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Mom and all my siblings, I love each and every one of you. My sincere gratitude goes to you for believing in me.

I dedicate this humble work to you all.

Tromsø - May 2013

Eslaem Almahi

ABSTRACT[1]

Background: Protein kinases and phosphatases persistently regulate various signal pathways that mediate many cellular processes. The mitogen activated protein kinases (MAPKs) are key components in the transduction of extracellular stimuli to biological responses. The dysregulation of MAPKs activity promotes the occurrence of diverse diseases, including cancer, making MAPKs signaling pathways attractive targets for developing new potential drugs. The extracellular signal-regulated kinase 3 (ERK3) is a unique atypical MAPK when it comes to regulation and functions. Much less is revealed about ERK3 pathophysiological functions in cancer pathogenesis. The MAPK-activated protein Kinase 5 (MK5) has lately been identified as the first downstream target for ERK3. A recent study has found that ERK3 can regulate the activity of the oncogenic steroid receptor coactivator 3 (SRC-3), which in turn promotes lung cancer cells migration and invasiveness.

Aim: In this study, we investigate the identity of the phosphor-donor of SRC-3 at Ser857, and examine the effect MK5, ERK3, and SRC-3 have on MMP2, MMP9, and MMP10 gene expressions, and on lung cancer cell proliferation and migration.

Methods: Subcloning, Western blot, qRT-PCR, luciferase assay, protein purification, *in vitro* kinase assay, colonogeneic assay, and scratch assay.

Results: MK5 seems to phosphorylate SRC-3 at Ser857 *in vitro*. SRC-3 and MK5 overexpression and co-overexpression increase MMP2 and MMP10 promoter activities. MK5, SRC-3, and ERK3 overexpressions enhance MMP9 promoter activity. The siRNA-mediated knockdown of MK5 and ERK3 results in reduced MMP2, MMP9, and MMP10 promoter activity in A549 cells. Endogenous MMP2 and MMP9 mRNA levels significantly decrease by expressing shRNAs targeting MK5 and ERK3 in H1299 lung cancer cells.

Conclusion: MK5 phosphorylates SRC-3 at the S857 *in vitro*. MMP2, MMP9, and MMP10 promoter activities and MMP2 and MMP9 mRNA expressions diminish by RNAi-facilitated knockdown (either by siRNA or shRNA) of ERK3 and MK5 in lung cancer cells.

Key words: Protein kinase, MAPKs, SRC-3, ERK3, MK5, MMP2, MMP9, MMP10, and lung cancer cells

ABBREVIATIONS

AP-1	Activator Protein 1
Bcr-Abl	Breakpoint Cluster Region-Abelson
bp	Base Pair
BSA	Bovine Serum Albumin
CBP	CREB-Binding Protein
CID	CBP-Interaction Domain
CP	Current Protocol
Ct	Cycle Threshold
CTD	Carboxyl Terminal Domain
dH_2O	Distilled MilliQ water
DNA	DeoxyriboNucleic Acid
EDTA	Ethylene Di-amine Tetra Acetic acid
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EGTA	Ethylene Glycol Tetra Acetic acid
ER	Estrogen Receptor
ERK	Extracellular signal Regulated Kinase
FAK	Focal Adhesion kinase
FBS	Fetal Bovine Serum
GPCR	G-protein Coupled Receptor
GR	Glucocorticoid Receptor
Her2	Human Epidermal Growth Factor Receptor 2
IGF-I	Insulin-like Growth Factor 1
IPTG	Isopropyl β-D-1-Thio-Galactopyranoside
IRS-1	Insulin Receptor Substrate 1
JAK	Janus Kinase
JNK	c-Jun N-terminal Kinase
kb	KiloBase
LB	Lysogeny Broth
MAPK	Mitogen-Activated Protein Kinase
MK5	MAPK-activated protein Kinase 5
MMP	Matrix MetalloProteinase

NF-κB	Nuclear Factor Kappa-light-chain-enhancer of activated B cells
NLK	Nemo-Like Kinase
NTC	No Template Control
PAK	p21-Activated Kinase
PBS	Phosphate Buffered Saline
PEA-3	Polyomavirus Enhancer Activator 3
PKA	Protein Kinase A
PKB	Protein Kinase B
PKC	Protein Kinase C
RNA	RiboNucleic Acid
Rpm	Rounds Per Minute
RTK	Receptor Tyrosine Kinase
SDS-PAGE	Sodium Dodecyl Sulfate Poly-Acrylamide Gel Electrophoresis
shRNA	Small/short hairpin RNA
siRNA	Small/short interfering RNA
SOC	Super Optimal broth with Catabolite repression
SRC-3	Steroid Receptor Coactivator 3
STAT	Signal Transducer and Activator of Transcription
TEA	Tris base, EDTA, and Acetic acid
TK	Tyrosine Kinase
Tris	Tris-(hydroxymethyl)-aminomethane
WT	Wild Type
β-Act	Beta-Actin
β-ME	2-Mercaptoethanol

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INTRODUCTION

Signal transduction is a vital process in living cells. Normally, the extracellular signals are transmitted into intracellular responses as a consequence of environmental changes, through receptor proteins or by transmembranal passing. Not only that cells perceive signals, cells produce signals too, for instance, as a mean of communication with other cells. This harmonized network of interactions is crucial in maintaining a stable, relatively constant condition in cells, as well as in cell growth, division, metabolism, apoptosis, differentiation, and motility.[1]

When a ligand, first messenger, binds to a receptor, a change in a certain intrinsic enzymatic activity is formed, and subsequently a so-called signal cascade is often created starting by the activation of a second messenger. Stimulation of intracellular transducing proteins, activation and inhibition of downstream effector proteins, and alternations in gene expression are examples of this kind of change. This phenomenon ends up with the acquisition of new functional properties. [2, 3]

Phosphorylation by protein kinases is one of the most central enzymatic activities in intracellular signaling and in signal transmission.[1]

1. Protein kinases

Protein phosphorylation is a reversible posttranslational modification that controls several physiological aspects of cells fate, such as cell division, proliferation, differentiation, and apoptosis. Accordingly, the balance between activation of kinases and phosphatases is tightly regulated in order to maintain cellular homeostasis.[4] Kinases and phosphatases reciprocal relationship is illustarted in Figure 1.

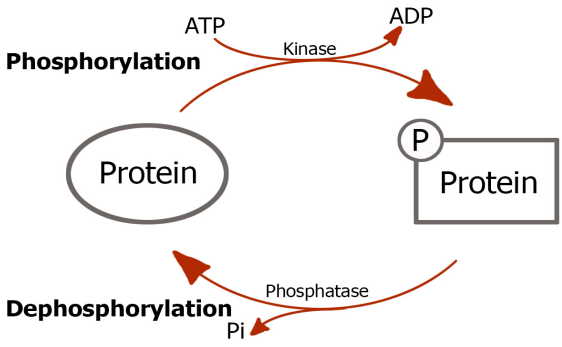


Figure 1: The action of protein kinases and phosphatases. A conformational change subsequent to phosphorylation is indicated as shape alternation.

Protein kinases are a group of enzymes that catalyze the transfer of a γ-phosphate from a donor, e.g. ATP, to a hydroxyl acceptor in an amino acid residue. Kinases are often classified depending on the phospho-acceptor amino acid. Mainly, there are the conventional Ser/Thr and tyrosine kinases. By phosphorylation, a protein is either activated or inhibited. [1, 5]

There are several mechanisms by which kinases phosphorylate their substrates. The serine/threonine or tyrosine specificity is predetermined by the structure of the catalytic domain of the kinase and binding interactions between kinase and the substrate.[6]

Phosphatases facilitate the reversible reaction, removing phosphate modification; and thenceforth the previous activity status is restored.[5]

1.1. Tyrosine kinases

Tyrosine kinases (TKs) are a class of protein kinases that have been divided into two main categories: receptor TKs, and non-receptor TKs. It has been discovered recently that our human kinome encodes 90 TKs.[7]

Receptor tyrosine kinases RTKs are transmembrane receptor proteins constitute of an extracellular binding domain, a transmembrane-spanning region, and an intracellular tyrosine kinase site containing the catalytic domain.[8]

In their unbound form most of RTKs exist in a monomeric and non-phosphorylated form. When a ligand binds to the extracellular domain of RTKs, it induces and stabilizes oligomerization through conformational changes, thus increasing the RTK activity. Subsequent to oligomerization of RTKs, the ATP-binding site catalyzes RTKs activation via autophosphorylation of cytoplasmic tyrosine residues. Consequently, further binding sites are formed for substrate proteins such as Shc, Grb2, and Src. These proteins in turn recruit other effector molecules, and intracellular signal cascades are then activated. The most common downstream signaling pathways activated by RTKs include Ras–ERK, extracellular signal regulated kinase, –mitogen activated (MAP) kinase pathway, the phosphoinositide 3-kinase (PI 3-kinase)–Akt, and the JAK/STAT signaling pathway. Eventually, the amplified signal that is triggered by RTKs leads to the regulation of gene expression as a response to the signal that initiated the cascade. [7, 8]

The second category of TKs is non-receptor, or cytosolic, TKs which lack both extracellular and transmembrane regions, and so their activation by signals causes either their dissociation from inhibitors or the phosphorylation of tyrosine residues. [7]

Cancers that originate from RTKs signal pathways occur by several mechanisms, e.g. gene amplification, overexpression of RTK proteins, and mutations in the corresponding genes.

These factors contribute to constitutive RTK signaling, sooner or later leading to the manifestation of dysregulated cell growth and cancer.[8]

1.2. Protein kinase signal pathways as drug targets

The conventional treatment of cancer consists of anti-metabolites, DNA topoisomerases, agents directed towards DNA (e.g. alkylating agents), agents directed towards hormonal signaling and against microtubule stabilization. [9]

This lasted from 1910s to the late 1990s. Now new novel therapeutic approaches are being developed based on the molecular mechanisms behind cancer for better-targeted therapies.

In cancer research large number of identified oncogenes encode protein kinases, therefore protein kinases play a major role in cancer initiation and maintenance.[4]

Abnormal protein phosphorylation that is caused by mutant alleles of protein kinase genes or oncogenes that signal through these protein kinase cascades might cause or lead to diseases. This occurs as a consequence of deregulation of cellular processes.

The establishment of the association between protein kinase Bcr-Abl and chronic myelogenous leukemia (CML) has revolutionized the treatment of CML through the discovery of imatinib (Gleevec®).[10] Next generation of Bcr-Abl tyrosine-kinase inhibitors followed imatinib. Hence the newly discovered nilotinib (Tasigna®) and dasatinib (Sprycel®) were approved as drugs for the treatment of CML. As for receptor kinase inhibitors, several epidermal growth factor receptor (EGFR) kinase inhibitors were developed, such as gefitinib (Iressa®). Additionally, trastuzmumab (Herceptin®), a monoclonal antibody against the Her2 extracellular domain was approved for treating breast cancer.[9]

Protein kinase inhibitors have also been assigned for treating other diseases, such as cardiovascular disease and vascular complications in diabetes mellitus [11], chronic inflammatory diseases, [12] and neurodegenerative diseases.[13]

2. Mitogen activated protein (MAP) kinase family

MAPKs are one of the most studied kinase families, and they play critical roles as signal mediators in mammalian cells.[14] MAPK family consists of ubiquitous proline-directed, protein-serine/threonine kinases. MAPKs take part in signal transduction pathways that modulate various cellular functions, for instance responses to hormones.[15] These biological functions affect diverse cellular programs, such as embryogenesis, proliferation, differentiation, and apoptosis depending on the metabolic state and cell environment.[16]

So far fourteen MAP kinase genes have been distinguished in our human kinome, of which define 7 distinct MAPK signaling pathways.[17]

2.1. The MAPK signaling cascades

The core of these cascades comprises at least three sequentially protein kinases that culminate in the activation of a multifunctional MAP kinase.[15] Each cascade starts upstream when an extracellular ligand, such as growth factor, stimulates RTK, the small GTPase RAS is activated, which in sequence recruits a MAP kinase kinase kinase (MAPKKK, MAP3K, or MEKK) such as Raf, which in turn activates a MAP kinase kinase (MAPKK, MAP2K or MEK), and then the effector MAPK itself is activated by the phosphorylation on two residues within a motif (Thr-Xaa-Tyr).[14, 17] The MAPKs activate by phosphorylation a vast array of substrate proteins on conserved (Ser-Pro) and (Thr-Pro) motifs. The substrate specificity is partly mediated by the selective docking of MAPKs to the substrate proteins. [14]

The signal pathway Ras-Raf-MAP2K-MAPK/ERK in vertebrates can be mobilized by extracellular ligands binding to either RTKs or GPCRs,[1] as shown in Figure 2.

