptor Family

Since TrkA receptors and EGFRs are both members of the receptor tyrosine kinase family of receptors [1], it can be speculated that they may be activated by similar mechanisms and that Neu1 is a common master enzyme involved in the activation process of all RTK receptors.

1.11.2 The Known Presence of Sialic Acids

The EGFR is a highly glycosylated molecule. In fact, 20% of its mass is considered due to its glycosylation [19]. Additionally, it is known that ~35-50% of the glycosylation present on the EGFR contains between one and two α -2,3-linked sialic acids attached to terminal-end galactose residues [34]. Since it has been hypothesized that that receptor glycosylation modification may play an important role in linking the EGF-EGFR binding event with the subsequent EGFR dimerization [43], it is interesting to investigate whether or not desialylation of the receptor glycosylation is the real culprit in this scenario.

1.11.3. A Novel Cellular Location for Neu1 Sialidase

One might question how Neu1, a sialidase traditionally classified as lysosomal, would be able to cleave sialic acids from receptors embedded in the plasma membrane. Post-ligand binding, both Jayanth *et al.* and Amith *et al.* observed a very rapid activation of Neu1, suggesting that there would be insufficient time for Neu1 to be recruited from the lysosomes to the plasma membrane and act on the Trk/Toll-like receptor [62, 64, 65]. Recently, it has been discovered that Neu1 is able to be sorted to the plasma membrane. In the lysosome, it forms a multienzyme complex consisting of Neu1, elastin binding protein (EBP), and protective protein-cathepsin A (PPCA). PPCA expresses mannose-6-phosphate on its surface, which directs the multienzyme complex towards the mannose-6-phosphate receptors on the cell's plasma membrane [66, 67] in LAMP-2-negative, MHC-II-positive vesicles [68]. Once at the plasma membrane, PPCA remains in complex with Neu1, where it is in charge of keeping the sialidase in an activation-ready conformation [46]. Clinical studies have shown that the cells of sialidosis patients often express Neu1 sialidases which exhibit mutations in the region where it would normally complex with PPCA or EBP, further supporting the notion that this complex is crucial to the proper function of Neu1 sialidase [69].

1.12 Proposed Model of EGFR Activation

An EGFR activation model can be proposed based on our knowledge of traditional EGFR activation schemes, our appreciation of the importance of EGFR glycosylation and sialylation in receptor activation, and our study of the role of Neu1 sialidase in TrkA and TLR activation:

Upon stimulation of the EGFR with EGF, Neu1 sialidase becomes activated via an unknown mechanism and desialylates the EGFR receptor glycosylation. This sialidase activity is proposed to be critical for proper receptor dimerization, and thus, activation, as is indicated by tyrosine phosphorylation and the initiation of subsequent signalling pathways.

1.13 Specific Aims of Research Project

Aim 1) To examine if a sialidase is activated upon EGF stimulation of the EGFR, and if so, which sialidase is activated (Neu1, -2, -3, or -4).

Aim 2) To determine if sialidase activity is required for EGFR activation, as is indicated by tyrosine phosphorylation on the intracellular domain of the EGFR

Aim 3) To investigate a possible mechanism of Neu1-dependent EGFR activation

1.14 Overall Hypothesis

Upon ligand stimulation of the EGFR, Neu1 sialidase becomes activated via an MMP-9 mediated pathway. The desialylation of receptor glycosylation by Neu1 sialidase is hypothesized to be essential for EGFR activation.

Chapter 2: Materials & Methods

2.1 Cell lines

The NIH3T3-hEGFR cell line is a mouse fibroblast cell line which overexpresses human EGFRs (described previously by [70, 71]) on its plasma cell membrane (provided by Dr. L. Raptis, Queen's University, Canada). A wild-type NIH3T3 mouse fibroblast cell line which expresses undetectable levels of EGFR was incorporated into these experiments as a negative control. The PANC-1 cell line (ATCC) is a human pancreatic duct epithelial carcinoma cell line which overexpresses EGFR on its surface. The above cell lines were grown in 1x DMEM (Gibco) enriched with 10% FBS (HyClone), and were maintained at 5% CO₂ and 37°C. The WG544 and 1140F01 cell lines are human fibroblast cell lines which are Neu1-deficient (provided by Dr. A. Pshezhetsky, Montreal University, Quebec). They were originally isolated from sialidosis patients. These human fibroblast lines were grown in 1xDMEM (Gibco) enriched with 5% horse serum (Gibco) and 3% fetal calf serum (HyClone) and were maintained at 5% CO₂ and 37°C.

2.2 Ligands

Epidermal growth factor (human EGF; Sigma), the natural ligand of the EGFR, was purchased as a lyophilized powder, reconstituted in sterile 1xPBS at a concentration of 1 mg/mL, and stored at -20°C. EGF was used in these experiments to stimulate the EGFRs at a concentration of 30 ng/mL. This concentration was determined to produce

optimal ligand-induced sialidase activity based on the results of our sialidase assays. Incubation times vary between experiments and thus are as indicated.

2.3 Inhibitors

Oseltamivir phosphate (Hoffmann-La Roche), a broad-range sialidase inhibitor which inhibits Neu1, -2, -3, and -4, was used at 200 µg/mL unless otherwise indicated. Oseltamivir phosphate binds to the sialidase and acts as a competitive inhibitor against sialic acids. Piperazine (Calbiochem) is a broad-range inhibitor of matrix metalloproteinases (MMP) -1, -3, -7, and -9 and was used to inhibit cells at a concentration of 1.25 ng/mL to 125 µg/mL for the incubations times indicated. Galardin (Calbiochem) is a hydroxamic acid which functions as a broad-range inhibitor of MMP-1, -2, -3, -8, and -9. It was used to inhibit cells at a concentration of 12.5 ng/mL to 125 µg/mL for the incubations times indicated. MMP-3-inhibitor (MMP3i; Calbiochem) and MMP-9-inhibitor (MMP-9i; Calbiochem) are specific, potent, and reversible inhibitors of MMP-3 and MMP-9, respectively. They were used as inhibitors of MMP-3 and MMP-9 activity at the indicated concentrations and incubation times.

2.4 Primary Antibodies

We employed the use of four neutralizing antibodies in order to inhibit sialidase function: rabbit-anti-human Neu1 IgG antibody (Santa Cruz), mouse-anti-human Neu2 IgG antibody (Santa Cruz), mouse anti-human Neu3 IgG antibody (MBL) and rabbit anti-human Neu4 IgG antibody (ProteinTech). In this work, EGFR activation was measured

as a function of tyrosine phosphorylation on the intracellular domain of the receptor. This phosphorylation was quantified through the use of rabbit anti-human-phosphoEGFR IgG antibody (Santa Cruz), which is specific against the phosphorylated Tyr1173 residue on the EGFR. Additional primary antibodies used include goat-anti-MMP9 IgG antibody (Santa Cruz) and rabbit-anti-human-EGFR IgG antibody (Santa Cruz).

2.5 Secondary Antibodies

The horseradish peroxidase-conjugated secondary antibodies used during the Western blot experiments were goat anti-rabbit-HRP antibody (Santa Cruz), donkey anti-goat-HRP antibody (Santa Cruz), and streptavidin-HRP (Cedarlane). CleanBlot IP Detection Reagent (Pierce) was used as a secondary antibody in the immunoprecipitation experiments. CleanBlot is a conjugated HRP antibody which optimally binds to the primary antibody and not denatured antibody fragments from the immunoprecipitation step. The immunocytochemistry experiment employed the use of F'(ab)₂ goat-anti-rabbit AlexaFluor 594 (Molecular Probes) as a secondary antibody.

2.6 Miscellaneous Reagents

The sialidase substrate, 4-MUNANA (2'-(4-methylumbelliferyl)- α -D-N-acetylneuraminic acid; Biosynth Intl.), was used at 0.318 mM, a concentration which was previously optimized by Woronowicz *et al.* (2004) for use in our specific sialidase assay [63, 72]. 4-MUNANA is a sialic donor molecule that, upon cleavage of its sialic acid by

an active sialidase, forms 4-MU which emits blue fluorescence at 450 nm after excitation at 365 nm. The mean fluorescence was quantified using the ImageJ program.

2.7 Sialidase Assay in live 3T3-hEGFR cells or PANC-1 cells

NIH3T3-hEGFR or PANC-1 cells were grown overnight on glass coverslips in a sterile 24-well tissue plate until they reached ~70% confluence. Cells were either treated with EGF at the indicated concentrations, or treated with both EGF and inhibitor/neutralizing antibody (oseltamivir phosphate, antiNeu1-4 neutralizing antibodies, MMP inhibitors, etc) at the indicated concentrations. Control cells were neither stimulated nor inhibited. The fluorescence substrate 4-MUNANA (2'-(4-methylumbelliferyl)- α -D-N-acetylneuraminic acid) was added to the cells and the slides were viewed after 60 seconds at 40X magnification by epi-fluorescence microscopy. 4-MUNANA is a sialic donor molecule that, upon cleavage of its sialic acid, forms 4-MU which emits blue fluorescence at 450 nm after excitation at 365 nm. The mean fluorescence was quantified using the ImageJ program.

2.8 Sialidase Assay in live human Neu1-deficient fibroblast cells

Human wildtype and Neu1-deficient fibroblast cells (WG544, 1140F01) were grown overnight on glass coverslips in a sterile 24-well tissue plate until they reached ~70% confluency. Cells were either left untreated or were treated with EGF at 30 ng/mL. The fluorescent substrate 4-MUNANA (2'-(4-methylumbelliferyl)- α -D-N-acetylneuraminic acid) (Biosynth International, Illinois, USA) was added to the cells and

the slides were viewed after 60 seconds at 40X magnification by epi-fluorescence microscopy. The mean fluorescence was quantified using the ImageJ program.

2.9 Immunocytochemistry for pEGFR in 3T3hEGFR cells

NIH3T3-hEGFR cells were grown overnight on glass coverslips in a 24-well tissue culture plate at 37°C until cells reached ~70% confluence. Cells were stimulated with 30 ng/mL EGF for 5 mins, treated with 100 µg/mL oseltamivir phosphate for 30 mins followed by stimulation with 30 ng/mL EGF for 5 mins, or treated with 100µg/mL neutralizing antibody (Neu1, -2, -3, -4) for 30 mins followed by stimulation with 30 ng/mL EGF for 5 mins. ‘No ligand’ cells were neither inhibited nor stimulated. Cells were fixed with 4 µg/mL paraformaldehyde, permeabilized with TritonX, and blocked with 4% bovine serum albumin (BSA) in 0.1% Tween-TBS for 20 minutes on ice. Cells were incubated with 4 µg/mL rabbit anti-human pEGFR for 60mins at 37°C, followed by incubation with 4 µg/mL goat anti-rabbit AlexaFluor 594 secondary antibody for 60 mins at 37°C. ‘Control’ cells were treated solely with secondary antibody in order to account for background non-specific fluorescence. Cells were mounted onto microscope slides using 3 µl mounting medium (DAKO) and viewed at 40X magnification by fluorescence microscopy. Red fluorescence is indicative of EGFR tyrosine phosphorylation. The density of red fluorescence was calculated using the Corel Photo Paint 8.0 program.

2.10.1 Preparation of cell lysates

3T3-hEGFR and 3T3 cells were grown in 75cm² flasks at 37°C until they reached ~80% confluence. Cells were treated with ligand, or with inhibitor and ligand at the indicated concentrations and incubation times. Control cells were left neither stimulated nor inhibited. Cells were removed from the flask, pelleted down, and resuspended in 99µl of prepared lysis buffer (50 mM Tris, 150 mM NaCl, 1 mM PMSF, 1% NP-40, 0.2 mg/ml leupeptin, 1% β-mercaptoethanol) and 1 µl protease inhibitor cocktail on ice for 30 mins. Cells were pelleted at 13000 rpm for 10 mins at 4°C. The supernatant was retained and stored at -80°C.

2.10.2 Bradford Assay

The protein concentration of each cell lysate sample was determined by performing a Bradford Assay. A standard curve was prepared using known concentrations of bovine serum albumin (BSA; Sigma) diluted in double-distilled water. Protein samples (2 µl of a 1:10 dilution) were then diluted in 798 µl double-distilled water. 200 µl of Bradford Reagent (Sigma) was added to each tube and the tubes were incubated at room temperature for 30 mins. Absorbance was measured on a spectrophotometer set to 595 nm. The protein sample values were determined by comparison to the standard curve data using Graph Pad Prism 4.0.