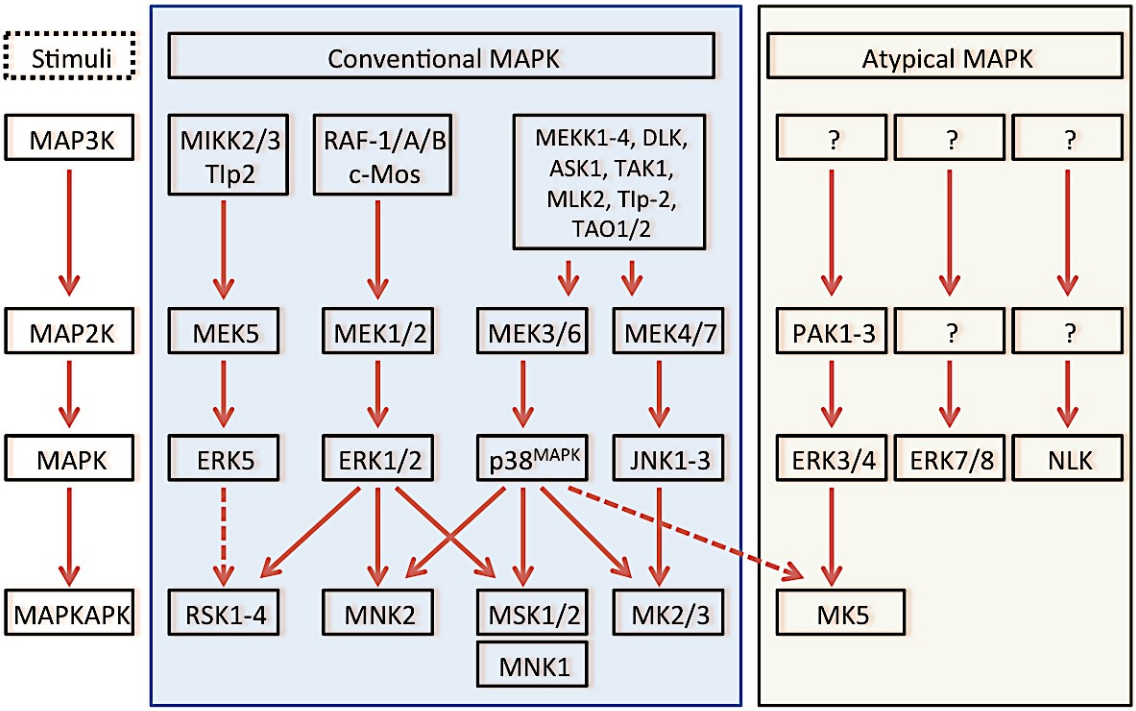


Figure 2: Conventional and atypical MAPK signaling pathways and the activation of downstream MAPKAPKs – The dotted lines indicate that the respective kinase reaction remains to be investigated. Figure is modified from Kostenko et al, 2012 [18]

2.2. Classification of MAPKs

MAPKs have been classified into conventional and atypical MAPKs. This categorization is based on their ability to get phosphorylated and activated by the previously mentioned MAPKKs/MAP2Ks.

The conventional MAPK family consists of ERK5, ERK1/2, p38 MAPK, and JNK1-3 that are protein substrates of several MAP2Ks. While the atypical MAPK family consists of ERK3/4, NLK, and ERK7. Little is revealed about their substrates but there are evidences indicating that PAK1-3 are phosphorylating and activating ERK3/4. The regulation, substrate specificity and physiological functions of atypical MAP kinases are not fully understood yet. (17, 19)

2.3. Conventional MAPKs

2.3.1. ERK1/2

Extracellular signal-regulated kinase 1 and 2 (ERK1/2) were first identified 20 years ago. They are 43 and 41 kDa, ubiquitously expressed, and share 83% amino acid identity.[16] The two kinases ERK1/2 are excessively expressed in tissues, and play vastly important roles in the regulation of meiosis and mitosis processes in differentiated cells. Various types of stimuli, e.g. growth factors, cytokines, virus infection, and carcinogens, trigger the ERK1/2 signaling pathway.[20] Upstream this cascade, the MAP2K/MAP3K kinases phosphorylate two serine residues or a serine and a threonine residue in the MAP2K1/2. The MAP3Ks in the ERK1/2 pathway are the isoform of Raf (c-Raf1, B-Raf, or A-Raf), Mos, and Tp12.[16] The so-called oncogenic Ras predominantly activates the ERK1/2 pathways, which in order contributes to the increased proliferation of tumor cells.[20] The mitogen-activated protein kinase (MAPK) kinases MAP2K1/2 phosphorylate ERK1/2 on tyrosine and threonine residues in the (Thr-Glu-Tyr) motif that is a part of the activation loops.[14] Downstream, ERK1/2 phosphorylate several protein kinases, such as the protein kinases p90 ribosomal S6 kinase (RSK), mitogen and stress activated kinase (MSK), MAPK interacting kinase (MNK), and the transcription factors Elk1, c-Fos, c-Myc, and Ets domain factors. RSK, MASK, and MNK participate in cell attachment and migration.[16]

2.3.2. ERK5

ERK5, also known as BMK-1 (big mitogen-activated protein kinase-1), is a MAPK that is coded by the ERK5-/MAPK7-gene. It was studied back in 1995 as the MEK5 interacting binding partners were investigated using yeast two-hybrid screen. ERK5 was also called

BMK-1 because of its large size compared to ERK1/2. At the same time it was revealed that ERK5 is particularly overexpressed in the heart, skeletal muscle, placenta, lungs, and kidneys. Further studies have shown that ERK5 is localized in both nucleus and cytoplasm, and that ERK5 signaling pathway is crucial for the development of the cardiovascular system, as well as having a potential role in tumor angiogenesis.[21]

The MEK5-ERK5 cascade starts when MAP3K2/3 is activated by cytokines, growth factors, neurotransmitters, hormones, or various cell stressors through RTK, G-coupled receptor protein or hormone receptor. When MAP3K2/3 binds MEK5 the whole confirmation of MEK5α protein is altered in away that enables Ser311 and thr315 to be come accessible for phosphorylation. Subsequently, MEK5 phosphorylates the conserved Thr218 and Tyr220 in the (Thr-Glu-Tyr) motif of ERK5. MAP3K2/3 and ERK5 bind to the same N-terminus extension of MEK5α. The suggested hypothesis is that MEKK-MEK5 complex dissociates to allow ERK5 interaction with MEK5.[22]

Accordingly, the activated ERK5 is able to phosphorylate various downstream substrates such as Sap1, cFOS, c-Myc, and MEF2. EKR5 can also autophosphorylate its carboxyl-terminal region, on the NLS region (nuclear localization signal), which allows ERK5 to shuttle from the cytosol to the nucleus and function as a transcription factor.[22]

Two of the downstream substrates, that ERK5 induces the phosphorylation of, are Bad and Foxo3a. This process could be conducted in either an Akt/PKB (protein kinase B)-independent or dependent manner. In both cases, the consequence of phosphorylation is blocking the apoptotic effect in the cell. [23]

2.3.3. p38-MAPK

The p38 protein is a tyrosine-phosphorylated protein and an enzyme that is well characterized and found to be expressed in the majority of cell types. There are four p38-kinases α (MAPK14), β (MAPK11), γ (MAPK12), and δ (MAPK13). The p38 MAPKs are involved in the regulation of gene expression of many cytokines and the activation of the immune response. On the other hand, inflammatory cytokines, hormones, and numerous environmental and cellular stressors, such as UV radiation, osmotic shock, heat shock, and hypoxia activate p38-MAPKs.[16, 20] The p38-MAPKs are also activated via dual phosphorylation on the (Thr-Glu-Tyr) motif within its activation loop by the MAP2K3/6.[16] On the contrary, MAP2K4 activates both p38-MAPKs and JNKs. [14]

The p38-MAPKs regulate the activity for several transcription factors, such as p53, activating transcription factor 2 (ATF2), ElK1, myocyte-specific enhancer factor 2 (MEF2) and

C/EBPβ. In addition, there are protein kinases that are also regulated by the p38-MAPKs, such as MAPK-activated kinase 2 (MK2 or MAPK2), mitogen- and stress-activated protein kinase 1 (MsK1), MAP kinase-interacting serine/threonine kinases 1 and 2 (MNK1/2). It is thought that p38α has a tumor suppressor role, due to its negative regulation of cell cycle progression and inducing apoptosis. Yet, there is a contra-hypothesis indicating that p38α might have oncogenic functions as well. [24]

2.3.4. JNK1-3

c-Jun N-terminal kinases (JNKs), also known as stress activated protein kinases (SAPK) is a family that consists of three distinct genes encode JNK/SAPKs: JNK1/SAPKβ, JNK2/SAPKα, and JNK3/SAPKγ. They are also termed MAPK8/9/10 respectively.[16, 24] The JNK1/2 are widely expressed in almost all of the body cells, however, JNK3 is mainly found in the brain, heart, and testis.[14]

The discovery of the JNKs was upon their binding and phosphorylating the DNA binding protein c-Jun. c-Jun associates with the AP-1 (activator protein 1) transcription complex that plays a critical role in the regulation of gene expression, such as cytokine genes as a consequence of environmental stress and growth factors.[20] It´s suggested that AP-1 activating by JNKs is ascribing JNKs to oncogenic functions. At the same time, it is believed that they have tumor suppressive functions due to their pro-apoptotic activity.[24] On the other hand, JNKs are thought to be involved in apoptosis by mitochondrial pathway mechanism.[14]

Inflammatory cytokines; environmental stresses, such as heat shock, ionizing radiation, oxidant stress, and DNA damage; DNA and protein synthesis inhibition; and growth factors trigger the activation of JNK/SAPKs. Accordingly, JNK proteins participate in several physiological events, such as cytokine production, inflammatory responses, stress-induced and developmentally programmed apoptosis, actin reorganization, cell transformation and metabolism.[16]

The JNK group of MAP kinases is activated by many of the same stimuli that cause activation of the p38 MAP kinases, including the exposure of cells to inflammatory cytokines and environmental stress. Due to this mode of activation, both the JNK and p38 MAP kinases are often collectively called stress-activated protein kinases (SAPK). [14]

Different MAP3Ks activate MAP2K4/7, including MAP3Ks (MAP3K1-4), mixed lineage kinases (MLKs, including MLK1-3 and DLK), Tpl2, ASKs, TAOs and TAK1. MAP2K4/7 phosphorylate and activate JNKs on (Thr-Pro-Tyr) motif within the activation loop, with phosphorylation selectivity for MAP2K4 and MAP2K7 on tyrosine and threonine residue,

respectively. MAP2K7 is a specific activator of the JNK pathway, as MAP2K4 activates both p38-MAPKs and JNKs.[14] Upstream MAP3K enzymes activate these two MAP2Ks through the phosphorylation of serine or threonine residues. JNKs phosphorylate and regulate many transcription factors, such as c-Jun, ATF-2, p53, Elk-1, and nuclear factor of activated T cells (NFAT), which in order regulate the expression of specific sets of genes that mediate cell proliferation, differentiation or apoptosis. [16]

2.4. Atypical MAPKs

Atypical MAP kinases include ERK3/ERK4, Nemo-like kinase NLK and ERK7. Much less is known about the regulation, substrate specificity and physiological functions of atypical MAP kinases.

Unlike other MAPKs, the atypical MAPKs ERK3/4 and NLK differ in the distinct (Thr-Xaa-Tyr) motif that serves for phosphorylation activation by MAP2K1-7. The unlikeness is the tyrosine residue is replaced by either glycine or glutamic acid. The ERK7 contains however the (Thr-Glu-Tyr) motif with a glutamic acid and tyrosine in place. This structural feature is indicating that ERK7 catalyzes autophosphorylation itself, without the participation of any MAP2Ks. [17]

2.4.1. ERK3/4

ERK3 was discovered 15 years ago, when it was cloned and studied side by side with the extensively studied MAP kinase ERK2. Later on, another human kinase with high homology to ERK3 was cloned at 1992, due to the interest of its homology to ERK1, and it was named ERK4.

Recent comparative genomic analyses showed that MAPK6 and MAPK4 (human ERK3 and ERK4 gene official names) display a similar organization of exon/intron boundaries, which is different from that of genes encoding conventional MAP kinases and related protein kinases.[17]

A structural comparison between ERK3/4 and other classical and atypical MAPKs are demonstrated in Figure 3.

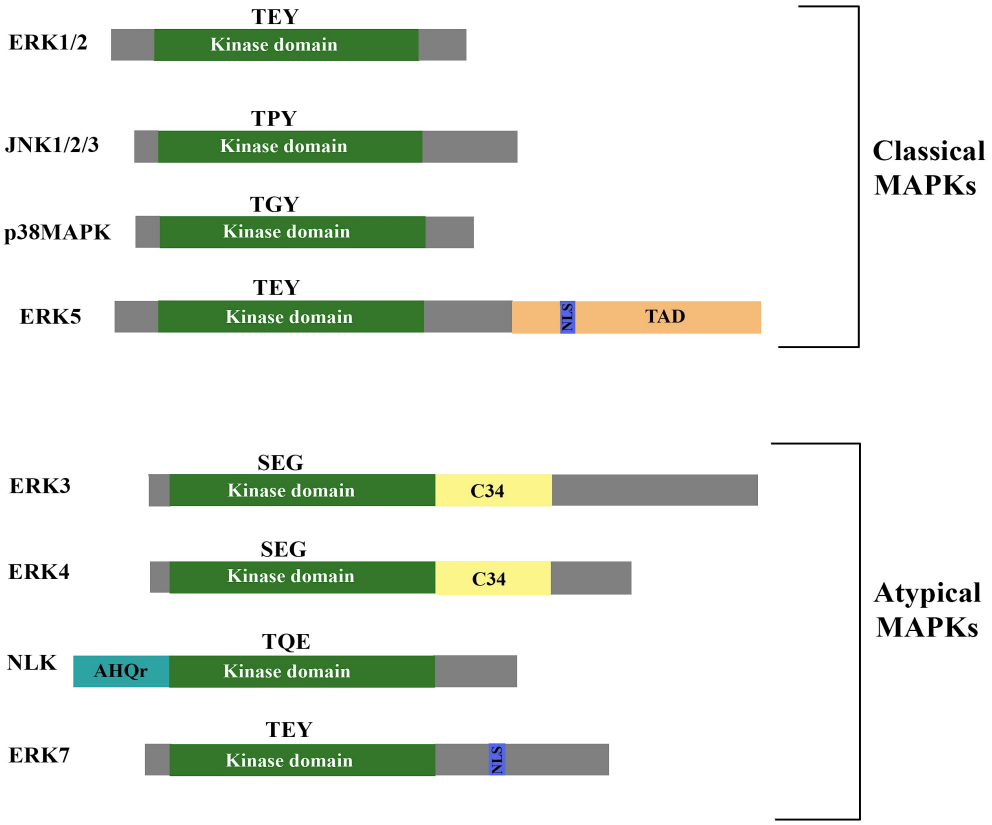


Figure 3: Schematic illustrations of human MAPKs structure on scale: Phosphorylation sites within kinase domains are indicated, along with some important domains. NLS: Nuclear localization sequence. TAD: Transactivation domain. C34: Conserved region in ERK3/4. AHQr: Ala/His/Gln-rich domain. Figure is modified from Coulombe et al, 2007 [17]

There is high amino acid identity, up to 73% within the kinase catalytic domain, between ERK3 and ERK4. Therefore, there are strong evidences suggesting that the MAPK6 and MAPK4 genes arose by duplication of a common ancestor.[17, 25]

Structurally, ERK3 and ERK4 contain a single phospho-acceptor site (Ser-Glu-Gly) in their activation loop, instead of the conserved dual-specificity (Thr-Xaa-Tyr) motif in other MAPK family members. Additionally, the ERK3 and ERK4 orthologs have the sequence (Ser-Pro-Arg) instead of (Ala-Pro-Glu) in subdomain VIII of the kinase domain. Interestingly, ERK3 and ERK4 are the only kinases in the kinome to have an arginine residue at this position.[17]

Unlike ERK4, ERK3 has a short half-life in vivo, due to the rapid processing by ubiquitin-mediated degradation. The physiological function of ERK3 and ERK4 still remains unclear. However, recent studies have indicated that one substrate of ERK3/4 is MAPKAPK5 (MK5)[14]

ERK3 mRNA is highly expressed in mammalian tissues, and it's mostly found in brain,

skeletal muscles and gastrointestinal tract. While ERK4 mRNA expression is found to be restricted to the brain, colon, eye, heart, kidney, lung, ovary, pancreas, placenta, prostate, and skin, with highest expression in the brain. Much less is known about the regulation mechanisms of ERK4 gene expression. Metabolic labeling and immunoblotting analysis with a phospho-specific antibody have shown that ERK3 is phosphorylated on activation loop Ser189 in cells. At the same time, it has been found that recombinant ERK3 protein, purified from E. coli, is capable of autophosphorylating itself on Ser189 in vitro. Several studies suggest that ERK3 plays a role in the control of cell differentiation as a negative regulator of cell cycle progression under certain cellular conditions, thus facilitating cell differentiation. Besides, it has been recently suggested that ERK3 contribute in glucose-induced insulin secretion.[17] ERK4 is, on the other hand, phosphorylated on activation loop residue Ser186, leading to its catalytic activation.[26]

2.5. PAK1-3

The p21-activated kinase family (PAKs) is one of the early discovered direct kinase targets of Ras-related small GTPases, and they are suggested to be central players in growth factor signaling networks that regulate morphogenetic processes. It has been established that PAKs control cell proliferation in some circumstances; however, they are widely involved in establishing cell polarity and promoting cellular plasticity via changes in the actin cytoskeleton. PAKs have great influence on cancer processing, brain function, and virus infection.[27]

The PAKs have been identified as ERK3/ERK4 activation loop kinases. Evidences show that I PAKs phosphorylate ERK3 and ERK4 on Ser-189 and Ser-186, respectively, both in vitro and in vivo, and that expression of activated Rac1 boosts this response. As a consequence of PAK-mediated phosphorylation of ERK3/ERK4, downstream MAP kinase-activated protein kinase 5 (MK5) is activated. [26]

2.6. MAPK-activated protein kinases (MAPKAPKs)

Downstream the MAPKs signal pathway, there are three structurally related MAPK-activated protein kinases (MAPKAPK2/3/5 or MK2/3/5) that modulate signal to various cellular targets. Although there is no known common function for all three MKs, these kinases share similar structural features. Moreover, MKs regulate gene expression at the transcriptional and post-transcriptional level, control cytoskeletal architecture and cell-cycle progression, and are

implicated in inflammation and cancer.[28]

In the same review it was mentioned that binding to p38α prior to stress activates MK2/3, whereas MK5 is unique in its interaction with and long-term activation by ERK3. The MKs exist in different cellular compartments from time to time, and this feature is regulated by conformational changes of the MKs themselves, as well as by nuclear export for MK2/3 and by protein–protein interactions and cytoplasmic anchoring for MK5.[28]

2.6.1. MK5

MAP kinase-activated protein kinase 5 (MK5) was first characterized as a protein kinase activated downstream of the p38 MAP kinase, hence is also named p38-regulated/activated protein kinase (PRAK). The physiological functions of MK5 depend on its activation and complex formation with the atypical MAP kinases ERK3/4. Accordingly, MK5 is thought to be unique in being regulated downstream of signaling pathways other than the classical MAP kinases p38 and ERK1/2 unlike other MAPKAPs.[29] Structural domain within MK5 are illustrate din Figure 4.

ERK3 specifically interacts with MK5 in vitro and in vivo. Subsequent, the expression of ERK3 in mammalian cells leads to nuclear-cytoplasmic translocation and activation of MK5 and to phosphorylation of both ERK3 and MK5. The activation of MK5 is independent of ERK3 enzymatic activity, but depends also on its own catalytic activity as well as on a region in the C-terminal extension of ERK3.[30]

Figure 4: Schematic illustration of MK5 structure: Catalytic domain with phosphor-acceptor site based on human sequence. NES: Nuclear export sequence. NLS: Nuclear localization sequence. ER3/4: ER3/4 interaction domain. Figure is adapted from Perander et al, 2008 [29]

Besides ERK3, ERK4 binds and activates MK5 too. In contrast to the classical MAPKs, the interaction between ERK3/4 and MK5 is strictly dependent on phosphorylation of the SEG motif of these kinases.

Åberg et al defined a novel MK5 interaction motif (FRIEDE) within ERK3/4, which is crucial for binding to the C-terminal region of MK5. [31]

3. SRC-family

Nuclear receptor co-regulators (NRCs), also known as steroid receptor co-activators (SRCs), are a family that constitute of ligand-regulated and orphan transcription factors. SRCs play an important role in the body's ability to transduce steroid, retinoid, thyroid, and other lipophilic endocrine hormones.[32]

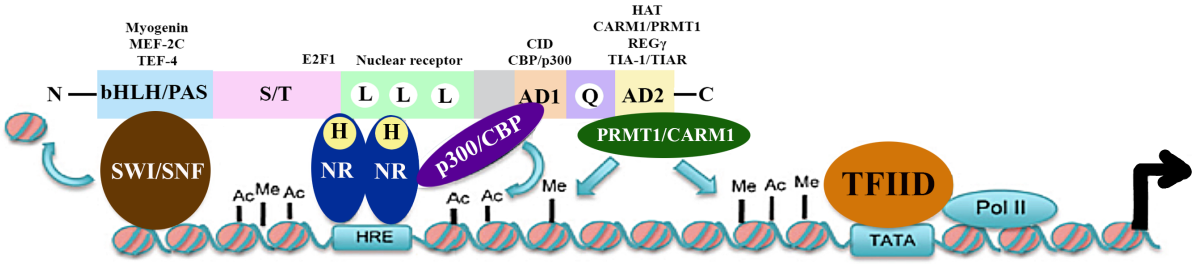


Figure 5: The activation of nuclear receptors, here SRC-3. SRC proteins act as coactivators of hormone bound nuclear receptors bind through their LXXLL motifs, of which they have three. Afterwards, SRCs recruit multiple secondary coactivator complexes that bind to their three activation domains (ADs). These secondary coactivators modify the chromatin and bridge the nuclear receptor complex with transcription machinery to start transcriptional activation. bHLH/PAS: basic helix-loop-helix/Per-Arnt-Sim. S/T: Ser/Thr-rich region. NR: nuclear receptor. Ac: acetylation. Me: methylation. HRE: hormone response element. L: LXXLL motifs. Figure is modified from Johnson and O´Malley, 2012.[33]

Nuclear receptors (NRs) are a large protein superfamily that binds as homo- or heterodimers to specific DNA elements leading to transcriptional activation of target genes, as shown in Figure 5. For instance, SRC-1 overexpression enhances ligand-induced transcriptional activation by progesterone receptor (PR), estrogen receptor α (ERα), glucocorticoid receptor (GR), thyroid receptor (TR), and retinoid X receptor (RXR).[33]

3.1. SRC-3

Steroid receptor coactivator-3 (SRC-3), also known as AIB1, is a member of the p160 steroid receptor coactivator family. Already in 1997, it was found that SRC-3 is amplified in breast cancer, so was named amplified in breast cancer 1 (AIB1). Originally, SRC-3 was identified as a transcriptional coactivator for nuclear receptors such as the estrogen receptor (ER), involved in the proliferation of hormone-dependent cancers. Since then the role of SRC-3 in cancer has been widely investigated.[34] SRC-3 was defined as a bona fide oncogenic protein overexpressed in multiple human cancers.[35]

SRC-3 is also known as nuclear receptor coactivator-3 (NCoA-3), receptor associated coactivator-3 (RAC3), activator of thyroid hormone and retinoid receptor (ACTR), thyroid

hormone receptor activating molecule-1 (TRAM1), and p300/CBP interacting protein (p/CIP).[34]

SRC-3 is a transcriptional coactivator for several transcription factors, such as E2F1, AP-1, NF-κB, STAT6, and PEA3. Besides it is thought that SRC-3 acts as a translational repressor to regulate proinflammatory cytokine mRNA translation.[34]