2.11 Western Blot for pEGFR

For each sample, 60 µg protein, 10 µl Loading Buffer (Sample Buffer + 5% β-mercaptoethanol), and up to 10 µl lysis buffer (described above) were added to eppendorf tubes to make a final volume of 20 µl per tube. Samples were boiled at 80°C for 10 mins and loaded into an 8% SDS-PAGE gel. The gel was run for 2 hrs at 100 V. Proteins were then transferred onto PVDF via semi-dry blotting for 80mins at 100 mA. The blot was blocked in 2% BSA in 0.1% Tween-TBS for 60 mins at 20°C, followed by incubation with rabbit anti-human pEGFR antibody (Santa Cruz) overnight at 4°C. The following day, the blot was incubated with 8 µg/mL goat anti-rabbit for 75 mins at 20°C. The blot was treated with chemiluminescent substrate for 5 mins and was subsequently developed on X-ray film (Sigma). NIH3T3 cells served as a negative control in this experiment. After development, blots were stripped and re-probed with rabbit anti-human panEGFR (Santa Cruz) as a loading control.

2.12 Co-Immunoprecipitation of EGFR and MMP-9, -Neu1, -Neu2, -Neu3, -Neu4

For each sample condition, 100 µg of lysate was immunoprecipitated with 1 µg of antibody overnight at 4°C. Samples were incubated with 10 µl Protein A or Protein G (as indicated) magnetic beads for 90 mins at 4°C. Samples were washed with the assistance of a magnetic rack, loaded into an 8% SDS-PAGE gel, and run at 100 V for 2 hours. The proteins were transferred onto PVDF via semi-dry blotting for 80 mins at 100 mA. The blot was blocked in 2% BSA in 0.1% Tween-TBS for 60mins at 20°C, followed by incubation with primary antibody (as indicated) overnight at 4°C. The following day, the

cells were incubated with a CleanBlot HRP-conjugated secondary antibody (Pierce) for 75 mins at 20°C. The blot was treated with chemiluminescent substrate for 5 mins and then developed on X-ray film (Sigma). NIH3T3 cells, as well as NIH3T3-hEGFR cells which were not incubated with anti-hEGFR antibody prior to the immunoprecipitation step, served as negative controls in these experiments. In the co-immunoprecipitation experiments of EGFR with Neu2, -3, or 4, a western blot of naïve 3T3-hEGFR cells was run simultaneous to the immunoprecipitation experiment in order to determine if the 3T3-hEGFR cells expressed detectable levels of Neu2, -3, and -4 protein.

2.13 Biotinylation and Immunoprecipitation: EGFR expression on the surface of 3T3-hEGFR cells

3T3-hEGFR cells were grown in 75cm² flasks until they reached ~80% confluence. Cells were left untreated (control), stimulated with 30 ng/mL EGF for 5 mins, or inhibited with 200 µg/mL oseltamivir phosphate, 100 µg/mL anti-Neu1 neutralizing antibody, or 50 µg/mL MMP-9 inhibitor for 30 mins prior to 30 ng/mL EGF stimulation for 5 mins. Cells were washed three times in cold 1xPBS. Cell concentration for each sample was adjusted to 5 X 10⁶ cells/mL. A biotin solution was prepared which consisted of 1 mg NHS-SS-Biotin (Pierce), 14 µl dimethyl sulfoxide (DMSO; Biorad), and 151 µl 1xPBS. 80 µl of the prepared biotin solution was added to 1 mL of cell suspension and incubated on ice for 30 mins. Cells were washed 4 times in 1xPBS. Each cell sample was re-suspended in 99 µl prepared lysis buffer (50 mM Tris, 150 mM NaCl, 1 mM PMSF, 1% NP-40, 0.2 mg/ml leupeptin, 1% β-mercaptoethanol) and 1 µl protease

inhibitor cocktail, and then allowed to incubate on ice for 30 mins. Cells were spun down at 13000 rpm for 10 mins. The supernatant was retained and protein concentration was determined via the Bradford Assay.

For each sample condition, 100 µg of lysates were immunoprecipitated with 1 µg of goat anti-EGFR antibody (Santa Cruz) overnight at 4°C. Samples were incubated with 10 µl of Protein G magnetic beads (New England Biolabs) for 90 mins at 4°C. Samples were washed with the assistance of a magnetic rack, loaded into an 8% SDS-PAGE gel, and run at 100 V for 2 hours. The proteins were transferred onto PVDF via semi-dry blotting for 80 mins at 100 mA. The blot was blocked in 2% BSA in 0.1% Tween-TBS overnight at 4°C, followed by incubation with streptavidin-HRP (Cedarlane) for 90 mins at room temperature. The blot was treated with chemiluminescent substrate for 5 mins and then developed on X-ray film (Sigma). NIH3T3 cells, as well as unstimulated NIH3T3-hEGFR cells which were not incubated with goat anti-EGFR antibody prior to the immunoprecipitation step, served as negative controls in this experiment.

2.14 Statistical Analysis

Statistical analysis was carried out using GraphPad Prism 4.0. Results were compared by a one-way ANOVA at 95% confidence using Bonferroni's Multiple Comparison Test.

Chapter 3: Results

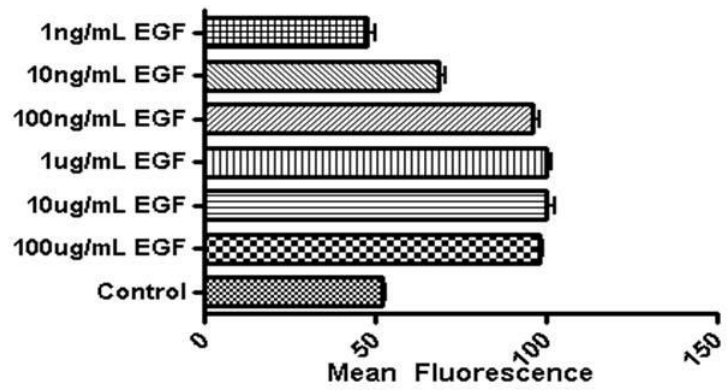
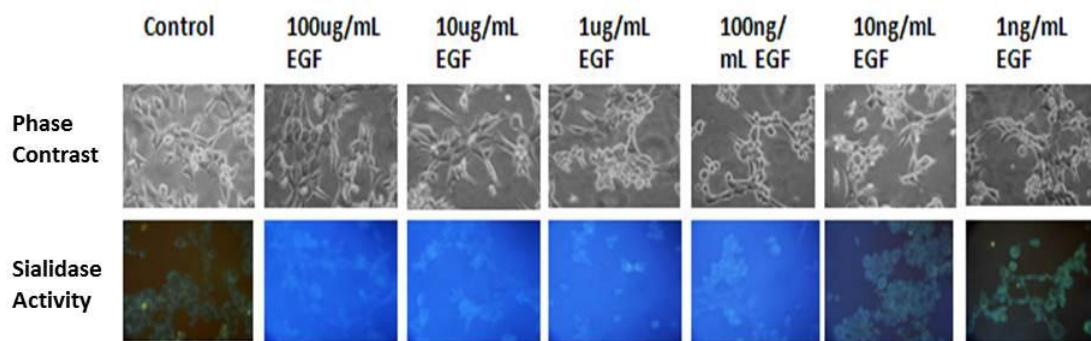
3.1 Induction of sialidase activity upon ligand stimulation of EGFRs in live 3T3-hEGFR cells

The first step of this project was to determine if one of the four mammalian sialidases became activated upon stimulation of the EGFR with its natural ligand, EGF. A sialidase assay (see section 2.7) was performed on live 3T3-hEGFR cells which were stimulated with doses of EGF ranging from 1 ng/mL to 100 µg/mL. Blue fluorescence surrounding the live cells indicates that an active sialidase has cleaved 4-MUNANA to form 4-MU. EGF stimulation of the EGFR was shown to activate sialidase in a dose-dependent manner. The 1 ng/mL dose of EGF did not provide significant results in comparison with the unstimulated control, but doses from 10 ng/mL to 100 µg/mL showed significant sialidase activation ($P < 0.05$) (Figure 3.1).

3.2 Oseltamivir phosphate inhibition of ligand-induced sialidase activity in live 3T3-hEGFR cells

Our results (Sec 3.1) indicated that one of the four mammalian sialidases was becoming activated upon ligand stimulation of the EGFR (Section 3.1). Therefore, we hypothesized that if we pre-treated cells with the broad-range sialidase inhibitor oseltamivir phosphate (inhibitor of Neu1, -2, -3, -4) prior to EGF stimulation, we should be able to block this ligand-induced sialidase activation. Consequently, in order to confirm the results observed in Section 3.1, a sialidase assay was performed in which live

Figure 3.1. EGF stimulation of the EGFR induces sialidase activity in a dose-dependent manner in live NIH3T3-hEGFR cells. NIH3T3-hEGFR cells were grown overnight on glass coverslips in a sterile 24-well tissue plate until they reached ~70% confluency. Live cells were treated with EGF at concentrations ranging from 1ng/mL to 100µg/mL. Control cells were neither stimulated nor inhibited. The fluorescence substrate 4-MUNANA (2'-(4-methylumbelliferyl)- α -D-N-acetylneuraminic acid) was added to the cells, coverslips were mounted onto microscope slides with 1µl mounting medium, and the slides were viewed after 60 seconds at 40X magnification by epifluorescence microscopy. 4-MUNANA is a sialic donor molecule that, upon cleavage of its sialic acid, forms 4-MU which emits blue fluorescence at 450nm after excitation at 365nm. The mean fluorescence was quantified using the ImageJ program. The data are a representation of one out of three independent experiments showing similar results.



3T3-hEGFR cells were inhibited with increasing doses of oseltamivir phosphate ranging from 25 ng/mL to 250 µg/mL prior to 30 ng/mL EGF stimulation. These results were compared to the sialidase activity present in an unstimulated control and a 30 ng/mL EGF-stimulated sample. Thymoquinone stimulation was added as an additional positive control as it has been shown to activate Neu4 sialidase [73, 74]. Blue fluorescence surrounding the live cells is indicative of sialidase activity. Therefore, oseltamivir phosphate inhibits EGF-induced sialidase activity in a dose-dependent manner (Figure 3.2).

3.3 Determination of the sialidase (Neu-1, -2, -3, -4) activated upon ligand stimulation of the EGFR in 3T3-hEGFR cells

The results from section 3.1 and 3.2 suggest that a sialidase becomes activated upon stimulation of the EGFR with EGF in live 3T3-hEGFR cells. In order to determine which of the four mammalian sialidases (Neu1, -2, -3, -4) was becoming activated, a sialidase assay was performed in which live 3T3-hEGFR cells were inhibited with specific neutralizing antibodies against Neu1, -2, -3, and -4 (50 µg/mL) prior to 30 ng/mL EGF stimulation. While the addition of anti-Neu1 neutralizing antibody was able to inhibit EGF-induced sialidase activity, the addition of anti-Neu2, anti-Neu3, or anti-Neu4 neutralizing antibody was unable to significantly alter the level of sialidase activity present within the cell in comparison to the EGF-stimulated sample. Therefore, these results suggest that the sialidase activated upon EGF stimulation of the EGFR is Neu1 sialidase (Figure 3.3).

Figure 3.2. Oseltamivir inhibition of EGF-induced sialidase activity live NIH3T3-hEGFR cells. NIH3T3-hEGFR cells were grown overnight on glass coverslips in a sterile 24-well tissue plate until they reached ~70% confluency. Cells were treated either with 30 ng/mL EGF alone or with oseltamivir phosphate (Tami) at the indicated concentrations. Control cells were neither stimulated nor inhibited. Thymoquinone (TQ) was added as an additional positive control as it is a known activator of Neu4 sialidase. The fluorescence substrate 4-MUNANA (2'-(4-methylumbelliferyl)- α -D-N-acetylneuraminic acid) was added to the cells, the coverslips were mounted onto microscope slides with 1 μ l mounting medium, and the slides were viewed after 60 seconds at 40X magnification by epi-fluorescence microscopy. 4-MUNANA is a sialic donor molecule that, upon cleavage of its sialic acid, forms 4-MU which emits blue fluorescence at 450nm after excitation at 365nm. The mean fluorescence was quantified using the ImageJ program. The data are a representation of one out of three independent experiments showing similar results.

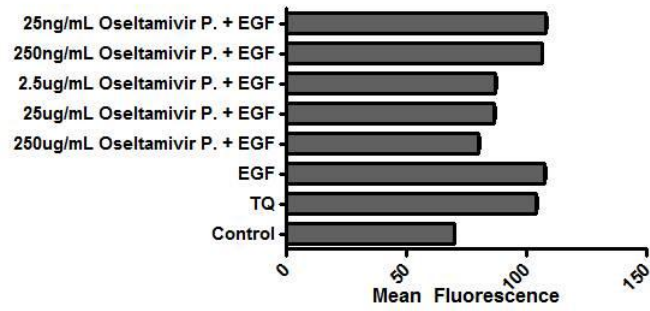
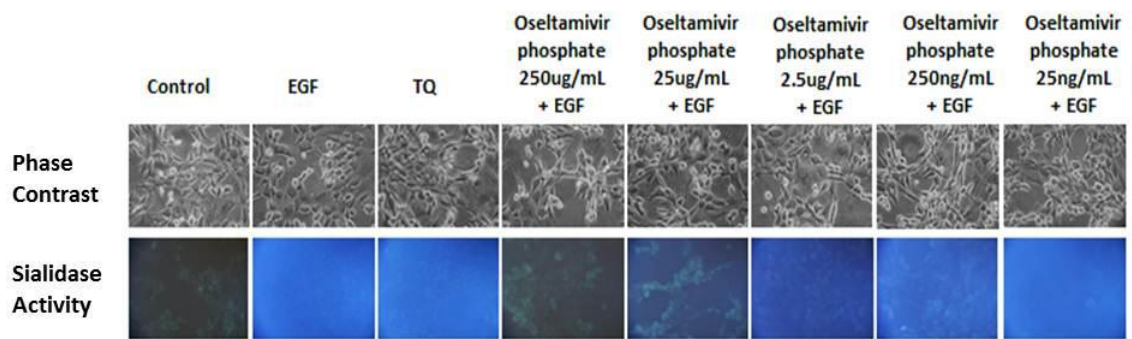
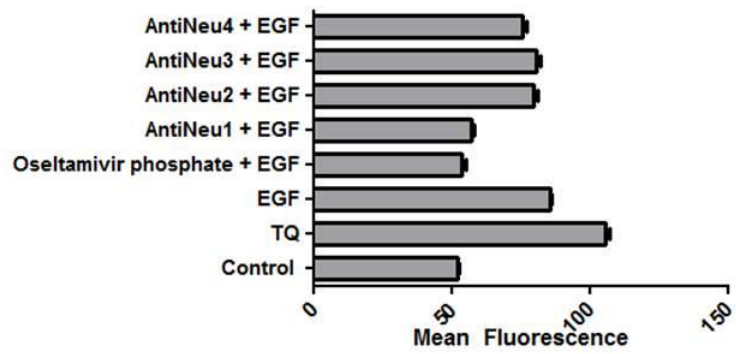
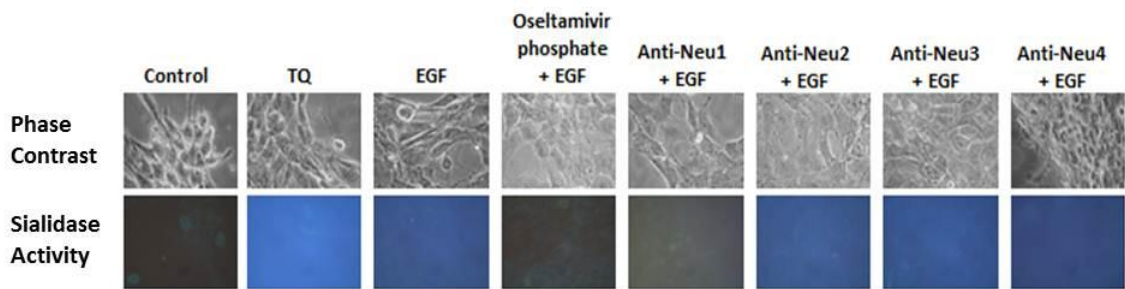


Figure 3.3. EGF stimulation of the EGFR induces Neu1 sialidase activity (not Neu2, -3, -4) in live NIH3T3-hEGFR cells. NIH3T3-hEGFR cells were grown overnight on glass coverslips in a sterile 24-well tissue plate until they reached ~70% confluency. Cells were either treated with 30 ng/mL EGF alone, in combination with 200 µg/mL oseltamivir phosphate, or in combination with 100 µg/mL antiNeu1, -2, -3, or -4 neutralizing antibody. Thymoquinone (TQ), a known activator of Neu4 sialidase, was added as an additional positive control. Control cells were neither stimulated nor inhibited. The fluorescence substrate 4-MUNANA (2'-(4-methylumbelliferyl)- α -D-N-acetylneuraminic acid) was added to the cells, the coverslips were mounted on microscope slides with 1µl mounting medium, and the slides were viewed after 60 seconds at 40X magnification by epi-fluorescence microscopy. 4-MUNANA is a sialic donor molecule that, upon cleavage of its sialic acid, forms 4-MU which emits blue fluorescence at 450 nm after excitation at 365 nm. The mean fluorescence was quantified using the ImageJ program. The data are a representation of one out of four independent experiments showing similar results.



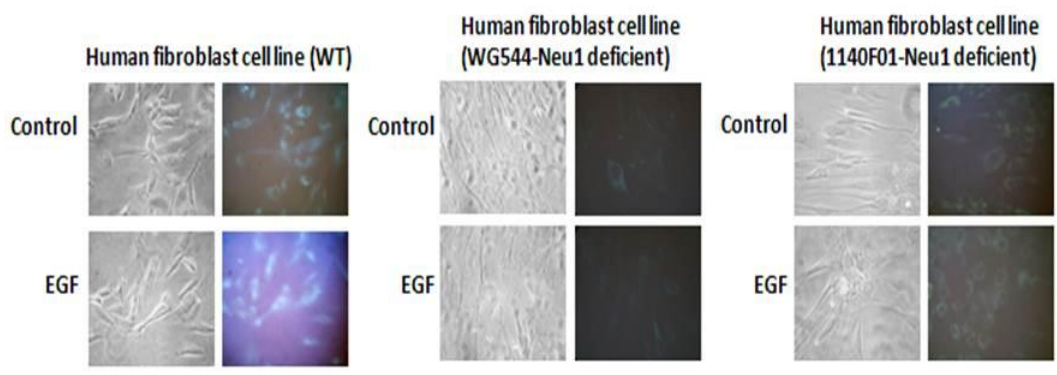
3.4 Induction of sialidase activity by EGF in wildtype and Neu1-deficient human fibroblasts

In order to confirm the results collected in section 3.3, a sialidase assay (see section 2.8) was performed in which a wild-type human fibroblast cell line and two Neu1-deficient human fibroblast cell lines (WG544 and 1140F01) were stimulated with EGF or left unstimulated as a control. If Neu1 sialidase is indeed the sialidase which becomes activated upon ligand stimulation of the EGFR, we expect that we would not observe sialidase activity, as is indicated by blue fluorescence, when the Neu1-deficient fibroblast cell lines are stimulated with EGF. The results of this assay confirm this hypothesis: the wild-type fibroblast cells showed sialidase activity upon ligand stimulation of the EGFRs, whereas the Neu1-deficient cells (WG544 and 1140F01) did not (Figure 3.4).

3.5 Induction of sialidase activity by EGF in human PANC1 cells

We were interested to see if EGF stimulation of the EGFR would induce Neu1 sialidase activity in a human carcinoma cell line in the same way that it does in the NIH3T3-hEGFR mouse fibroblast cell line. PANC-1, a cell line derived from the ductal epithelium of a cancerous human pancreas, were stimulated with 30 ng/mL EGF or treated with oseltamivir phosphate or anti-Neu1 neutralizing antibody prior to EGF stimulation before being analyzed for sialidase activity via a sialidase assay (see section 2.7). Our results correlated with the results seen in the 3T3-hEGFR cells, with EGF-stimulation of the EGFR inducing sialidase activity in PANC-1 cells and this activity

Figure 3.4. EGF stimulation of the EGFR is unable to induce sialidase activity in Neu1-deficient human fibroblasts. Human wildtype and Neu1-deficient fibroblast cells (WG544, 1140F01) were grown overnight on glass coverslips in a sterile 24-well tissue plate until they reached ~70% confluency. Cells were either left untreated (control) or were treated with EGF at 30 ng/mL. The fluorescence substrate 4-MUNANA (2'-(4-methylumbelliferyl)- α -D-N-acetylneuraminic acid) was added to the cells, the coverslips were mounted on microscope slides with 1 μ l mounting medium, and the slides were viewed after 60 seconds at 40X magnification by epi-fluorescence microscopy. 4-MUNANA is a sialic donor molecule which, upon cleavage of its sialic acid, forms 4-MU which emits blue fluorescence at 450 nm after excitation at 365 nm. Experiment was performed by Dr. Preethi Jayanth.



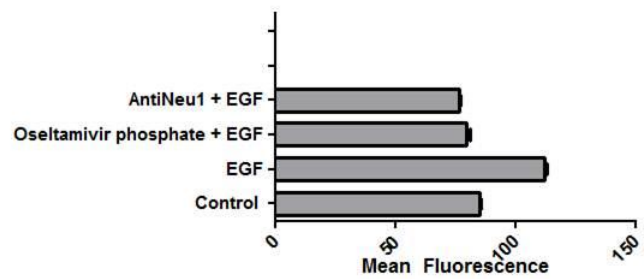
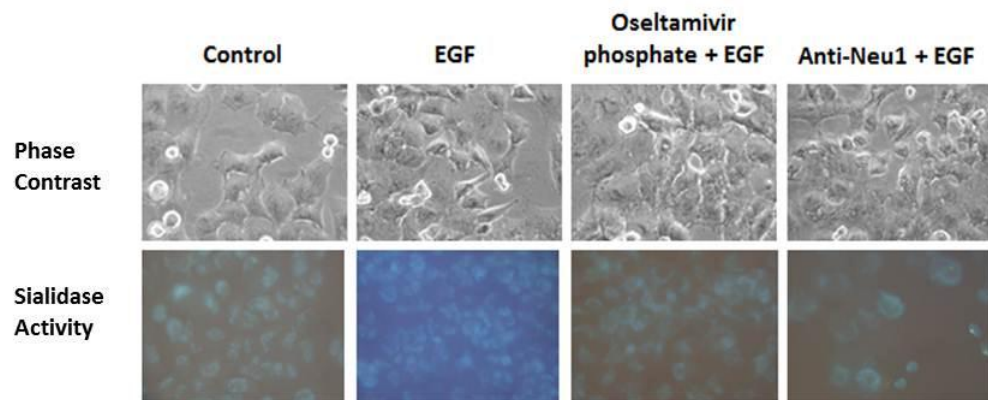
being blocked by pre-treatment with oseltamivir phosphate and anti-Neu1 neutralizing antibody (Figure 3.5).

3.6 Effects of Neu1 inhibition on EGFR tyrosine phosphorylation in 3T3-hEGFR cells

Jayanth *et al.* (2010) observed that Neu1 sialidase activity upon NGF stimulation of the TrkA receptor was necessary for proper receptor activation, as is indicated by tyrosine phosphorylation [64]. Since the TrkA and the EGFR are members of the same receptor family (RTK), we hypothesize that they may be activated by similar mechanisms. A Western blot was performed in order to determine if EGF-induced Neu1 sialidase activity is in fact necessary for EGFR tyrosine phosphorylation (see section 2.10-11). Cells were left unstimulated, stimulated with 30 ng/mL for 5 minutes, pre-treated with 200 µg/mL oseltamivir phosphate for 30 mins prior to EGF stimulation for 5 mins, or pre-treated with 100 µg/mL anti-Neu1 neutralizing antibody for 30 mins prior to EGF stimulation for 5 mins (Figure 3.6A). Unstimulated NIH3T3 cells were included in this experiment as a negative control. The blots were probed with an antibody against the phosphorylated Tyr 1173 residue on the EGFR's intracellular domain.

Two additional versions of this Western blot experiment were performed, each with a slight modification on the procedure described above. First, this experiment was repeated with 10 min EGF stimulations instead of 5 min stimulations (Figure 3.6B). Secondly, this experiment was repeated as described above with the following modification: instead of pre-treatment of the cells with either 200 µg/mL oseltamivir phosphate or 100 µg/mL anti-Neu1 neutralizing antibody for 30 mins prior to EGF

Figure 3.5. EGF stimulation of the EGFR induces Neu1 sialidase activity in live PANC-1 cells. PANC-1 cells were grown overnight on glass coverslips in a sterile 24-well tissue plate until they reached ~80% confluency. Cells were either treated with 30 ng/mL EGF alone, or in combination with 200 µg/mL oseltamivir phosphate or 100 µg/mL antiNeu1, -2, -3, or -4 neutralizing antibody. Control cells were neither stimulated nor inhibited. The fluorescence substrate 4-MUNANA (2'-(4-methylumbelliferyl)- α -D-N-acetylneuraminic acid) was added to the cells, the coverslips were mounted on microscope slides with 1µl mounting medium, and the slides were viewed after 60 seconds at 40X magnification by epi-fluorescence microscopy. 4-MUNANA is a sialic donor molecule which, upon cleavage of its sialic acid, forms 4-MU which emits blue fluorescence at 450 nm after excitation at 365 nm. The mean fluorescence was quantified using the ImageJ program. The data are a representation of one out of three independent experiments showing similar results.



stimulation for 5 mins, the inhibitors and ligand underwent simultaneous addition for a 5 min incubation period (Figure 3.6C).