The SRC-3 gene is located on chromosome 20q12-12. The SRC-3 protein is approximately 160 kDa and contains three basic structural domains, shown in Figure 6, consistent with the other two SRC family members (SRC-1 and SRC-2). The N-terminal basic helix-loop-helix-Per/ARNT/Sim domain (bHLH-PAS) mediates the interaction between SRC-3 and other DNA-binding proteins. The receptor-interaction domain (RID) contains three LXXLL motifs, by which SRC-3 binds to the ligand-activated nuclear receptors. The C-terminal domain contains two intrinsic transcriptional activation domains, AD1 and AD2, by which SRC-3 interacts with histone acetyl-transferases and histone methyl-transferases, respectively. [34]

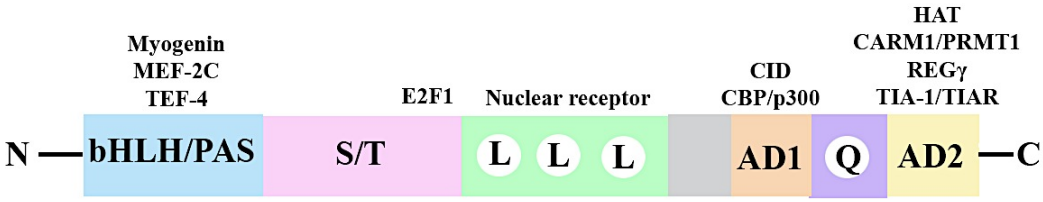


Figure 6: Structural domains of SRC-3. AD1/2: Activation domains. CID: CBP/p300 interacting domain. S/T: Ser/Thr-rich region. bHLH: Basic helix-loop-helix domain. L: LXLL α-helix motif. PAS: Per/ARNT/Sim homologous domain. Q: Gln-rich domain. RID: Receptor interacting domain. Figure modified from Tien and Xu, 2012

3.1.1. SRC-3 in cell cycle control

The role of SRC-3 in cell cycle has been extensively studied. Some suggested that SRC-3 acts as transcriptional coactivator for the G1/S phase transition in association with the transcriptional factor E2F1. This supports the suggested theories that SRC-3 overexpression induced cell proliferation and transformation of tumors in away that is E2F1-dependent.

Other results suggested that SRC-3 is involved in regulating cell cycle, but through the activation of Akt signaling pathway.

A third suggested mechanism supporting the fact that SRC-3 is a cell cycle regulator is through the regulation of IGF-I that is shown to be influencing the signaling in cancer. It was observed that SRC-3 is tightly correlated with IGF-I signaling in cancers. Some other studies predicted that SRC-3 regulates IGF-I signaling through binding to the transcription factor AP-1, and AP-1 on its order enhances the transcription of IGF-I and IRS-1. [34]

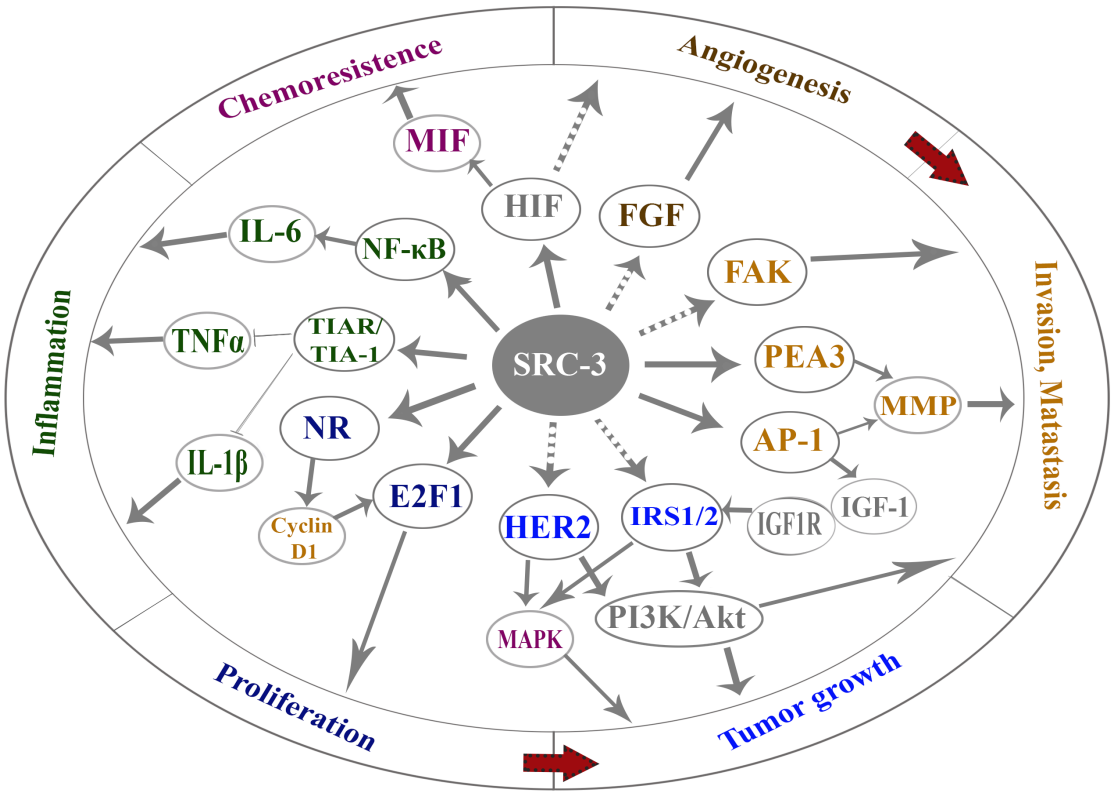


Figure 7: SRC-3 affects various cell aspects by through multiple signaling pathways. SRC-3 is shown to act as a co-activator for different nuclear receptors and transcription factors, which lead to promote cancer. Several SRC-3 interaction partners are illustrated. Figure is modified from Tien and Xu, 2012 [36]

3.1.2. SRC-3 in tumorigenesis

A number of clinical studies showed that SRC-3 overexpression correlated with expression of the EGFR family member HER2 in breast cancer specimens, and that SRC-3 depletion completely inhibited mammary tumorigenesis induced by the oncogene MMTV-HER2/neu. One recent in vitro study showed that SRC-3 knockdown in lung, pancreatic and breast cancer cell lines reduced EGF-mediated phosphorylation of EGFR and HER-2, and therefore inhibited the activation of EGF signaling. [34]

It has been shown in several molecular studies that SRC-3 might lead to an increase in the expression of MMPs, which in turn break down the extracellular matrix enabling the tumor cell to invade into stromal compartment. [36]

3.1.3. SRC-3 in apoptosis

Some investigators suggested that overexpression of SRC-3 reduced H_2O_2-mediated apoptosis in the HEK293 cell line. Several studies indicate that SRC-3 possessed anti-apoptotic properties. It is established that NF-κB signaling inhibits apoptosis by regulating the transcription of many anti-apoptotic proteins, such as Bcl-2 and the FLICE inhibitory

protein (FLIP). On the other hand, SRC-3 activated NF-κB signaling in coordination with IκB kinase (IKK) and acted as a transcriptional coactivator for NF-κB in cancer cells. Allover, SRC-3 affects apoptosis through NF-κB signaling. Furthermore, SRC-3 is influencing apoptosis through Akt signaling, which is involved in multiple apoptosis signaling pathways involving for instance the kinases JNK and the regulator protein B-cell lymphoma 2 (Bcl-2). [34]

3.1.4. SRC-3 in invasion and metastasis

Many studies predicted that SRC-3 promotes cancer invasion and metastasis. The mechanisms by which SRC-3 is encouraging invasion and metastasis have been broadly examined, and there are strong evidences suggesting the involvement of matrix metalloproteinases (MMPs). SRC-3 is thought to regulate the expression of MMP2/9 by functioning as a transcriptional coactivator for PEA3. Additionally, SRC-3 also was shown to activate focal adhesion kinase (FAK) signaling, and so enhance cell motility as a part of cancer invasion and metastasis. [34]

3.2. SRC-3 in cancer pathogenesis

SRC-3 is a key co-regulator of ERα activity and has been shown to have a critical role in breast cancer. Subsequently, it is of a great interest to define the potential diagnostic and prognostic role of SRC-3. Nowadays, many research groups are investigating the phosphorylation mechanisms of SRC-3, as well as phosphorylation sites that are important for modulating SRC-3 activity and its role in breast cancer.[37] A study was conducted utilizing mice harboring a dominant-negative mutant thyroid hormone receptor-β (TRβPV/PV mice) and SRC-3$^{-/-}$mice to investigate the role of SRC-3 in thyroid cancer. The TRβPV/PV mice spontaneously develop follicular thyroid carcinoma similar to human cancer. While, the TRβPV/PV mice deficient in SRC-3 (TRβPV/PVSRC-3$^{-/-}$ mice) had significantly increased survival, decreased thyroid tumor growth, delayed tumor progression and lower incidence of distant metastasis as compared with TRβPV/PV mice with SRC-3 (TRβPV/PVSRC-3$^{+/+}$ mice). Along with in vivo and in vitro analyses, it was established that SRC-3 is involved in regulating multiple target genes and signaling pathways during thyroid carcinogenesis.[38] Cai et al have studied the role of SRC-3 in lung cancer development. Their study showed that SRC-3 is overexpressed in non–small cell lung cancer (NSCLC), which correlates with poor disease-free and overall survival and, in some cases, is associated with DNA amplification. Knockdown of SRC-3 in lung cancers leads to reduced cell growth,

decreased colony formation ability, and increased apoptosis in NSCLC cell lines with high endogenous levels of SRC-3. In addition, the study shows that SRC-3 knockdown can potentiate the effect of gefitinib in EGFR tyrosine kinase inhibitor (TKI)–resistant cells. [39]

4. Matrix metalloproteinases (MMPs)

MMPs are the main matrix-degrading proteinases, of the class endopeptidases, that can practically cleave most of the components in extracellular matrix (ECM), hence the name. MMPs play central roles in embryogenesis, organ morphogenesis, and wound healing. Many studies examined and characterized the abnormal activation of MMP genes, and it´s suggested that this activation is accounted for the development of many diseases, such as arthritis, atherosclerosis, and tumorigenesis. This indicated that MMPs are important in inflammation and carcinogenesis. Until today, up to around 20 MMPs have been identified. In a pathophysiological context, most MMPs take part in cell adhesion, angiogenesis, and metastasis. By chopping ECM components, MMPs regulate cell growth, differentiation, apoptosis, and migration. It became clearer now that MMPs participate in tumorigenesis in both early and late stages, by influencing malignant transformation, angiogenesis, and tumor growth in early stages, and tumor progression, invasion, and metastasis in late stages.[40]

SRC-3 has been found to regulate both MMP2 and MMP9 in human MDA-MB-231 and PyMT cells. That is by binding and stimulating the PEA3 transcription factor. Other studies suggested that SRC-3 is regulating MMP7 and MMP10 in MDA-MB-231 cells by co-activating AP-1. In addition, SRC-3 has been found to co-activate AP-1 and PEAs, leading to up-regulating the expression of MMP2 and MMP13. There are many transcriptional factors that are being co-activated by SRC-3, promoting cancer cell invasion to the surroundings.[36]

All of the above make MMPs central game players in cancer pathogenesis.

A recent study carried out by Long et al revealed that ERK3 interacted with and phosphorylated SRC-3 at Ser857. This phosphorylation is believed to be essential for SRC-3 interaction with the ETS transcription factor PEA3, which in turn promotes upregulation of MMP gene expression and invasive activity in lung cancer cells. This study identifies a role for ERK3 in promoting lung cancer cell invasiveness by phosphorylating and regulating SRC-3 proinvasive activity. As such, ERK3 protein kinase may be an attractive target for therapeutic treatment of invasive lung cancer.[35]

AIM OF THESIS

We wish to investigate whether MK5 phosphorylates SRC-3 at Ser857 in the absence of ERK3 and whether MK5 and ERK3 have impact on MMP2, MMP9, and MMP10 gene expression in both promoter transcription activity and mRNA level. We wish also to examine if MK5 and ERK3 are influencing lung cancer cell proliferation and migration.