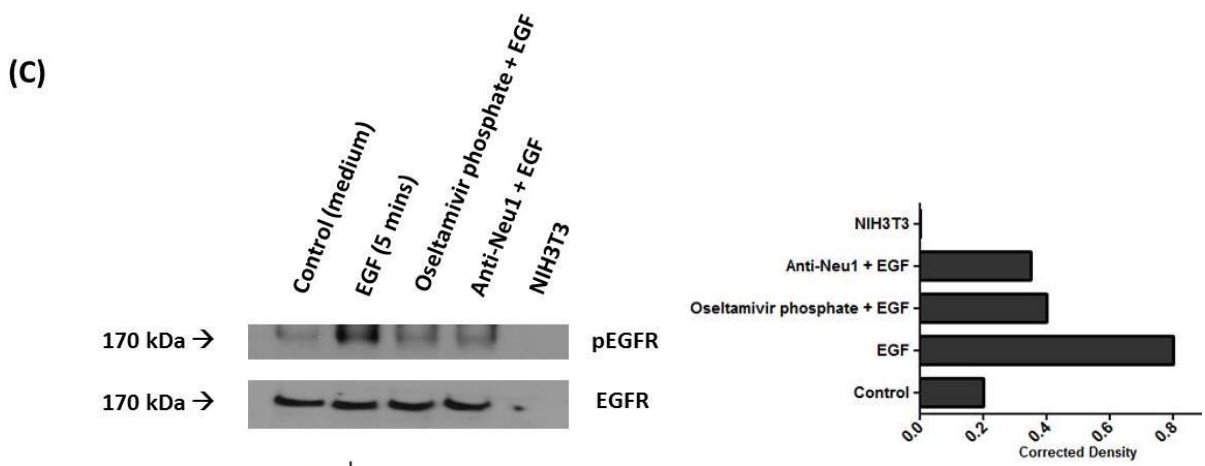
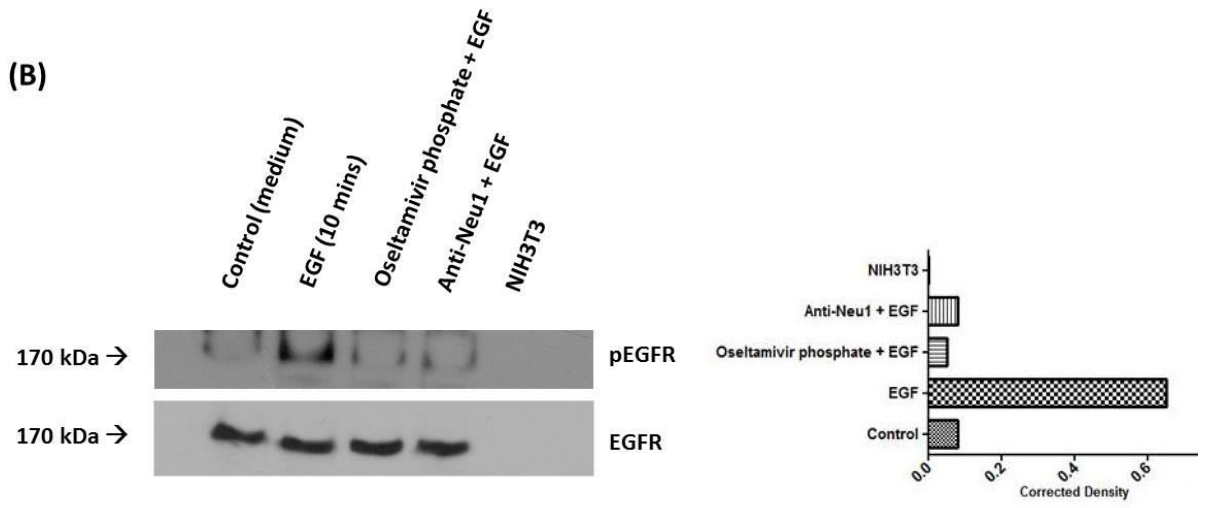
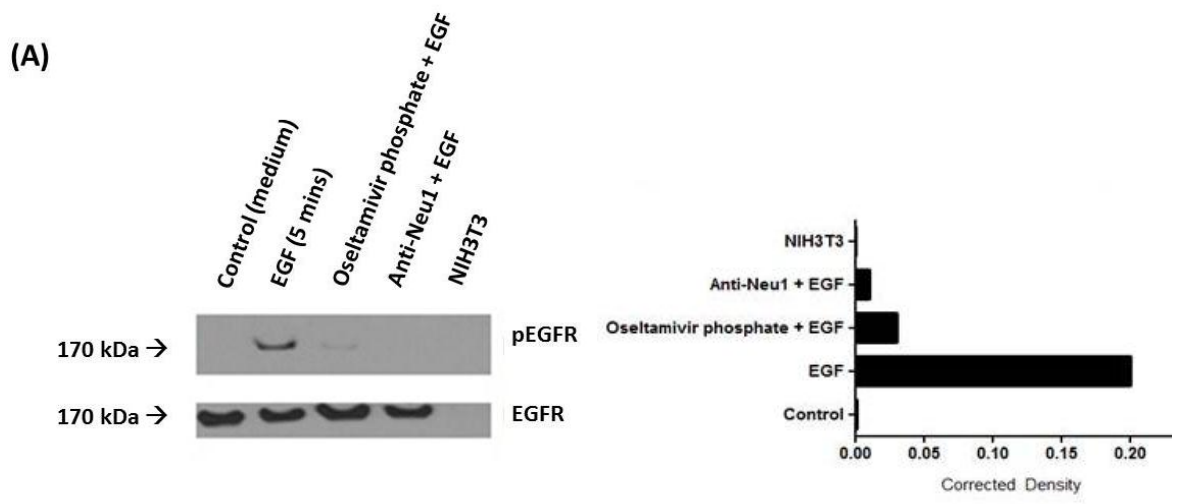
Inhibition of EGF-induced tyrosine phosphorylation was observed in the samples which were pre-treated with either oseltamivir phosphate or anti-Neu1 neutralizing antibody prior to EGF stimulation for 5 mins. The same trends were observed when the EGF incubation periods were increased from 5 mins to 10 mins. When the cells were simultaneously treated with oseltamivir phosphate/anti-Neu1 antibody and EGF, we also observed inhibition of ligand-induced tyrosine phosphorylation. However, upon quantification of this data, we noted decreased inhibition of tyrosine phosphorylation when the cells were simultaneously treated with inhibitor and EGF. In all three versions of this Western blot, it is apparent that EGFR phosphorylation is decreased when the Neu1 sialidase is inhibited. Collectively, these results suggest that Neu1 sialidase activity is an important step in the EGFR activation mechanism.

3.7 Effects of Neu1 inhibition on EGFR tyrosine phosphorylation in 3T3-hEGFR cells:

Immunocytochemistry

In order to confirm the results observed in section 3.5, an immunocytochemistry experiment was performed on 3T3-hEGFR cells, which measured tyrosine phosphorylation under various treatment conditions (see section 2.9). Cells were left untreated ('no ligand'), stimulated with 30 ng/mL for 5 min, treated with 200 μ M oseltamivir phosphate prior to EGF stimulation, or treated with 100 μ g/mL neutralizing antibody against Neu1, -2, -3, or -4 prior to EGF stimulation. Cells were immunostained

Figure 3.6. Inhibition of EGF-induced EGFR tyrosine phosphorylation by broad-range sialidase inhibitor oseltamivir phosphate and specific anti-Neu1 neutralizing antibody. (A) NIH3T3-hEGFR cell lysates were prepared for the following treatment conditions: unstimulated (control), 30 ng/mL EGF for 5mins, or pre-treatment of the cells with 200 µg/mL oseltamivir phosphate or 100 µg/mL antiNeu1 neutralizing antibody for 30 mins prior to stimulation with 30 ng/mL EGF for 5 mins. Unstimulated NIH3T3 cells were added as a negative control. For each sample, 60 µg of protein was loaded into an 8% SDS-PAGE gel. The gel was run for 2 hrs at 100 V. Proteins were then transferred onto PVDF via semi-dry blotting for 80 mins at 100 mA. The blot was blocked in 2% BSA in 0.1% Tween-TBS for 60 mins at 20°C, followed by incubation with 0.14 µg/mL rabbit anti-human pEGFR antibody overnight at 4°C. The following day, the blot was incubated with 0.04 µg/mL goat anti-donkey for 75 mins at 20°C. The blot was treated with chemiluminescent substrate for 5 mins and developed on X-ray film. NIH3T3 cells served as a negative control in this experiment. After development, blots were stripped and reprobed with rabbit anti-human panEGFR as a loading control. (B) A western blot was performed as described above, with one modification: NIH3T3-hEGFR cells were stimulated with 30 ng/mL EGF for a 10 min period instead of 5 mins in all treatment samples (C) A western blot was performed as described above, with one modification: instead of pre-treatment for 30 mins, oseltamivir phosphate/anti-Neu1 antibody were added to the NIH3T3-hEGFR cells simultaneously with 30 ng/mL EGF for a 5 min incubation period. The data are a representation of one out of three (A) or two (B, C) independent experiments showing similar results.



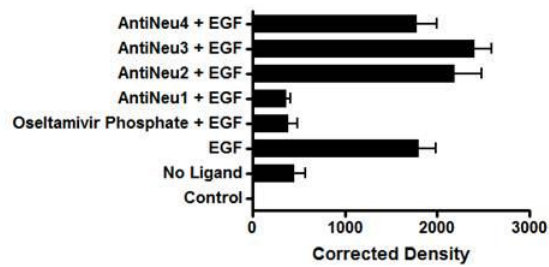
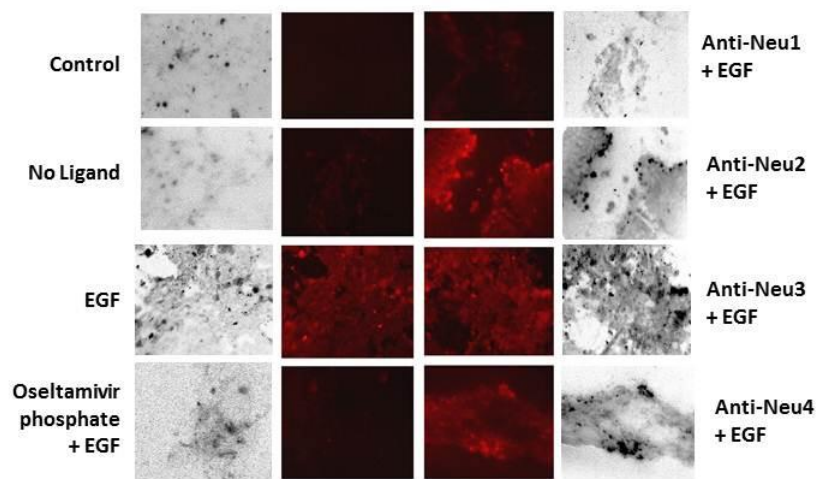
with rabbit anti-pEGFR antibody, followed by goat anti-rabbit Alexa 594 secondary antibody. Therefore, red fluorescence is indicative of tyrosine phosphorylation.

In support of the western blot results described in section 3.5, we observed inhibition of EGF-induced tyrosine phosphorylation in the cell samples which were pre-treated with oseltamivir phosphate and anti-Neu1 neutralizing antibody prior to EGF stimulation, but not in the samples pre-treated with anti-Neu2, -3, or -4 neutralizing antibody (Figure 3.7). This experiment provides further evidence supporting a Neu1-mediated mechanism of ligand-induced EGFR activation, as is measured through tyrosine phosphorylation.

3.8 Co-immunoprecipitation of EGFR with mammalian sialidases (Neu1,-2,-3,-4) in naïve and ligand-stimulated 3T3-hEGFR cells

The results of our sialidase assays (described in section 3.1-3.3) suggest a rapid induction of Neu1 sialidase activity (<30 secs) after EGF stimulation of the EGFRs. Therefore, we hypothesize that Neu1 must exist either in close proximity or in complex with the EGFR on the cell surface in order to account for this rapid activation. Jayanth *et al.* observed that Neu1 co-immunoprecipitates with the TrkA receptor [64]. Since the TrkA receptor and the EGFR are both members of the RTK receptor family, we hypothesize that they may form complexes with similar molecules. In this set of experiments, co-immunoprecipitation techniques were utilized in order to determine whether or not Neu1 and the EGFR exist in complex (see section 2.12). Additionally, co-immunoprecipitation experiments were performed to test if the EGFR forms a complex with any of the other three mammalian sialidases (Neu2, -3, or -4).

Figure 3.7. Inhibition of EGF-induced EGFR phosphorylation by oseltamivir phosphate and anti-Neu1 neutralizing antibody in NIH3T3-hEGFR cells. NIH3T3-hEGFR cells were grown overnight on glass coverslips in a 24-well tissue culture plate at 37°C until cells reached ~70% confluence. Cells were stimulated with 30 ng/mL EGF for 5 mins, treated with 100 µg/mL oseltamivir phosphate (Tami) for 30 mins followed by stimulation with 30 ng/mL EGF for 5 mins, or treated with 100 µg/mL neutralizing antibody (Neu1, -2, -3, -4) for 30 mins followed by stimulation with 30ng/mL EGF for 5 mins. ‘No ligand’ cells were neither inhibited nor stimulated. Cells were fixed with 4 µg/mL paraformaldehyde, permeabilized with TritonX, and blocked with 4% bovine serum albumin (BSA) in 0.1% Tween-TBS for 20 mins on ice. Cells were immunostained with rabbit anti-human pEGFR for 60 mins at 37°C, followed by incubation with goat anti-rabbit AlexaFluor 594 secondary antibody for 60mins at 37°C. ‘Control’ cells were treated solely with secondary antibody in order to account for background non-specific fluorescence. Cells were mounted onto microscope slides using 3 µl mounting medium and viewed at 40X magnification by fluorescence microscopy. Red fluorescence is indicative of EGFR tyrosine phosphorylation. The density of red fluorescence was calculated using the Corel Photo Paint 8.0 program. The data are a representation of one out of three independent experiments showing similar results.



Neu1 and EGFR were found to co-immunoprecipitate in naïve cells, EGF-stimulated cells, as well as cells which were pre-treated with oseltamivir phosphate prior to EGF stimulation (Figure 3.8). Neu2 and EGFR were not found to co-immunoprecipitate with each other, despite there being appreciable levels of Neu2 and EGFR present in a western blot of naïve 3T3-hEGFR cells (Figure 3.9A). Neu3 and EGFR were not found to co-immunoprecipitate with each other, although upon follow up with a western blot, there was not a detectable level of Neu3 present in the cell lysates prepared from naïve 3T3-hEGFR cells (Figure 3.9B). Interestingly, and quite unexpectedly, Neu4 and EGFR were found to co-immunoprecipitate in both naïve and EGF-stimulated 3T3-hEGFR cells (Figure 3.9C).

3.9 Inhibition of EGF-induced sialidase activity by broad-range MMP inhibitors piperazine and galardin in live 3T3-hEGFR cells

Our results suggest that Neu1 sialidase activity is an important mediator in the EGFR activation mechanism. Our attention must now turn to elucidating how Neu1 sialidase becomes activated upon EGF stimulation of the EGFR. Jayanth *et al.* (2010) suggested an MMP-9-mediated mechanism in which the MMP-9 acted upon the EBP in the tripartite complex, removing it and resulting in the activation of the Neu1 sialidase [64]. Since the TrkA and EGFR are both members of the RTK family, we hypothesize that their ligand-induced Neu1 sialidase activity may be dependent on a similar MMP-mediated mechanism.

Figure 3.8. EGFR and Neu1 co-immunoprecipitate on the cell surface of naïve and EGF-stimulated NIH3T3-hEGFR cells. NIH3T3-hEGFR cells were left unstimulated (control), stimulated with 30 ng/mL EGF for 5 mins, or inhibited with 200 µg/mL oseltamivir phosphate for 30 mins prior to 30 ng/mL EGF stimulation for 5 mins. Cell lysates were prepared. For each sample condition, 100 µg of lysates were immunoprecipitated with 1 µg of goat anti-EGFR antibody overnight at 4°C. Samples were incubated with 10 µl Protein G magnetic beads for 90 mins at 4°C. Samples were washed with the assistance of a magnetic rack, loaded into an 8% SDS-PAGE gel, and run at 100 V for 2 hours. The proteins were transferred onto PVDF via semi-dry blotting for 80 mins at 100 mA. The blot was blocked in 2% BSA in 0.1% Tween-TBS for 60 mins at 20°C, followed by incubation with rabbit anti-Neu1 antibody overnight at 4°C. The following day, the cells were incubated with CleanBlot HRP-conjugated secondary antibody for 75 mins at 20°C. The blot was treated with chemiluminescent substrate for 5 mins and then developed on X-ray film. NIH3T3 cells, as well as NIH3T3-hEGFR cells which were not incubated with anti-Neu1 antibody prior to the immunoprecipitation step, served as negative controls in this experiment. Blot was stripped and reprobbed with anti-panEGFR antibody to determine equal loading. The data are a representation of one out of four independent experiments showing similar results.

IP: EGFR
Blot: Neu1

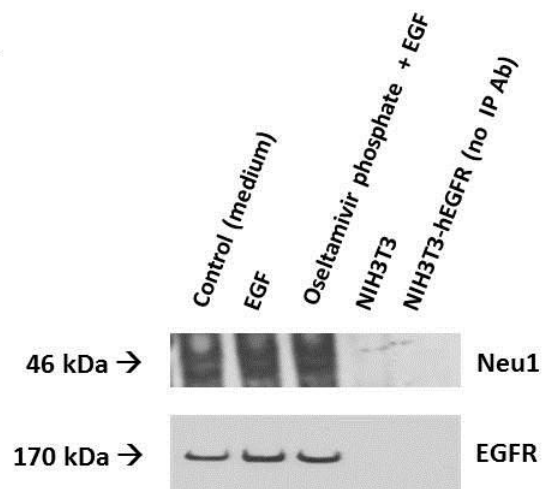
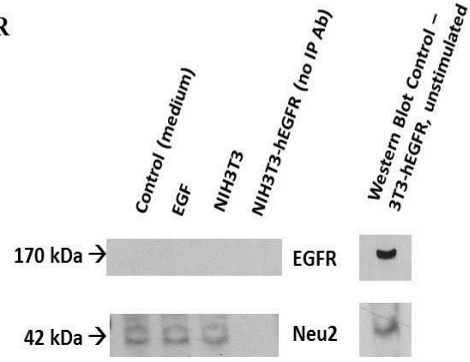


Figure 3.9. Neu4, but not Neu2 or -3, co-immunoprecipitates with the EGFR on the cellular surface of naïve and EGF-stimulated NIH3T3-hEGFR cells. NIH3T3-hEGFR cells were left unstimulated (control) or were stimulated with 30 ng/mL EGF for 5 mins. Cell lysates were prepared. For each sample condition, 100 µg of lysates were immunoprecipitated with 1µg of (A) mouse anti-Neu2 antibody (B) mouse anti-Neu3 antibody (C) rabbit anti-Neu4 antibody overnight at 4°C. Samples were incubated with 10µl Protein A/G magnetic beads for 90mins at 4°C. Samples were washed with the assistance of a magnetic rack, loaded into an 8% SDS-PAGE gel, and run at 100 V for 2 hours. The proteins were transferred onto PVDF via semi-dry blotting for 80 mins at 100 mA. The blot was blocked in 2% BSA in 0.1% Tween-TBS for 60 mins at 20°C, followed by incubation with goat anti-EGFR antibody overnight at 4°C. The following day, the cells were incubated with CleanBlot HRP-conjugated secondary antibody for 75 mins at 20°C. The blot was treated with chemiluminescent substrate for 5 mins and then developed on X-ray film. NIH3T3 cells, as well as NIH3T3-hEGFR cells which were not incubated with anti-Neu2/3/4 antibody prior to the immunoprecipitation step, served as negative controls in this experiment. Blots were stripped and reprobed with anti-Neu2, -Neu3, -Neu4 antibody to determine equal loading. Additionally, a western blot of unstimulated 3T3-hEGFR cells was run simultaneous to the immunoprecipitation experiment in order to determine if the 3T3-hEGFR cells expressed detectable levels of Neu2, -3, and -4 protein. The data are a representation of one out of three independent experiments showing similar results.

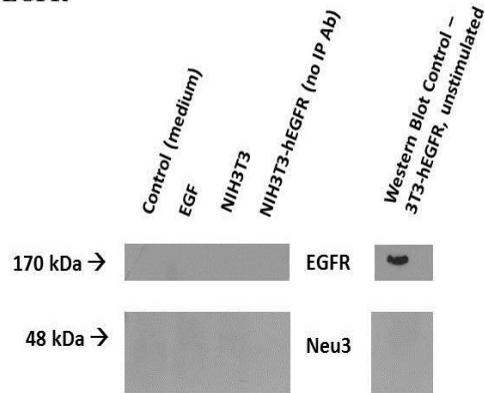
A)

IP: Neu2
Blot: EGFR



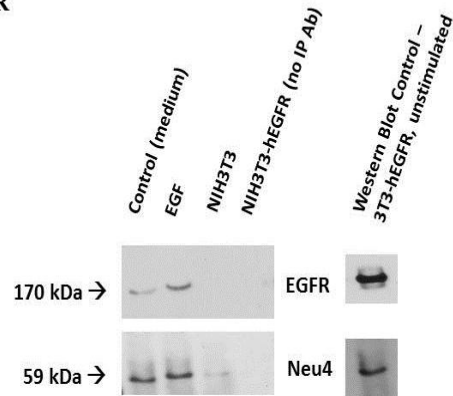
B)

IP: Neu3
Blot: EGFR



C)

IP: Neu4
Blot: EGFR



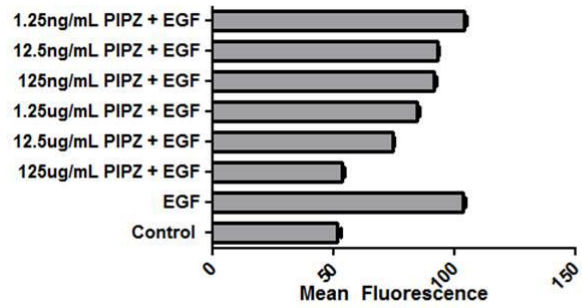
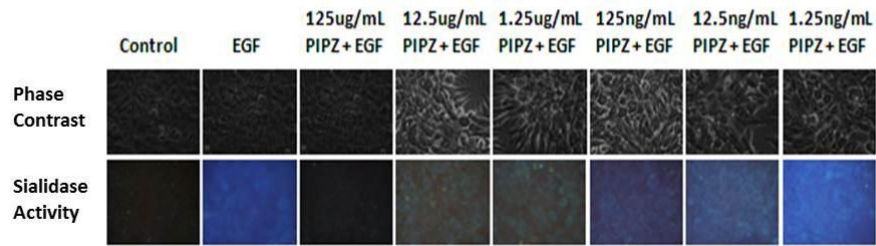
Two sialidase assays were performed in which live 3T3-hEGFR were treated with 30 ng/mL EGF, as well as increasing doses of two broad-range MMP inhibitors: piperazine (PIPZ) or Galardin. PIPZ inhibits MMP-3, -7, and -9 and was used to treat the cells in doses ranging from 1.25 ng/mL to 125 μ g/mL prior to 30 ng/mL EGF stimulation (Figure 3.10A). Galardin inhibits MMP-1, -2, -3, -8, and -9 and was used to treat the cells in doses ranging from 12.5 ng/mL to 125 μ g/mL prior to 30 ng/mL EGF stimulation (Figure 3.10B). Blue fluorescence surrounding the live cells is indicative of sialidase activity. Both PIPZ and Galardin were able to inhibit EGF-induced Neu1 sialidase activity in a dose-dependent manner. PIPZ and Galardin were able to significantly inhibit ligand-induced sialidase activity ($P < 0.05$) at doses greater than 12.5 μ g/mL and 125ng/mL, respectively. Based on the process of elimination between the types of MMPs that are inhibited by both PIPZ and Galardin, we hypothesized that the MMP exerting its effects upon EGF-stimulation of the EGFR must either be MMP-3 or MMP-9.