In this study, we take into consideration the results published in the previously mentioned Long et al article.[35] The main findings in this paper indicate that ERK3 binds and phosphorylates SRC-3, and the ERK3-induced phosphorylation of SRC-3 promotes metastasis through increasing transcriptional activities of MMP2, MMP9, and MMP10. In this context, we choose to test MK5 in the kinase assay, in order to check for SRC-3 phosphorylation by MK5 at Ser857. The argument for this is based on preliminary data from kinase assays, conducted by PhD candidate Ellen Tømte, in our research group, indicating that MK5 is responsible of the phosphorylation activity of SRC-3 and ERK3.

Thereupon, in this context the main the questions to be addressed are:

- Does MK5 phosphorylate SRC-3 at Ser857?
- Do the overexpression of SRC-3, ERK3, and MK5 influence MMP2, MMP9, and MMP10 promoter activity?
- Will the siRNA knockdown of ERK3 and/or MK5 influence MMP2, MMP9, and MMP10 promoter activity?
- Will the shRNA knockdown of ERK3 and/or MK5 influence endogenous MMP2 and MMP9 gene expression and affect lung cancer cells ability to proliferate and migrate?

MATERIALS

Vectors

Plasmid	Source
pCR®2.1-TOPO® MMP2-promoter	Cloned by Ellen Tømte, Pharmacology research group, University of Tromsø
pGL3 basic	Promega®, Madison, WI, U.S.A
pGL3-basic-MMP2 promoter	Mathod no.1
pCH110	Phamacia, GE Healthcare Biosciences®, Pittsburgh, PA, U.S.A
GR	Strøm et al, 2010[41]
pcDNA3	Invitrogen, Life Technologies™, Carlsbad, CA, U.S.A
pcDNA3-SRC-3	Kindly provided by G. Mellgren University of Bergen
pcDNA3-ERK3-myc	Seternes et al, 2004[42]
pEGFP	Clontech Laboratories, Inc., Takara Bio™, Shiga, Japan
pEGFP-MK5	Seternes et al, 2002[43]
pGL3-basic-MMP9 promoter	A gift from I. Mikkola, Pharmacology research group, University of Tromsø
pGL3-basic-MMP10 promoter	Kindly provided by R. Bassel-Duby, UT Southwestern Medical Center, Dallas, TX, U.S.A

SiRNA

Silencer® Negative Control #1 siRNA	Catalog# AM4635	Ambion® Invitrogen, Life Technologies™, Carlsbad, CA, U.S.A
Silencer® Validated ERK3 (MAPK6) siRNA	Catalog# AM51331	Ambion® Invitrogen, Life Technologies™, Carlsbad, CA, U.S.A
Silencer® MK5 (PRAK) siRNA	Catalog# AM16706	Ambion® Invitrogen, Life Technologies™, Carlsbad, CA, U.S.A

Bacterial strains

E.coli, DH5α competent cells	Inoue et al, 1990[44]	Pharmacology research group, University of Tromsø
E.coli, BL21 competent cells		Novagen®, Merck KGaA, Darmstadt, Germany

Cell lines

H1299	Carcinomic human non-small cell cancer, lung	LGC standards	ATCC® Number: CRL-5803™
A549	Carcinomic human alveolar basal epithelial cell, lung	LGC standards	ATCC® Number: CCL-185™
HeLa S3	Adenocarcinoma human cell, cervix	LGC standards	ATCC® Number: CCL-2.2™

The shRNA knockdown cell lines

Bjarne Herold Johansen, Pharmacology research group, University of Tromsø, kindly provided these H1299 and A549 cell pools with stable knockdown of ERK3 and MK5, by retroviral expression of shRNAs targeting ERK3 or MK5.

H1299-shRNA	H1299-shLuc	H1299-shERK3	H1299-shMK5
A549-shRNA	A549-shLuc	A549-shEKR3	A549-shMK5

Growth culture for bacteria

LB-broth	10 g/l Bacto™ tryptone, Difco 5 g/l Bacto™ yeast extract, Difco 5 g/l NaCl pH adjusted to 7.4	SUMP section, University hospital in Northern Norway (UNN), Tromsø
LB-agar plate, 100 µg/ml ampicillin	10 g/l Bacto™ tryptone, Difco 5 g/l Bacto™ yeast extract, Difco 5 g/l NaCl pH adjusted to 7.4 10 g/l agar Ampicillin Sodium salt, Sigma-Aldrich®	SUMP section, University hospital in Northern Norway (UNN), Tromsø
Ampicillin Sodium salt	Catalog# A9518	Sigma-Aldrich®, St. Louis, MO, U.S.A.
S.O.C. medium	20 g/l Bacto™ tryptone, Difco 5 g/l Bacto™ yeast extract, Difco 8.6 mM NaCl 2.5 mM KCl 10 mM MgCl 10 mM $MgSO_4$ 20 mM Glucose pH adjusted to 7.4	SUMP section, University hospital in Northern Norway (UNN), Tromsø

Cell culturing media

RPMI-1640 Medium	Catalog# R8758	Sigma-Aldrich®, St. Louis, MO, U.S.A
Dulbecco's Modified Eagle Medium (DMEM)	Catalog# D5796	Sigma-Aldrich®, St. Louis, MO, U.S.A

Buffers, solutions, chemical reagents, and enzymes

Method		Composition / Catalog#	Source
Cloning	1×TAE buffer	40 mM Tris-acetate 1 mM EDTA Ad 5 L dH_2O	Pharmacology research group, University of Tromsø
	NEBuffer1	Catalog# B7001S	New England Biolabs® inc, Ipswich, MA, U.S.A
	T4 DNA Ligase Reaction Buffer	Catalog# B0216L	
	T4 DNA Ligase	Catalog# M0202S	
	XhoI	Catalog# R0146S	
	KpnI	Catalog# R0142S	
	Ampicillin Sodium salt	Catalog# A9518	Sigma-Aldrich®, St. Louis, MO, U.S.A.
	SeaKem® LE agarose	Catalog# 50005	Lonza™, Basel, Switzerland
	SeaKem® GTG agarose	Catalog# 50071	
	BSA	Catalog# B9001S	New England Biolabs® inc, Ipswich, MA, U.S.A
	Gel loading Dye blue 6 ×	Catalog# B7021S	
	1kb Plus DNA ladder	Catalog# 10787-018	Invitrogen, Life Technologies™, Carlsbad, CA, U.S.A
	EB buffer	Catalog# 19086	QIAGEN®, Gmbh Hilden, Germany
Cell culturing	Dulbecco's Phosphate Buffered Saline (PBS)	Catalog# D8662	Sigma-Aldrich®, St. Louis, MO, U.S.A.
	0.25% trypsin-EDTA solution	Catalog# T4049	
	Streptomycin-Penicillin (10 mg /ml - 10000 U/ml)	Catalog# P4333	
	Puromycin di-hydrochloride	Catalog# P9620	
	Fetal Bovine Serum superior (FBS)	Catalog# S 0415	Biochrom AG®, Berlin, Germany
Cell transfection	Opti-MEM® Reduced Serum Medium	Catalog# 31985047	Gibco®, Invitrogen, Life Technologies™, Carlsbad, CA, U.S.A
Luciferase assay	Dithiothreitol (DTT)	Catalog# 43816	Fluka analytica, Sigma-Aldrich®, St. Louis, MO, U.S.A.
	Dexamethasone,	Catalog# D4902	Sigma-Aldrich®, St. Louis, MO, U.S.A.
	Tropix® Lysis buffer	Catalog# T1004	Applied biosystems®, Bedford, MA, U.S.A
	Buffer A (Dual-light® reagent)		
	Buffer B (Dual-light® reagent)		
	Tropix® Galacton-Plus Tropix® Accelerator II		

Buffers, solutions, chemical reagents, and enzymes *(Continued)*

Method		Composition / Catalog#	Source
RNA extraction	β-Mercaptoethanol	Catalog# A1108.0100	Applichem®, Darmstadt, Germany
	Ethanol	Catalog# 24106	Sigma-Aldrich®, St. Louis, MO, U.S.A.
Western blots	MKK lysis buffer	20 mM Tris (pH 7.0), 1% Triton X-100 5 mM Tetra-sodium pyrophosphate (NaPPi) 50 mM NaF 1 mM EDTA 1 mM EGTA 1 mM vanadate (VO_4^{2-}) 0.27 M sucrose 10 mM β-glycerophosphate Ad 100 ml dH_2O	Pharmacology research group, University of Tromsø
	Complete, Mini, EDTA-free protease inhibitor cocktail tablet	Catalog# 11836170001	Roche™, Switzerland
	NuPage® LDS sample buffer 4×	Catalog# NP0008	Invitrogen, Life Technologies™, Carlsbad, CA, U.S.A
	NuPage® Sample reducing agent 10×	Catalog# NP0009	
	NuPage® MES SDS Running buffer ×20	Catalog# NP0002-02	
	NuPage® Novex® 4-12% Bis-Tris Precast Gel	Catalog# NP0301BOX and NP0302BOX	
	MagicMark™ XP Western Protein Standard	Catalog# LC5603	
	SeeBlue® Plus2 Pre-Stained Protein Standard 1×	Catalog# LC5925	
	Blotting buffer	29 g Tris base 144 g glycerin 1 L methanol Ad 5 L dH_2O	Pharmacology research group, University of Tromsø
	Odyssey® Nitrocellulose membrane	Catalog# P/N 926-31092	Li-cor biosciences Odyssey, Lincoln, NE, U.S.A
	Odyssey® Blocking buffer	Catalog# P/N 927-40003	
	TBS buffer 10×	200 ml Tris 1 M, pH 7.5 400 g NaCl 10 g KCl Ad 5 L dH_2O	Pharmacology research group, University of Tromsø
	TBS-T buffer 1×	500 ml TBS buffer 10× 5 ml Tween 20 Ad 5 L dH_2O	

Buffers, solutions, chemical reagents, and enzymes *(Continued)*

Method		Composition / Catalog#	Source
GST-tagged protein purification	IPTG	Catalog# 9030	Takara Bio™, Shiga, Japan
	Glutathione Sepharose™ 4B Media	Catalog# 17-0756-01	GE Healthcare Biosciences, Pittsburgh, PA, U.S.A
	5 M NaCl	292.2 g NaCl Ad 1 L dH_2O	Pharmacology research group, University of Tromsø
	1 M DTT	Catalog# D0632	Fluka analytica, Sigma-Aldrich®, St. Louis, MO, U.S.A.
	Lysis buffer	1× PBS 1 % Triton X-00 Ad 15 mL dH_2O	Pharmacology research group, University of Tromsø
	Wash Buffer	20 mM Tris, pH 7.5 0.25 M NaCl 2 mM EDTA 2 mM EGTA 0.03% Brij-35 Ad 1 L dH_2O	
	Elution Buffer	Wash Buffer 20 mM glutathione, pH 8.0 (61 mg glutathione/10 ml buffer) 30 µl 1 M NaOH Ad 50 mL dH_2O	
	PageBlue™ Protein staining solution	Catalog# 24620	Thermo-scientific, Rockford, IL, U.S.A
	Dialysis Buffer:	50 mM Tris, pH 7.5 0.1 mM EDTA 0.1% 2-mercaptoethanol 50% glycerol Ad 2 L dH_2O	Pharmacology research group, University of Tromsø
Measurement of protein concentration	Bio-Rad® DC Protein Assay Kit 2	Catalog# 500-0112-MSDS	Hercules, CA, U.S.A.
Kinase assay	Assay buffer:	50 mM Tris-HCl 0.1 mM EGTA 1 mM sodium vanadate 1 mM DTT Ad 1 mL dH_2O	Pharmacology research group, University of Tromsø
	ATP mix:	3 % (v/v) 10 mM ATP 5 % (v/v) 1 M $MgCl_2$ 92 % Assay buffer	
Colonogenic assay	Colonogenic reagent	50% Ethanol 0.25% 1,9-dimethyl methylene blue Ad 12 mL dH_2O	Pharmacology research group, University of Tromsø

Plasmid purification kits

QIAgen Plasmid Mini Kit	Catalog# 27106	QIAGEN®, Gmbh Hilden, Germany
QIAgen Plasmid Midi Kit	Catalog# 12643	QIAGEN®, Gmbh Hilden, Germany
QIAquick Gel Extraction Kit	Catalog# 28704	QIAGEN®, Gmbh Hilden, Germany

RNA extraction kit

RNeasy® Plus Mini Kit	Catalog# 74134	QIAGEN®, Gmbh Hilden, Germany

Cell transfection kits

Lipofectamine™ LTX with Plus™ Reagent kit	Catalog# 15338-100	Invitrogen, Life Technologies™, Carlsbad, CA, U.S.A
Lipofectamine™ 2000™ Reagent kit	Catalog# 11668-019	Invitrogen, Life Technologies™, Carlsbad, CA, U.S.A

qRT-PCR assay kits

Reverse transcriptase core kit	Catalog# RT-RTCK-05	Eurogentec®, Seraing, Belgium
qPCR MasterMix Plus low Rox	Catalog# RT-QP2X-03+WOULR	Eurogentec®, Seraing, Belgium
Brilliant II SYBR® Green QPCR Master Mix	Catalog# 600828	Agilent Technologies, Inc., Santa Clara, CA, U.S.A

qPCR Primers

	Forward	Reverse
MMP2	5'TGGGACAAGAACCAGATCACATA3'	5'CGAGCAAAGGCATCATCCA3'
MMP9	5'CCACCACAACATCACCTATTGG3'	5'GAAGGCGCGGGCAAA3'
ERK3	5'ATGGATGAGCCAATTTCAAG3'	5'CTGACAATCATGATACCTTTCC3'
MK5	5'CCCGACTCTTAATTGTAATGG3'	5'TATCTGCTTTGTTACTTGGC3'
β-Act	Hs_ACTB_1_SG, RefSeq# NM_001101, Catalog# QT00095431 QIAGEN®, Gmbh Hilden, Germany	

Kits used in GST-tagged protein purification

Novagen® D-Tube™ Dialyzer Midi	Catalog# 71507-3	Novagen®, Merck KGaA, Darmstadt, Germany

Proteins used in kinase assay

Protein	Source
Active His-MK5	Division of Signal Transduction Therapy (DSTT), University of Dundee, Dundee, Scotland
GST	O.M. Seternes, Pharmacology research group, University of Tromsø
GST-SRC-3 CID wt	Method no. 3 – GST-tagged protein purification
GST-SRC-3 CID S857A	Method no. 3 – GST-tagged protein purification

Antibodies used in western blot

Primary antibodies	Source	Secondary antibodies	Source
Anti-phospho SRC-3, 2nd bleed (1:500)	Custom made, Division of Signal Transduction Therapy, (DSTT), University of Dundee, Dundee, UK	Anti-sheep IgG (H&L) Donkey IRDye 800CW P/N 613 732 168 (1:10000)	Rockland™ Immunochemical, Gilbertsville, PA, U.S.A
Anti-actin Polyclonal Rabbit Catalog# #4968 (1:1000)	Cell Signaling Technology, Inc. ®, Boston, MA, U.S.A.	Anti-rabbit Donkey IRDye 800CW Catalog# 926 32213 (1:10000)	Li-cor biosciences Odyssey, Lincoln, NE, U.S.A
Anti-ERK3 MAPK6 (612-721) Catalog# H00005597-M02 (1:500)	Abnova™, Taipei City, Taiwan	Anti-mouse Donkey IRDye 800CW Catalog# 926 32212 (1:10000)	Li-cor biosciences Odyssey, Lincoln, NE, U.S.A
Anti-MK5 PRAK - A7 Mouse monoclonal IgG (1:500)	Santa Cruz Biotechnology, Inc. Santa Cruz, CA, U.S.A	Anti-mouse Donkey IRDye 800CW Catalog# 926 32212 (1:10000)	Li-cor biosciences Odyssey, Lincoln, NE, U.S.A

METHODS

1. Construction of reporter gene plasmid vector by subcloning

In molecular biology, genetically modified plasmids are essential in many experiments. In order to obtain this, many approaches have been utilized within the field of recombinant DNA to create a single DNA construct that is capable of being replicated and expressed in a given host. Destination genetic carriers are mostly plasmid or bacteriophage cloning vectors, while insert sequences are often derived from any organism. In cases where insert is a previously cloned DNA segments, the procedure is termed sub-cloning, and followed by ligation of DNA fragments in gels. [45]

In this study we wanted to construct a reporter gene plasmid to be used in luciferase assays, by using the sub-cloning technique. First cutting the requested insert and vector by restriction enzymes, and then separating them on gel electrophoresis. Afterwards both insert and vector were ligated together, and transformed into competent bacterial cell, and then purified.

1.1. Cutting of vector and insert with restriction enzymes

Restriction enzymes, or endonucleases, recognize and bind to specific sequences of double-stranded DNA and subsequently catalyze the cleavage at specific sites.

The following was pipetted into 1.5 ml microcentrifuge tube for each sample:

DNA sample: 1 µg plasmid DNA
1 µl of 10× restriction enzyme buffer
1 U/µg of restriction enzymes
dH_2O to 10µl total sample volume

The mix was vortex, and then incubated on a warm plate at 37°C for 2 hours.[46]

1.2. Separation of DNA fragments on agarose gel electrophoresis

Agarose gel electrophoresis is a common and widespread technique in molecular biology. It is simple and highly effective for separating, identifying, and purifying DNA fragments ranging between 0.5-25 kb. The method is based on exposing a porous agarose gel to an electrical field in order to separate DNA fragment according to size. A commercial ladder is often used for sizing the fragments, as well as a DNA intercalating agent that helps detecting the migrated fragments. [47]

The procedure was conducted according to the following steps:

- 0.7 % agarose gel was made by adding 100 ml of 1× TEA buffer to 0.7 g of agarose in suitable flask, and mix was heated in microwave oven for 90 seconds. The flask was swirled every 30 seconds to make sure that agarose is dissolved.
- After 5 min the mix was cooled to about 55°C, and 2 µl ethidium bromide (10 mg/ml) was added.
- The agarose mix was poured into a gel tray with the well comb in place
- The gel was left at room temperature for 30 minutes, until it has completely solidified.
- The gel was transferred to a running chamber filled with 1× TEA buffer
- The DNA samples were added 2 µl of 6× loading buffer prior to adding into wells.
- 5 µl of 1kb Plus DNA ladder was added to the first well.
- 10 µl of each digested DNA sample was added to wells.
- The gel was run at 90V, 400 mA electric field for 45 min.

The DNA fragments were visualized using the 2000 UV transillluminator Bio-Rad®, and the software Quantity One® 4.30. And then a picture of the gel is taken. This is for analysis purposes.

But, when we were interested in separating DNA fragments the DNA was visualized on a UV-lamp and the fragments of interest were cut using a scalpel and transferred to new 1.5 ml microcentrifuge tubes.

1.3. DNA purification from electrophoresis gel

The aim of this experiment is to extract and purify DNA fragments from SeaKem® GTG agarose gel slices.

- The purification was conducted in concordance with the QIAquick Gel Extraction Kit protocol from QIAGEN®, cat.no 28704.
- DNA concentrations of the fragments were measured using NanoDrop® ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, U.S.A.).
- The concentrations were estimated in ng/µl in the software NanoDrop 1000 V3.8.
- 1% SeaKem® LE agarose gel was used to confirm the result.

1.4. Ligase reaction

Since 1967, DNA ligases were utilized and widely used in molecular biology and biotechnology applications. DNA ligases are endogenously found as replication and repair enzymes, and nowadays they are used for this exact propose in, for instance, cloning and next-generation DNA sequencing. The ligase enzymes catalyze the linking of neighboring 3¢-hydroxyl and 5¢-phosphorylated DNA terminal in double stranded DNA. DNA ligases process nicked dsDNA and homologous, cohesive ends as substrates. Some ligases, such as T4 DNA ligase, accept fully base-paired (blunt end) substrates as well. [48]

The ligase reaction consisted of the following:

1 µl of 10x buffer for T4 DNA ligase
1 µl of T4 DNA ligase
0.5 µg of the vector
1.5 µg of the insert

The mix was incubated at room temperature for 1.5 hour.[48]

1.5. Transformation

Introducing genetic elements to bacteria is known to the most efficient method for rapid multiplication for genetic materials.

Procedure for transformation using heats-shock as follows:

- DH5α competent cells were thawed on dry ice.
- 100 µl DH5α cells were added to new 15 ml falcon tubes.
- 2 µl of the ligase reaction mix were added to DH5α cells.
- The tubes were then incubated on ice for 15 min.
- The DH5α cells in the tubes were then heat shocked for 90 sec at 42°C.
- Afterwards, 400 µl SOC media were added to the tubes, and put into incubator at 37°C and 225 rpm shaking for 1 hour.
- In between, LB-plates with suitable selective antibiotic, were preheated at room temperature.
- 100 µl of falcon tubes components were added to each plate and spread onto surface.
- Plates were incubated over night at 37°C.

1.6. Overnight culture

One picked colony was inoculated to each growth culture.

The following amount LB media with appropriate antibiotics was prepared:

For miniprep purification: 3 ml

For midiprep purification: 25 ml LB for high copy plasmid, and 100 ml LB for low copy plasmid

The cultures were incubated in 37°C incubator with shaking (225 rpm) overnight (14-16 hours). The bacterial cells were harvested by centrifugation and processed for plasmid purification.

1.7. Plasmid purification

The used approach is depending on the desired plasmid yield.

- For miniprep plasmid purification we used the QIAprep Spin Miniprep Kit and a Vacuum Manifold from Qiagen (cat.no 27106).
- For midi-prep we used the QIAprep Midi Kit from Qiagen (cat.no 12643).
- All steps were curried out according to the bench protocol that is attached to the kit.
- The DNA concentrations were measured using NanoDrop® ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, U.S.A.).
- The concentrations were estimated in ng/µl in the software NanoDrop 1000 V3.8.

2. Mammalian cell culture techniques

2.1. Cell culture

The H1299 cells were cultured in RPMI-1640 supplemented with 10% FBS and 1% penicillin/streptomycin. A549 and HeLa S3 cells were cultured in DMEM supplemented with 10% FBS and penicillin/streptomycin. While working with the shRNA knockdown cell lines, puromycin (selection agent) was added and diluted to a final concentration of 1 µg/ml in the medium. Cell cultures were always incubated in Thermo-scientific nunc® EasyFlasks™ inside a HERAcell® 150i CO_2 incubator at 37°C, 5% CO_2 and 80-95% relative humidity.

2.2. Cell splitting and counting

Cell cultures were split 2-4 times a week, depending on cell confluence and planning of assays and experiments. Before starting medium and trypsin were rewarmed in 37°C heat incubator, while PBS was kept at room temperature all times.

- The old medium was discarded
- Cells were washed with 0.2 ml PBS per cm² of flask surface area.
- Flasks were rocked gently and the PBS was aspirated.
- 40 µl of 0.25% trypsin-EDTA per cm^2 culture flask surface area is then added to each flask and so the flasks were tilt side to side to cover cell monolayer.
- Flasks were put inside the humidified cell incubator at 37°C and 5% CO_2 until the monolayer was disappeared.
- Cells were then sub-cultivated in appropriate dilutions in fresh medium to a final volume of 0.2 mL medium per cm^2 flask surface area. Remaining cells were counted and used in assays.

A Bürker hemocytometer was used for cell counting.

- The assigned coverslip was affixed properly on the chamber.
- After resuspending the cells in the media by pipetting up and down, some cell culture was transferred into the chamber under the coverslip.

- The cells were counted in at least 3 of the 9 A-squares under the Zeiss AxioVert S100 inverted microscope.
- Cell concentration (cell/ml) was estimated according to the following formula:

$$\frac{cell\ count \times dilution\ factor \times 10^4}{number\ of\ counted\ A-squares}$$

2.3. Seeding cells

After estimating the cell concentration, an approximate number of cells were seeded in appropriate culturing well-system plates or dishes. The amount of cells seeded and the culturing vehicle was depended on assay for which cells were prepared for. Cells were always seeded a day before the planned assay.

- The amount of cell suspension that was required was determined in ml by the following formula:

$$\frac{number\ of\ wells \times desired\ cell\ number\ in\ each\ well}{the\ cell\ concentration\ \left(\frac{cell}{ml}\right)}$$

- The required amount of medium and the calculated volume of cell suspension were added to each well.
- The BD Falcon™ Multiwell Plates were used in this lab, with convenient number of wells.
- The well plates were then put into the humidified incubator at 37°C and 5% CO_2 overnight or until assays were conducted.