3.10 Inhibition of EGF-induced sialidase activity by specific MMP-3 and MMP-9 inhibitors in live 3T3-hEGFR cells

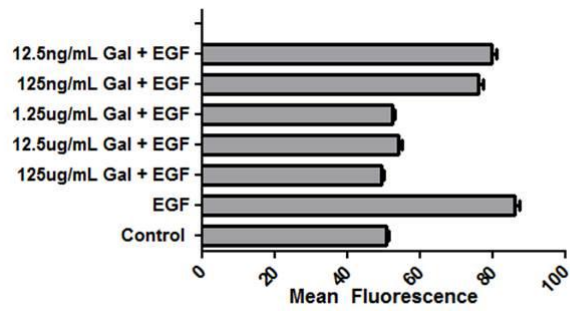
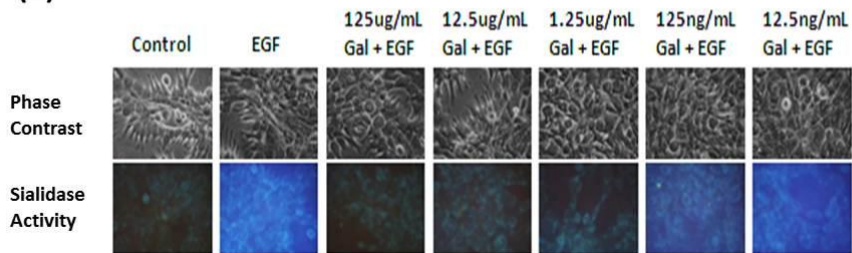
In order to test whether ligand-induced Neu1 sialidase activity was mediated by MMP-3 or MMP-9, we employed the use of specific inhibitors against these matrix metalloproteinases. Two sialidase assays were performed in which live 3T3-hEGFR were treated with 30 ng/mL EGF, as well as increasing doses of a specific MMP-3 inhibitor (50 ng/mL – 500 μ g/mL) or a specific MMP-9 inhibitor (5 ng/mL – 500 μ g/mL) prior to 30 ng/mL EGF stimulation. Blue fluorescence surrounding the live cells is indicative of sialidase activity. While pre-treatment of the cells with MMP3i was unable to block EGF-

Figure 3.10. Inhibition of EGF-induced Neu1 sialidase activity by broad-range MMP inhibitors piperazine and galardin in live NIH3T3-hEGFR cells. NIH3T3-hEGFR cells were grown overnight on glass coverslips in a sterile 24-well tissue plate until they reached ~70% confluency. Cells were either alone with 30 ng/mL EGF alone, or in conjunction with the broad-range MMP inhibitor (A) **piperazine** (inhibits MMP-3,-7,-9) or (B) **galardin** (inhibits MMP-1,-2,-3,-8,-9) at the indicated concentrations. Control cells were neither stimulated nor inhibited. The fluorescence substrate 4-MUNANA (2'-(4-methylumbelliferyl)- α -D-N-acetylneuraminic acid) was added to the cells, the coverslips were mounted on microscope slides with 1 μ l mounting medium, and the slides were viewed after 60 seconds at 40X magnification by epi-fluorescence microscopy. 4-MUNANA is a sialic donor molecule that, upon cleavage of its sialic acid, forms 4-MU which emits blue fluorescence at 450 nm after excitation at 365 nm. The mean fluorescence was quantified using the ImageJ program. The data are a representation of one out of three independent experiments showing similar results.

(A)



(B)



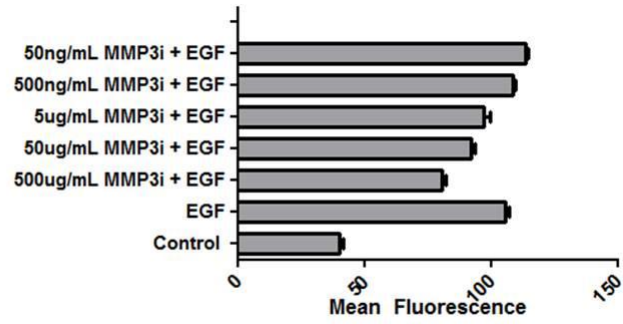
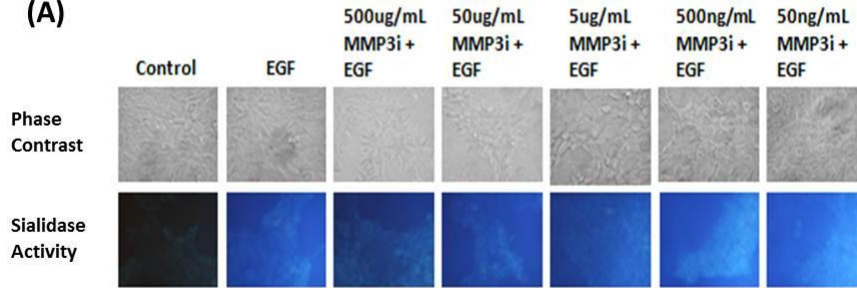
induced sialidase activity (Figure 3.11A), pre-treatment of the cells with MMP9i was able to inhibit EGF-induced sialidase activity in a dose-dependent manner (Figure 3.11B). Ligand-induced sialidase activity was significantly inhibited ($P < 0.05$) at doses greater than 5 $\mu\text{g}/\text{mL}$. These results suggest that MMP-9 is playing a key role in mediating the activation of Neu1 sialidase when the EGFR is stimulated with EGF.

3.11 Effects of MMP-9 inhibition on EGFR tyrosine phosphorylation in 3T3-hEGFR cells

A Western blot was performed in order to determine if MMP-9 activity is required in proper EGFR activation – specifically, tyrosine phosphorylation (see section 2.10-11). Cells were left unstimulated, stimulated with 30 ng/mL for 5 mins, pre-treated with 50 $\mu\text{g}/\text{mL}$ MMP-9i for 30 mins prior to EGF stimulation, or pre-treated with 75 $\mu\text{g}/\text{mL}$ MMP-9i for 30 mins prior to EGF stimulation. Unstimulated NIH3T3 cells were included in this experiment as a negative control. The blots were probed with an antibody against the phosphorylated Tyr 1173 residue on the EGFR. Inhibition of EGF-induced tyrosine phosphorylation was observed in the samples which were pre-treated with both the 50 $\mu\text{g}/\text{mL}$ and 75 $\mu\text{g}/\text{mL}$ MMP-9i prior to EGF stimulation, in comparison to the cells which were stimulated with 30 ng/mL EGF alone (Figure 3.12). Collectively, these results suggest that MMP-9 may play a critical role in mediating a Neu1 sialidase-dependent EGFR activation mechanism.

Figure 3.11. Inhibition of EGF-induced Neu1 sialidase activity by specific MMP-9 inhibitor, but not specific MMP-3 inhibitor, in live NIH3T3-hEGFR cells. NIH3T3-hEGFR cells were grown overnight on glass coverslips in a sterile 24-well tissue plate until they reached ~70% confluency. Cells were either alone with 30 ng/mL EGF alone, or in conjunction with (A) **specific MMP-3 inhibitor** or (B) **specific MMP-9 inhibitor** at the indicated concentrations. Control cells were neither stimulated nor inhibited. The fluorescence substrate 4-MUNANA (2'-(4-methylumbelliferyl)- α -D-N-acetylneuraminic acid) was added to the cells, the coverslips were mounted on microscope slides with 1 μ l mounting medium, and the slides were viewed after 60 seconds at 40X magnification by epi-fluorescence microscopy. 4-MUNANA is a sialic donor molecule that, upon cleavage of its sialic acid, forms 4-MU which emits blue fluorescence at 450 nm after excitation at 365 nm. The mean fluorescence was quantified using the ImageJ program. The data are a representation of one out of three independent experiments showing similar results.

(A)



(B)

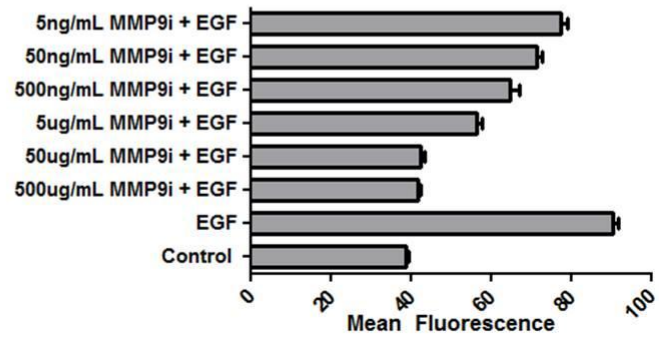
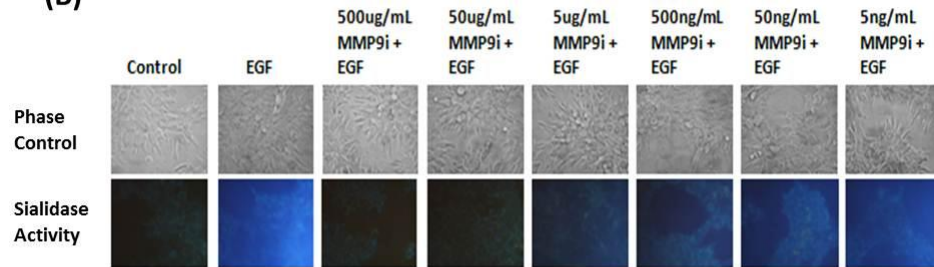
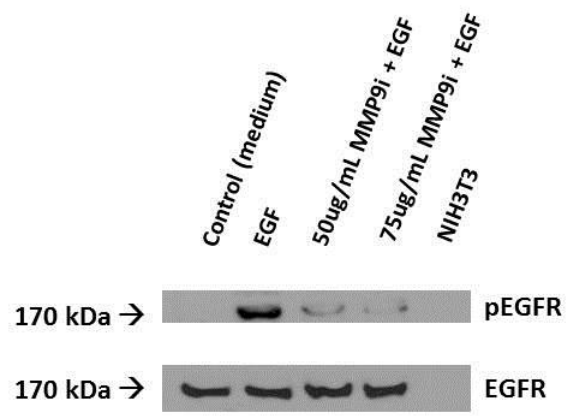


Figure 3.12. Inhibition of EGF-induced EGFR tyrosine phosphorylation by specific MMP-9 inhibitor in NIH3T3-hEGFR cells. NIH3T3-hEGFR cell lysates were prepared for the following treatment conditions: unstimulated (control), 30 ng/mL EGF for 5 mins, or pretreatment with 50 µg/mL or 75 µg/mL specific MMP9-inhibitor for 30 mins prior to stimulation with 30 ng/mL EGF. Unstimulated NIH3T3 cells were added as a negative control. For each sample, 60 µg of protein was loaded into an 8% SDS-PAGE gel. The gel was run for 2 hrs at 100 V. Proteins were then transferred onto PVDF via semi-dry blotting for 80 mins at 100 mA. The blot was blocked in 2% BSA in 0.1% Tween-TBS for 60 mins at 20°C, followed by incubation with 0.14 µg/mL rabbit anti-human pEGFR antibody overnight at 4°C. The following day, the blot was incubated with 0.04 µg/mL goat anti-donkey for 75 mins at 20°C. The blot was treated with chemiluminescent substrate for 5 mins and developed on X-ray film. NIH3T3 cells served as a negative control in this experiment. After development, blots were stripped and reprobbed with rabbit anti-human panEGFR as a loading control. The data are a representation of one out of three independent experiments showing similar results.



3.12 Co-immunoprecipitation of EGFR and MMP-9 in naïve and EGF-stimulated 3T3-hEGFR cells

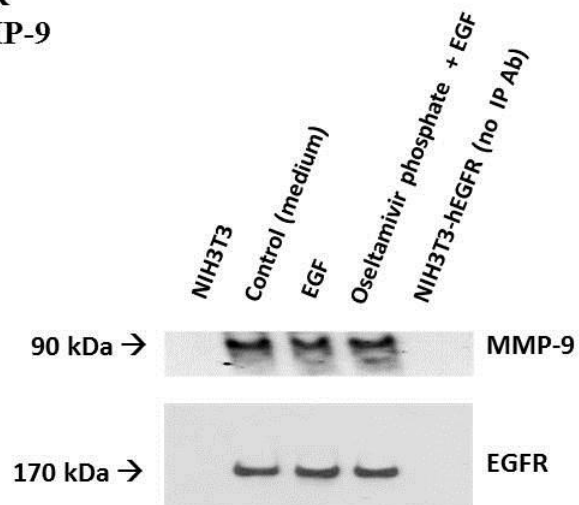
The results of our sialidase assays (section 3.1-3.3) suggest a rapid induction of Neu1 sialidase activity (<30 secs) after EGF stimulation of the EGFR. If MMP-9 does indeed act upon Neu1 sialidase after EGF stimulation of the EGFR, and Neu1 sialidase co-immunoprecipitates with the EGFR, we hypothesize that MMP-9 must also either exist in close proximity or in complex with the EGFR on the plasma membrane. Therefore, a co-immunoprecipitation experiment was performed to determine whether or not MMP-9 and the EGFR were complexed together (see section 2.12). MMP-9 and EGFR were found to co-immunoprecipitate in naïve and EGF-stimulated cells, as well as cells which were pre-treated with oseltamivir phosphate prior to EGF stimulation (Figure 3.13).

3.13 Expression of EGFR on the cell surface of naïve, stimulated, and inhibited 3T3-hEGFR cells

The validity of our collected data, and subsequently its proper analysis, requires us to determine the degree to which the expression levels of EGFR on the cell surface of NIH3T3-hEGFR cells are modified by our treatment protocols. In order to address this concern, a biotinylation-based experiment was utilized (see section 2.13). 3T3-hEGFR cells were stimulated with EGF, or inhibited with oseltamivir phosphate, anti-Neu1 neutralizing antibody, or specific MMP-9 inhibitor prior to EGF stimulation. Control cells were neither inhibited nor stimulated. Post-treatment, all cells were biotinylated.

Figure 3.13. EGFR and MMP-9 co-immunoprecipitate on the cellular surface of naïve and EGF-stimulated NIH3T3-hEGFR cells. NIH3T3-hEGFR cells were left unstimulated (control), stimulated with 30 ng/mL EGF for 5 mins, or inhibited with 200 µg/mL oseltamivir phosphate for 30 mins prior to 30 ng/mL EGF stimulation for 5 mins. Cell lysates were prepared. For each sample condition, 100µg of lysates were immunoprecipitated with 1 µg of goat anti-EGFR antibody overnight at 4°C. Samples were incubated with 10 µl Protein G magnetic beads for 90 mins at 4°C. Samples were washed with the assistance of a magnetic rack, loaded into an 8% SDS-PAGE gel, and run at 100 V for 2 hours. The proteins were transferred onto PVDF via semi-dry blotting for 80 mins at 100 mA. The blot was blocked in 2% BSA in 0.1% Tween-TBS for 60 mins at 20°C, followed by incubation with rabbit anti-MMP-9 antibody overnight at 4°C. The following day, the cells were incubated with CleanBlot HRP-conjugated secondary antibody for 75 mins at 20°C. The blot was treated with chemiluminescent substrate for 5 mins and then developed on X-ray film. NIH3T3 cells, as well as NIH3T3-hEGFR cells which were not incubated with anti-EGFR antibody during the immunoprecipitation step, served as negative controls in this experiment. Blot was stripped and reprobed with anti-panEGFR antibody to determine equal loading. The data are a representation of one out of three independent experiments showing similar results.

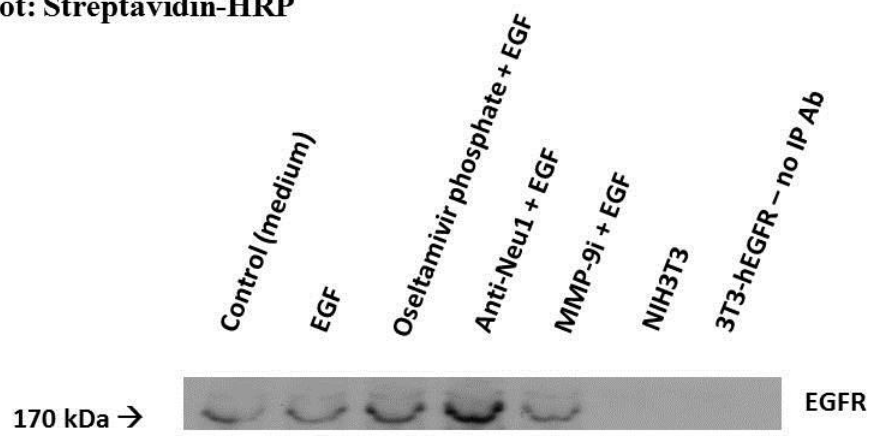
IP: EGFR
Blot: MMP-9



Therefore, any EGFRs still present on the cell surface post-treatment would become biotinylated. An immunoprecipitation experiment was performed in which EGFRs were immunoprecipitated out from the cell sample, run on a Western blot, and probed with streptavidin-HRP against the biotin. Our results indicate that EGFR expression was not significantly decreased after inhibition of the cells with oseltamivir phosphate, anti-Neu1 neutralizing antibody, or specific MMP-9 inhibitor, in comparison with the control and EGF-stimulated samples (Figure 3.14). In fact, we observed an increased level of EGFR expression in the case of treatment with anti-Neu1 antibody prior to EGF stimulation. Therefore, it can be concluded that the decrease in phosphorylated EGFR observed in the western blots in section 3.6-7,11 can be attributed with confidence to the inhibition of EGFR activation in the presence of specific treatments, and not to the down-regulation of EGFR expression on the cell surface as a result of those treatments.

Figure 3.14. Expression levels of EGFR on the cellular surface of NIH3T3-hEGFR cells in the presence of oseltamivir phosphate, anti-Neu1 neutralizing antibody, and specific MMP-9 inhibitor. NIH3T3-hEGFR cells were grown in 75cm² flasks until they reached ~80% confluence. Cells were left untreated (control), stimulated with 30 ng/mL EGF for 5 mins, or inhibited with 200 µg/mL oseltamivir phosphate, 100 µg/mL anti-Neu1 neutralizing antibody, or 50 µg/mL MMP-9 inhibitor for 30mins prior to 30ng/mL EGF stimulation for 5 mins. Whole cells were biotinylated with NHS-SS-Biotin , and then cell lysates were prepared. For each sample condition, 100 µl of lysates were immunoprecipitated with 1 µg of goat anti-EGFR antibody overnight at 4°C. Samples were incubated with 10 µl of Protein G magnetic beads for 90 mins at 4°C, washed, loaded into an 8% SDS-PAGE gel, and run at 100 V for 2 hours. The proteins were transferred onto PVDF via semi-dry blotting for 80 mins at 100 mA. The blot was blocked in 2% BSA in 0.1% Tween-TBS overnight at 4°C, followed by incubation with streptavidin-HRP for 90 mins at room temperature. The blot was treated with chemiluminescent substrate for 5 mins and then developed on X-ray film (Sigma). NIH3T3 cells, as well as NIH3T3-hEGFR cells which were not incubated with goat anti-EGFR antibody during the immunoprecipitation step, served as negative controls in this experiment. The data are a representation of one out of two independent experiments showing similar results.

IP: EGFR
Blot: Streptavidin-HRP



Chapter 4: Discussion

After decades of research devoted to this subject, much is known about the EGFR activation mechanism. However, as was discussed previously, the mechanistic link between the ligand-binding event and the receptor dimerization event still remains a mystery. Our results show, for the first time, a ligand-induced EGFR activation mechanism that is dependent on desialylation by Neu1 sialidase via an MMP-9-mediated pathway. We hypothesize that this desialylation acts early on in the EGFR activation mechanism and aids in the removal of a steric hindrance which was formerly preventing receptor dimerization. In this section, the plausible pathways of Neu1/MMP-9 activation and our final proposed EGFR mechanism of activation, as well as the clinical relevance of this body of work, will be addressed and discussed.

4.1 Understanding MMP-9 and Neu1 Crosstalk Within the EGFR Activation Schematic

4.1.1 Desialylation of EGFR by Neu1 Sialidase is a Key Mediator of EGFR Activation

Central to our novel signalling paradigm is the concept that Neu1 sialidase is a key mediator in the EGFR activation mechanism. The results of our sialidase assays (section 3.1-3.4) show that Neu1 sialidase is quickly activated upon ligand-stimulation of the EGFR (<30 seconds). Neu1 sialidase, therefore, must either exist in complex with the EGFR or in very close proximity. The results of our immunoprecipitation experiments

suggest that Neu1 indeed co-immunoprecipitates with the EGFR both pre- and post-ligand stimulation. While it may seem counter-intuitive that Neu1 sialidase, an enzyme traditionally classified as lysosomal, could exert such effects on a cell surface receptor, it has been recently well-documented that Neu1 sialidase can indeed be recruited to the plasma membrane of the cell and stably exist there. Its ability to do this is directly tied to its presence within the tripartite complex of elastin binding protein (EBP), protective protein cathepsin A (PPCA), and Neu1 sialidase [75, 76]. Mannose-6-phosphate structures on the PPCA are able to direct this complex towards the mannose-6-phosphate receptors on the cell surface [66].

As the EGFR is a highly glycosylated receptor, with its N-glycosylation each being sialylated with 1-2 sialic acids, we propose that the Neu1 sialidase functions to desialylate the receptor. Furthermore, we hypothesize that it is this desialylation that allows for proper receptor dimerization and thus, activation. The sialic acids may exert this effect by acting as a physical barrier to EGFR dimerization, either by acting as a steric hindrance themselves or by maintaining the unstimulated receptor in a conformation that is not conducive to receptor-receptor association. Alternatively, the sialic acids may instead exert their effects by taking advantage of their unique chemical properties – the acidic charge and hydrophobicity of the sialic acids may act as a repulsive force, preventing the two receptors from forming the close receptor-receptor complex necessary for proper EGFR activation.

The concept of Neu1 sialidase having a role in cell surface receptor activation is not a novelty. In fact, work by Duca *et al.* (2007) has shown that Neu1 exists in direct complex with the elastin receptor on the cell surface. Their results went on to suggest that

Neu1 sialidase activity may be required for proper elastin receptor activation, a phenomenon which they attributed to the generation of free sialic acids [77]. The importance of Neu1 and elastin receptor association is supported by Hinek *et al.*, who reported that this enzyme-receptor complex is crucial for elastin fiber formation, considering sialodosis patients who are Neu1-deficient are unable to maintain normal levels of elastogenesis [66]. Another receptor whose activation mechanism has been shown to be Neu1-mediated is the insulin receptor. Arabkhari *et al.* showed that Neu1 sialidase has the ability to act directly on the insulin-like growth factor and insulin receptor (in arterial smooth muscle cells and myoblasts, respectively), desialylating their extracellular glycosylation [78].

Neu1 desialylation of cell surface receptors has recently been implicated in the process of phagocytosis. In 2010, Seyrantepe *et al.* treated THP-1 derived macrophages with sialidase inhibitors, specific anti-Neu1 neutralizing antibody and Neu1 siRNA and set out to determine the effects of these treatments on phagocytosis. They observed that Neu1 inhibition and deficiency resulted in a lack of cellular differentiation from monocytes to macrophages, decreased IL-1 β and IL-6 production, as well as a decrease in the phagocytosis of foreign particles. These functions were restored upon addition of exogenous sialidase. Upon review of their results, they hypothesized that the role of Neu1 sialidase in phagocytosis was to desialylate multiple cell surface phagocytic receptors upon stimulation with ligand [79].

4.1.2 MMP-9 Facilitates Neu1 Activation after EGF Stimulation of the EGFR

MMP-9 is a zinc and calcium-dependent type IV collagenase, whose main purpose in the cell is to remodel the extracellular matrix by acting on common matrix proteins, such as elastin, collagen, and gelatin [80]. By altering the makeup of the extracellular matrix, MMP-9 has a profound effect on various physiological processes such as wound repair, angiogenesis, and embryogenesis [81].

As discussed above, our results indicate that desialylation of the EGFR by Neu1 sialidase plays a critical role in EGFR activation. Central to this mechanism is the concept that MMP-9 activity is able to activate Neu1 sialidase upon ligand stimulation of the EGFR. The results of our sialidase assays show that EGF-induced sialidase activity can be blocked by pre-treatment of the cells with the broad-range MMP inhibitors galardin and piperazine and specific MMP-9 inhibitor, but not a specific MMP-3 inhibitor (section 3.9-3.10). Therefore, these results suggest that ligand-induced Neu1 sialidase activity is dependent on the function of MMP-9. This was further supported by a Western blot which showed that ligand-induced EGFR tyrosine phosphorylation could be blocked by pre-treatment with specific MMP-9 inhibitor (section 3.11). Additionally, MMP-9 was found to co-immunoprecipitate with the EGFR on the cell surface of both naïve and EGF-stimulated 3T3-hEGFR cells (section 3.12), adding further support to the hypothesis that rapidly-induced MMP-9 activity is necessary for proper EGFR activation. Collectively, these results lay the foundation for understanding MMP-9's role in facilitating a Neu1-mediated EGFR activation mechanism.