2.4. Recovering cells from liquid nitrogen

Cells are stored in liquid nitrogen, which is one of the most common methods for cryopreserving. The cultured cells are frozen in complete medium in the presence of a cryoprotective agent such as dimethylsulfoxide (DMSO). The necessity of the cryoprotective agent lies in the fact that it reduces the freezing point of the medium, as well as allowing a slower cooling rate, which in turn reduces the risk of ice crystal formation that can damage cells and cause cell death. When cells are recovered from nitrogen tank, DMSO must be washed away, because of its cytotoxicity, especially in high concentrations.

The procedure used in this method is as follows:

1. About 75% mL of the total medium (i.e. medium + 15% FBS + 1% antibiotics) is put in appropriate cell culture flask and incubated for 30 min in cell incubator.
2. The requested cryovial is removed from the liquid nitrogen tank and placed immediately in 37°C water bath.

3. When the cells have thawed, the content is pipetted into suitable centrifuge tubes.
4. Warm medium is then carefully added drop wise to the cells, to a total volume of 25% of total flask medium.
5. The cell suspension is centrifuged at 13000 rpm for 10 min.
6. The supernatant is discarded, and cell pellet is then re-suspended in new medium (about 25% of total flask medium).
7. The cell suspension is distributed by swirling the flask gently and so the flask was placed in the incubator.

2.5. Cell transfection

Introducing various genetic elements into mammalian cells is a practical mean for investigating biological responses subsequent to alternations in regulation of gene expression, and hence in cell characteristic. The efficacy of cell transfection depends immensely on the cell type and the used technique. Throughout the recent decades, many approaches have been developed for optimizing the efficacy. The most common techniques are calcium phosphate, DEAE-dextran transfection, electroporation, and liposome-mediated transfection. In this context, two commercial liposome-mediated transfection kits were used, because they are optimal for our cell lines. The liposome-mediated transfection is based on cationic liposomes that contain neutral lipids. The positively charged surface of the liposome binds to negatively charged phosphate groups on DNA, and the residual positive charge then theoretically facilitates binding to negatively charged sialic acid residues on the cell surfaces.[49]

In this study, cells were transfected as part of luciferase assay, and for this purpose every well was cotransfected with requested plasmids along with luciferase vector of interest and pCH110 β-galactosidase vector for normalization.

2.5.1. HeLa S3 cells DNA transfection with Lipofectamine™ LTX

The transfection was conducted according to the protocol followed the Lipofectamine® LTX with Plus™ Reagents kit, MAN0001225, Invitrogen, Life Technologies™. Cells were always seeded a day ahead before transfection.

2.5.2. A549 cell DNA transfection with Lipofectamine 2000

In order to transfect DNA plasmids into A549 cells the protocol "Lipofectamine®2000 Reagent", MAN0000995, Invitrogen, Life Technologies™ was followed. Cells were always seeded a day ahead before transfection.

2.5.3. A549 cell siRNA transfection with Lipofectamine 2000

This transfection was performed following the protocol "Transfecting siRNA into Mammalian Cells Using Lipofectamine®2000", 200025-0621W, Invitrogen, Life Technologies™. Cells were always seeded a day ahead before transfection.

2.6. Harvesting cells

Many assays within molecular biology are conducted on mammalian cell components, for instance proteins and RNA. Therefore, there is a need for releasing those from cell membrane. For this purpose, many techniques have been developed with variations in used reagents and conditions depending on assay type.

2.6.1. Harvesting for Luciferase

1. 0.5 µl DTT was added to 1 ml of Tropix® Lysis buffer
2. Cells were washed once with PBS
3. 60 µl of DTT/Lysis buffer solution is added to each well
4. The plate was then put on rocking, 100 rpm, for 10 min
5. The well contents were pipetted to new microcentrifuge tubes, and centrifuged for 10 min at 13000 rpm

2.6.2. Harvesting for RNA extraction

- Cells were harvested according to the protocol attached to RNeasy® Plus Mini Kit, Catalog# 74136. The amount of RLT plus/β-ME solution was 350 µl, and the protocol for monolayer cell lysis directly.
- Cell lysate was homogenized by passing it 5 times through a 0.9 cm needle fitted to an RNase-free syringe.
- No further modifications were applied when following the protocol.
- The RNA concentrations were measured using NanoDrop® ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, U.S.A.).
- The concentrations were estimated in ng/µl in the software NanoDrop 1000 V3.8.

2.6.3. Harvesting for western blot and protein concentration measurement

1. The medium was discarded and cells were washed once with PBS
2. 250 µl of MKK lysis buffer was added per 6 cm surface area
3. The adherent cells were scraped off using a cell scraper
4. Cells lysate was transferred gently into a 1.5 ml pre-cooled microcentrifuge tube
5. The microcentrifuge tubes were put on ice for 15 min
6. Then tubes were spin at 13000 rpm for 10 minutes in a 4°C pre-cooled centrifuge
7. The tubes were gently removed from the centrifuge and placed on ice
8. The supernatant was transferred to a new tubes kept on ice, and pellet was discarded.

2.7. Luciferase assay

Dual luminescence-based reporter gene assay is typically used to measure the transcriptional activity for certain promoters by measuring both luciferase and β-galactosidase activity. This technology is based on the fact that many transcriptional activator protein bind to specific enhancer elements in promoters, which triggers the transcription of the nearby genes. Here the luminescent luciferin and Tropix® Galacton Plus substrates are incorporated for the detection of luciferase and β-galactosidase, respectively. [50]

- Some cells were transfected with DNA one day before conducting this assay, and other cells were transfected two days earlier with siRNA.
- 20 µl of the harvested cell lysate supernatant were pipetted into the designated wells in a Thermo-scientific nunc® 96 microwell white polystyrene plate.
- 20 µl of lysis buffer was used as blanks.
- The Luminoscan RT was used for this assay.
- Instrument hoses were washed with dH_2O before use.
- The automatized program in instrument added 15 µl of Buffer A (contains salts and other important reagents) to cell lysate for the luciferase reaction, and so the luciferase signal is measured immediately after the injection of 60 µl Buffer B (contains luciferin and Galacton-Plus, 1:100 mixed right before use, substrates).
- The light signal that follows the enhanced luciferase reaction decays with a half-life of about 1 minute.
- After 45 minutes after the luciferase reaction, the Accelerator-II was added (60 µl per well) in order to measure the light signal from the accumulated product of the β-galactosidase/Galacton-Plus reaction.
- The procedure was conducted according to the protocol P/N T1003, T1004, T1005, Applied biosystems®, Invitrogen and Luminoscan RT user manual.

2.8. The qRT-PCR assay

The quantitative real-time PCR (qRT-PCR) has transformed the world of gene expression analysis. In the past many mRNA detecting methods were used, but on the contrary, qRT-PCR is fast, precise, relatively inexpensive, and does not require radioactive substrates. qRT-PCR is often used to screen for certain gene transcripts, mRNA, and at the same time quantify amounts of mRNA by measuring the transcriptional activity of that gene, which in turn can be used to quantify any nucleic acid. qPCR can be performed on different substrates, including chromosomal DNA, mitochondrial DNA, or cDNA generated by reverse transcription of RNA. There are couples of detecting fluorescent dyes that are used in qRT-PCR, which monitor the double-stranded DNA without stopping the PCR, i.e., in real. One category of dyes are intercalating dyes, such as SYBR green that fluoresce solely when

intercalated into the minor groove of double-stranded DNA. Fluorescence intensity is proportional to the amount of double-stranded DNA present in a given well, due to the accumulation of the PCR product with each cycle. Therefore, as the amount of double-stranded DNA doubles with each round of PCR amplification, the intensity of SYBR fluorescence follows pace.[51]

- This assay was conducted in accordance with the protocol attached to the Reverse transcriptase core kit, (ref. RT-RTCK-05) and the protocol for MasterMix Plus low Rox, (ref. RT-QP2X-03+WOULR) from Eurogentec®, Seraing, Belgium with no modifications. The The Mx3000P QPCR System was used, and the instrument was utilized for use by MxPro® qPCR Software, Agilent Technologies, Santa Clara, CA, U.S.A
- The cycle threshold (Ct) values were analyzed in Excel (Microsoft office, 2011, mac) as presented in appendix. Two-sample student´s *t*-test was used to compare differences in means of two groups' in StatPlus (AnalystSoft Inc., 2009, mac).

2.9. Western blot

This technique is used to first, separate proteins on SDS-PAGE gel electrophoresis, and second, blot the proteins on a nitrocellulose membrane. Afterwards, the requested proteins are identified using specific antibodies, monoclonal or polyclonal, that recognize corresponding protein-fused antigens. Conjugated primary antibodies and corresponding secondary antibodies of non-conjugated primary antibodies are marked with IR element that enables the detection using infrared microscopy. [52]

2.9.1. SDS-PAGE gel electrophoresis

- 39 µl of the cell lysate were transferred to new microcentrifuge tubes
- 15 µl of NuPage LDS sample buffer ×4 and 6 µl of NuPage sample reducing agent were added. Samples were incubated on a hot plate at 70°C for 10 min
- 50 ml of 20× NuPAGE® MES SDS Running Buffer was added to 950 ml dH_2O in order to prepare 1× SDS Running Buffer
- The SDS-PAGE gel was prepared for sample loading as instructed by the manufacturer.
- 15 µl of each sample were added to each well in the 10 well SDS-PAGE gels, and 12 µl were added to each well of the 12 well SDS-PAGE gels.
- The gel chamber was connected to power supplier and run at 200 V for 45 min

2.9.2. Blotting

- When electrophoresis was complete, the gel cassette was removed from chamber, and opened using gel knife allowing the gel to remain on the top of the bottom plate of the cassette.
- Sponge bas were soaked in 1× blotting buffer until saturation
- Filter papers and membrane were also soaked in the same buffer prior to use
- The gel was then removed from cassette by gel knife
- One presoaked filter paper was placed on top of the gel
- The plate was turned over so the gel and filter paper are facing downwards on a sponge pad in the blotting chamber
- The gel "foot" was removed
- On the gel top a membrane was placed, and so another filter paper was put on top of the membrane
- Sponge pads were placed inside so blotting chamber is tight
- The blotting chamber/module was then assembled, and filled with blotting buffer. The blotting sandwich is illustrated in Figure 8.
- The outer chamber was filled with cool deionized water.
- The blotting was run at 25V for 2,5 hours

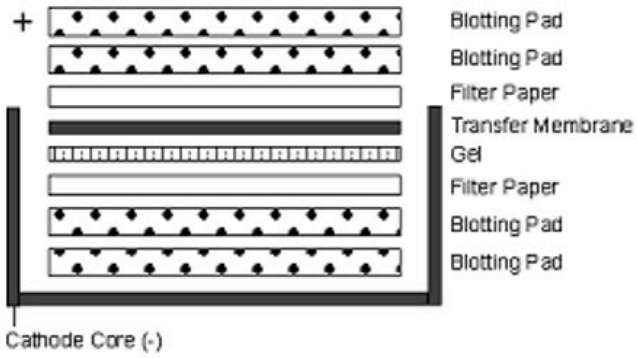


Figure 8: The blotting sandwich. The illustration is adapted from invitrogen.com

2.9.3. Blocking

- The membrane was removed from blotting sandwich after blotting was completed.
- Then it was soaked in 10 ml Odyssey Blocking buffer for 1 hour with gentle rocking.

2.9.4. Antibodies staining

- 5 ml of Odyssey Blocking buffer were added in a 50 ml centrifuge tube
- 5 µl of Tween 20 are added, and mixed by vortex
- Primary antibodies is then diluted in the solution
- Membrane is then placed inside the tube with bands towards the inside
- Membrane was then incubated over night at 4°C on the rotating wheel.
- The membrane is washed 4 times with TBS-T for 5 min each

- Afterwards, 5 ml of Odyssey Blocking buffer were added in a 50 ml centrifuge tube
- 5 µl of Tween 20 are added, and mixed by vortex
- Secondary antibodies is then diluted in the solution
- The membrane is then put inside the tube and incubated for 1 hour at room temperature on rotating wheel, while covered with aluminum foil paper.
- The membrane is washed 4 times with TBS-T for 5 min each
- The membrane is washed once with TBS for 5 min each

2.9.5. Detection and analysis

In The ODYSSEY® Sa Infrared Imaging System, the analysis of the western results were conducted in concordance with manufacturer's instructions.