When MMP-9 exerts its enzymatic properties on elastin, the end result is the production of elastin peptides. These peptides then have the ability to bind to the elastin

binding protein (EBP), which is already known to be located on the cell surface in a tripartite complex with Neu1 sialidase and PPCA [76]. Previous work has shown that when elastin peptide binds to the EBP located in this specific tripartite complex, it has the capacity to initiate Neu1 activation [77]. Therefore, it seems as though engaging the EBP is the most plausible and direct route to Neu1 sialidase activation on the cell surface. If MMP-9 were to become activated after ligand stimulation with the receptor, elastin peptides would be produced which could bind to EBP and consequently, activate Neu1 sialidase.

4.1.3 MMP-9 Activation: A Possible Role for G-protein Coupled Receptors

One must question how the MMP-9 becomes activated upon ligand stimulation of the epidermal growth factor receptor. One hypothesis is that it becomes activated by a G-protein coupled receptor (GPCR). GPCRs are no strangers to MMP activation, as they have been shown to activate multiple classes of MMPs, including MMP-2 [82], MMP-3, and MMP-9 [83]. Additionally, GPCRs have been linked to EGFR activation in past studies through the process of transactivation [84], which has shown that activation of a GPCR can result in EGFR tyrosine phosphorylation independent of ligand stimulation. This is believed to happen via an MMP-mediated pathway, as Galardin (broad-range MMP inhibitor) is able to block the usual transactivation of the EGFR by bombesin, a GPCR agonist [85].

Interesting to note, however, is the increasing scope of research observing that RTK receptors may actually require GPCR function as part of their primary activation mechanism [86]. The insulin and insulin-like growth factor are currently the key

protagonists in these novel studies. It has been shown that these two receptors must engage with G α i and G $\beta\gamma$ proteins in order to become fully activated [87].

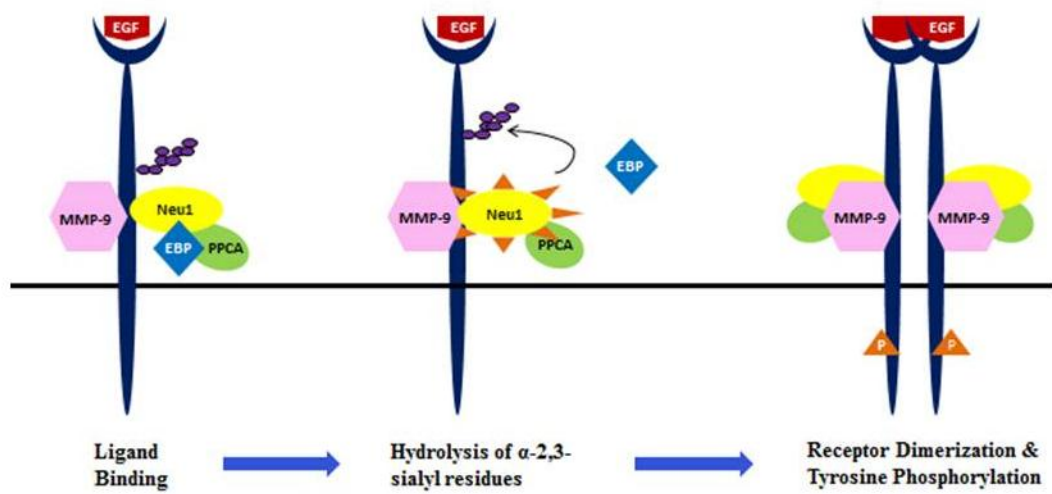
El Zein *et al.* lend further support to the GPCR-MMP9-EGFR concept by showing that when GPCRs are inhibited with cyclosporine H prior to EGF stimulation, EGF-induced ROS production, MMP-9 up-regulation, and tyrosine phosphorylation are blocked in monocytic cells [88]. Of course, central to this paradigm is the concept that a conformational change must occur within the receptor in order for the GPCR to become activated. In the case of the EGFR, it is known that a conformational change does occur upon ligand binding, with the receptor moving from 'closed' to 'open' formation, as well as the extension of a dimerization arm in order to facilitate receptor dimerization [27].

4.2 Proposed Model of EGFR Activation

Therefore, based on what is known about Neu1, MMP-9, and GPCR activity, as well as the data collected in this project, we hypothesize the following model of EGFR activation (Figure 4.1):

The EGFR is present on the plasma membrane and co-immunoprecipitates with MMP-9 and Neu1. Neu1 has been previously shown to exist in a tripartite complex with EBP and PPCA on the cell surface. Upon EGF binding to the EGFR, the MMP-9 molecule becomes activated. We hypothesize that MMP-9 activation may occur through GPCR signalling, initiated by the conformational change in the EGFR post-ligand binding. Once MMP-9 becomes activated, it will either act directly on the EBP, or it will act indirectly on the EBP by cleaving elastin in the extracellular matrix which will then go on to bind EBP. Once EBP has bound elastin peptides, Neu1 in its complex becomes

Figure 4.1. Proposed model of EGFR activation. The EGFR is located on the plasma membrane and exists in direct complex with Neu1 sialidase and MMP-9. Neu1 has been recruited from the lysosome and exists in a tripartite complex with elastin binding protein (EBP) and protective protein cathepsin A (PPCA). EGFR activation begins with EGF binding to the extracellular ligand-binding domain of the EGFR. Upon ligand binding, MMP-9 becomes activated via an unknown mechanism (GPCR?). MMP-9 exerts its elastase function, forming elastin peptides from the extracellular matrix. Upon binding of these elastin peptides, EBP detaches from the complex, initiating the activation of Neu1 sialidase. Neu1 hydrolyses α -2,3-linked sialic acids from receptor glycosylation, removing a barrier which was formerly preventing EGFR dimerization. Receptor dimerization is followed by tyrosine kinase activity, tyrosine phosphorylation, and the initiation of various cell signalling cascades within the cell.



activated. Neu1 will hydrolytically cleave α -2,3-linked sialic acid(s) from the non-reducing, terminal ends of N-glycosylation on the EGFR extracellular domain. Desialylation allows for the removal of a steric hindrance which was formerly keeping the receptor dimers apart, either by the direct repulsion of the acidic, charged sialic acids, or by the physical conformation of the receptors maintained by the presence of sialic acids. The receptor will then dimerize, autophosphorylate, and initiate its signalling network within the cell.

4.3 Clinical Relevance

Understanding the EGFR activation mechanism in complete detail has tremendous relevance in the fields of cancer biology and therapeutics. EGFR over-expression is often implicated in oncogenesis, where the downstream anti-apoptotic and pro-growth effects of EGFR signalling act to further reinforce the survival of cancerous cells. As such, analysis of EGFR expression and signalling is often incorporated into the clinical management of oncogenesis. For example, EGFR over-expression is routinely used as a biomarker in the analysis of basal-like breast tumours, where it acts as a predictor of poor prognosis and a high rate of relapse and metastasis [89].

Additionally, the presence of EGFR mutants on the cell surface can also have severe and negative effects on the health and well-being of the cell, and by extension, of the individual. One of the major EGFR mutants implicated in an array of tumours is the EGFRvIII mutant, which contains a 267 amino acid deletion in the extracellular domain of the receptor, including 4 N-glycosylation sites [10, 43, 89]. The issues with this

receptor stem from the fact that it remains constitutively active at all times, sending a continuous stream of pro-growth and division signals into the cell's interior.

Novel cancer therapeutics are built upon this knowledge and function to inhibit the EGFR with the hope of shutting down its aberrant signalling pathways. There are two major forms of therapeutics which target the EGFR activation mechanism: the first involves the administration of high-affinity antibodies (ie. cetuximab) to competitively bind to the ligand-binding site, thus preventing ligand binding, and the second involves the administration of small-molecule inhibitors (ie. erlotinib, gefinitib) which bind to the tyrosine kinase portion of the receptor and inhibit its phosphorylation activity [11, 90, 91].

If Neu1 sialidase is playing a major and critical role in the EGFR activation mechanism as our data suggest, Neu1 has the potential to be included as an alternative target for novel chemotherapeutics. By inhibiting the Neu1-mediated EGFR activation mechanism, pro-growth and division signals will not be sent into the tumourigenic cell, with potential positive effects in the clinical management of tumours. In this body of work, the PANC-1 cell line, a human carcinoma cell line derived from the pancreatic ductal epithelium, was used in a preliminary experiment to observe if Neu1 sialidase was also playing a role in EGFR activation within a human pancreatic cancer model. We observed the same results in this PANC-1 cell line as we had previously in the 3T3-hEGFR mouse fibroblast cell line, which is that EGF-stimulation of the EGFR rapidly induces Neu1 sialidase activity (section 3.5). Therefore, based on this preliminary experiment, we recommend future use of the PANC-1 cell model in the study of a Neu1-mediated EGFR activation mechanism, and support future research into the effects of

Neu1 sialidase inhibition on EGFR signalling (and by extension, oncogenesis) in an *in vivo* model.

4.4 Neu1: A Common Master Enzyme in the Regulation of RTK Activation

Given that Neu1 desialylation has been shown to be an important step in TrkA [64], insulin and insulin-like growth factor [78], and epidermal growth factor receptor activation and signalling, Neu1 sialidase may in fact act as a common master enzyme in the activation of all RTK receptors and warrants further exploration.

Chapter 5: Summary & Conclusions

The basic steps of the EGFR activation have been well elucidated. Receptor activation first requires ligand binding to the receptor, followed by a conformational change in each receptor and the extension of a dimerization arm to facilitate receptor-receptor association. After the receptor dimer has formed, the intracellular tyrosine residues of the EGFR will become phosphorylated, functioning as docking sites for intracellular signalling proteins which mediate signal transduction. While receptor glycosylation has been analyzed in terms of its importance for receptor structure and stability on the cell surface, a consensus has not been reached on the role of extracellular EGFR glycosylation in terms of receptor activation. The results of this project provide evidence for a novel mode of EGFR activation that is dependent on receptor glycosylation modification by Neu1 sialidase after ligand-binding.

To address the specific aims of this project (previously discussed in *1.13*):

Aim 1) To examine if a sialidase is activated upon EGF stimulation of the EGFR.

After employing a sialidase assay to test for the presence of active sialidase, we discovered that one of the four mammalian sialidases (Neu1, -2, -3, or -4) was becoming activated upon stimulation of the EGFR with EGF, its natural ligand. Through the use of specific neutralizing antibodies against each of the four sialidases, we were able to

deduce that it was Neu1 sialidase which was becoming activated, as the addition of this neutralizing antibody was the only one able to block EGF-induced sialidase activity.

Aim 2) To determine if sialidase activity is required for EGFR activation.

When NIH3T3-hEGFR cells were pre-treated with broad-range (oseltamivir phosphate) and specific (anti-Neu1 neutralizing antibody) sialidase inhibitors prior to EGF stimulation of the EGFR, the result was inhibition of EGFR tyrosine phosphorylation. Therefore, Neu1 sialidase activity appears to be required for EGFR activation.

Aim 3) To investigate a possible mechanism of Neu1-dependent EGFR activation.

It was previously known that MMP activity is able to activate Neu1 sialidase through its interaction with elastin binding protein which exists in complex with Neu1 sialidase on the cell surface. Therefore, we hypothesized that an MMP may be mediating EGF-induced Neu1 sialidase activity. Upon pre-treatment of the NIH3T3-hEGFR cells with both broad-range (galardin, piperazine) and specific MMP inhibitors (MMP-3i, MMP-9i) prior to EGF stimulation, we found that MMP-9 activity is critical for EGF-induced Neu1 sialidase activity, as well as EGFR tyrosine phosphorylation (and thus, activation). This concept is further supported by the finding that both Neu1 and MMP-9 co-immunoprecipitate with the EGFR on the plasma membrane in both naïve and EGF-stimulated NIH3T3-hEGFR cells, allowing for our proposed rapid activation and molecular interaction.

Understanding the EGFR activation mechanism in detail has potential for great clinical relevance and use. Due to the role of EGFR signalling and over-expression in oncogenesis, EGFR activation is a common therapeutic target in drug development. Therefore, by gaining a greater understanding of EGFR activation and its association with Neu1 sialidase activity, Neu1 sialidase may soon be included as another molecule that has the potential to be targeted therapeutically. Additionally, the finding that both the TrkA and EGFR activation pathways are dependent on Neu1/MMP-9 cross-talk provides us with clues towards the existence of a common activation scheme among the entire family of RTK receptors.

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