3. GST-tagged protein purification

The glutathione-*S*-transferase (GST) gene fusion system is widely used for protein expression and purification from bacterial lysates. The GST binds Glutathione with high affinity and specificity in chromatography column matrix under mild conditions. Next, the GST moiety is removed from the desired protein is obtained by a specific protease cleavage site located between the GST moiety and the recombinant polypeptide. [53]

Day 1:

- 5 ml culture in LB media was inoculated with one colony of transformed BL21 competent E. coli carrying the plasmid that encodes the desired fusion protein.
- This culture was incubated over night at 37°C and 255 rpm.

Day 2:

1. 100 ml culture was prepared using the 5 ml culture and incubated at 37°C until OD600 reached 0,5 (1-2 hours).
2. The IPTG (1:1000) was added to the media and incubate in room temperature 5 hours.
3. The samples were centrifuged at 10 000 RPM for 35 minutes.
4. The supernatants were discarded and pellets stored at -70°C until further preparation.

Day 3:

1. The pellets were thawed on ice and all buffers cooled down on ice.
2. The pellets were solubilized by vortex, and then resuspended in 5 ml lysis buffer.
3. The lysate was transferred to new sonication tubes
4. The samples were sonicated 3 times 45 seconds each.
5. Samples were centrifuged at 10000 rpm for 35 minutes
6. The Glutathione Sepharose beads were prepared. For 4 samples:

   a. 300 µl of Sepharose was pipetted in 2 tubes (for two samples).
   b. The Sepharose was washed three times in 1 ml lysis buffer, and then centrifuged at 3000 rpm for 1 minute.
   c. After last wash the Sepharose was dissolved in 300 µl of lysis buffer.
7. The sonicated supernatant was transferred to a new 50 ml tube and added 250 µl of 5 M NaCl (1:20) and 10µl 1 M DTT (1:500).
8. 100µl Glutathione Sepharose was added to each tube.
9. The tubes were incubate with agitation for 1 hour at 4°C.
10. Tubes were then centrifuged for 5 minutes at 500x g and supernatant was discarded.
11. The pellet was dissolved in 500 µl of washing buffer and transferred to spin column.
12. The spin column was washed 5 times by following the steps:
   a. 500 µl washing buffer was added
   b. Tubes were centrifuged at 1000 rpm for 1 minute
   c. The flow-though was discarded
13. The protein of interest was eluted in elution buffer by adding and collecting 200 µl at a time. After each fraction is collected the sample was placed immediately on ice. This step was repeated until 8 eluted fractions are collected from each sample.
14. To evaluate presence of protein in the eluted fractions, an SDS-PAGE gel was run (descried earlier) and colored with PageBlue Staining as describes by manufacturer.
15. Fractions with requested proteins was mixed together and dialyzed with D-Tube Dialyzer Midi, by following the steps:
   a. The cap was removed from D-tube and 800 µl of dH_2O is added and incubated upright for at least 5 minutes. The cap was put on and screwed gently.
   b. Cap was removed and water is discarded from the tube using a pipet.
   c. The sample is added and cap screwed on gently.
   d. The tube is placed in a floating rack. The rack was then put in beaker containing 100-1000 fold sample volume of dialysis buffer and a stir bar. The entire surface of the membranes was submerged in buffer.
   e. The samples was incubated in dialysis buffer over night and stirred for at least 3 hours at 4°C. The beaker was covered with parafilm and placed it in a hood.

Day 4:

1. The cap was removed from D-tube dialyzer.
2. The sample was transferred from D-tube Dialyzer to a clean microcentrifuge tube.
3. The sample was diluted and applied on a SDS-PAGE NuPage gel to determent the approximately concentration.
4. The protein was stored at -20°C when not in use.

3.1. Measurement of protein concentration

In some protein-utilizing assays, protein concentration needs to be measured to have a standard baseline. For instance, when comparing protein expression in different cell lines, or in cells with genetically modified protein expression.

- 20 µl of reagent S was added to each ml of reagent A. This working reagent is called A' and is stable for 1 week
- 8 dilutions of standard protein bovine serum albumin (BSA) were prepared with concentrations 0.078125, 0.15625, 0.3125, 0.625, 1.25, 2.5, 5, 10 mg/ml protein in MKK lysis buffer. This was performed in a serial twofold dilution manner. These standard solutions were used to prepare a standard curve for each assay.
- 3 dilutions of sample protein extract were prepared in MKK lysis buffer.
- 5 µl were pipetted of standards and samples into a new 96 well plate.
- 25 µl of reagent A' was added into each well.
- 200 µl of reagent B was added into each well.
- When bubbles formed, they were popped with a clean, dry needle
- After 15-30 minutes, the absorbance for each sample was read using the instrument VersaMax™ Plus micro-plate reader at 750 nm. The instrument is supplied with SoftMax® Pro Data Acquisition & Analysis Software.

3.2. In vitro kinase assay

This approach was used to determine whether an active protein phosphorylates another protein *in vitro*.

1. The assay buffer and ATP mix were prepared
2. The following component were added to the designated tubes:
    - x µl of specific substrate protein
    - x µl of active kinase
    - 10µl ATP mix
    - Ad 50 µl Assay buffer
3. The tubes were incubated at 30°C for 30 min
4. The reaction was stopped, by adding SDS sample buffer and sample reducing agent.
5. The samples were then analyzed using SDS-PAGE gel electrophoresis

4. Colonogenic assay

This is a simple method to measure the proliferation ability of mammalian cells. Cells are seeded in low density, so the proliferation will be measured by counting single colony forming units.

- The seeded cells was grown for 10 days, with medium replacement every three days
- On the 10^{th} and last day of the experiment, cells were washed once with PBS, and then stained with 350 µl of colonogenic reagent for 45 min
- Cells were afterwards washed twice with PBS, and visible cells were counted
- Data were analyzed in Excel (Microsoft office, 2011, mac) as presented in appendix. One-way analysis of variance (ANOVA) was used to compare differences means of several groups' and two-sample student´s *t*-test was used to compare differences in means of two groups' in StatPlus (AnalystSoft Inc., 2009, mac).

5. Scratch (wound healing) assay

This technique is a broadly used for investigating wound-healing processes, and giving indication about the migration ability. Cells are seeded in high density, so they are confluent when wound is made.

- Using the seeded cells the wound was created the next day, by manually scratching the cell surface with a pipette tip (yellow 10-200µl)
- Baseline pictures were taken using Zeiss AxioVert S 100, with DS-L1 Nikon camera, and the area was marked on the plate lid.
- After 28 hours pictures were taken at the same spot.

RESULTS

1. Optimizing and validating the new antibody: anti-phospho-SRC-3

Usually, when a new antibody is received, titration experiments need to be performed in the pursuance of determining the optimal antibody dilution for optimal results. In other words, finding the conditions and concentrations that give the best staining with minimum background and non-specific binding. Titration experiments are normally conducted by first selecting a fixed incubation time. Then a series of experimental dilutions of the antibody are tested on the same type of sample, so other conditions are standardized. Manufacturers often suggest a start concentration on product data sheet. Along with this concentration, it is advised to run a 2-fold dilution and a double concentration of the suggested concentration.

In this study we wanted to run a titration experiment on new phospho-specific antibodies against SRC-3 from 3 bleed-batches from the same sheep.

1.1. Testing primary antibodies from different bleeds

Anti-phospho-SRC-3 antibodies were designed to bind the phosphorylated SRC-3 protein at the residue Ser857. A study from last year [35] suggested that SRC-3 is phosphorylated by the MAP kinase ERK3 at Ser857. However, the residues surrounding Ser857 indicate that this phosphorylation site is not a site for a proline-directed kinase, but resembles a MAPKAP kinase substrate motif, e.g. MK5, as illustrated in Figure 9. [54]

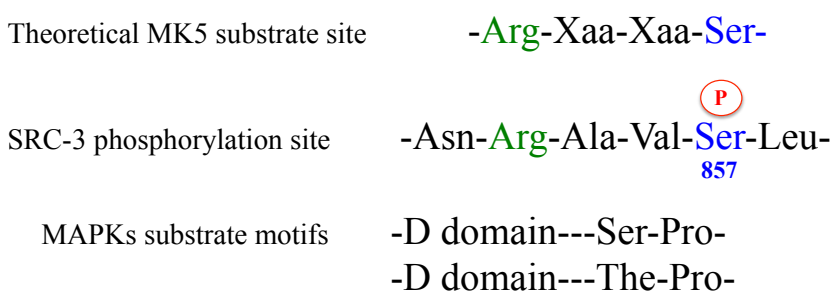


Figure 9: Illustration of substrate sites in MAPKs and MK5, and phosphorylation site of SRC-3. The SRC-3 phosphorylation site resembles the suggested MK5 substrate site, more readily than MAPKs substrate site. Xaa: any amino acid. P: Phosphate group. D-domain: Docking domain.

In support of this, preliminary data from our own lab (E. Tømte unpublished results, Figure 11) show that SRC-3 CID is indeed a very good *in vitro* substrate for MK5 and rather a poor substrate for ERK3. Thus, our hypothesis is that ERK3 induces phosphorylation of SRC-3 at Ser857 through the activation of MK5, which phosphorylates SRC-3 directly (Figure 10)

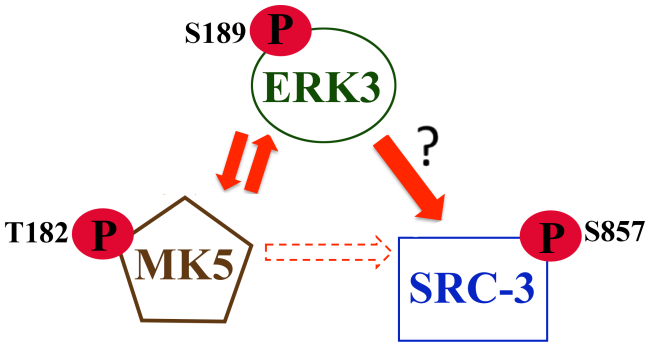


Figure 10: Illustration of suggested phosphorylation pathways involving ERK3, MK5, and SRC-3. In Long et al, 2012, ERK3-mediate phosphorylation of SRC-3 is described (question marked arrow. Our research group is more concerned about MK5´s role in SRC-3 phosphorylation (dotted arrow), and more data are verifying that MK5 is the phosphor-donor of SRC-3.

1.2. MK5 phosphorylates SRC-3

As previously mentioned, our main hypothesis is that MK5 is phosphorylating SRC3 at Ser857, and not ERK3. The kinase assay that was carried out prior to this study and served at a background is shown in Figure 11. We see that MK5 solely phosphorylates SRC-3 CID, and at the same time no signs for phosphorylation by ERK3.

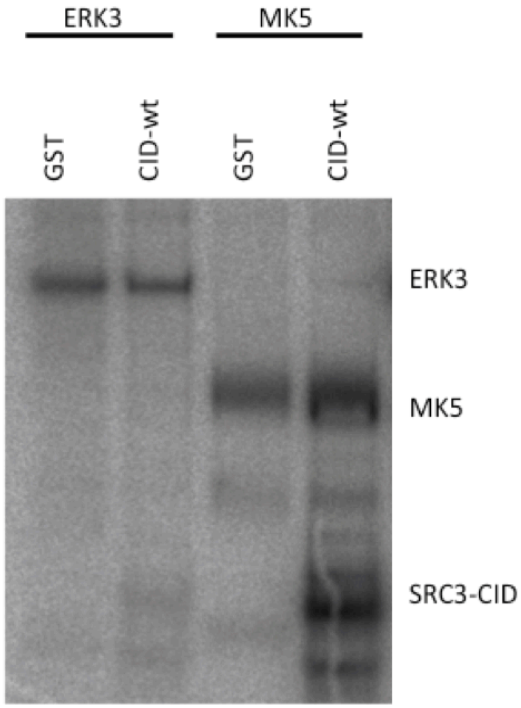


Figure 11: *In vitro* phosphorylation assay demonstrating that MK5, not ERK3, is phosphorylating SRC-3 CID. Kinase assay performed using 300 ng of purified ERK3 protein, 50 ng active his-MK5, 100 µM ATP, 15 mM $MgCl_2$, 2 µCi ^{32}P-ATP (Nerliens M.) in assay buffer. The reaction was carried out at 30°C for 15 min. Proteins were resolved by SDS-PAGE gel, and visualized by autoradiography with 13 hours exposure period using the phosphorimager Fuji BAS-5000.

In order to test this hypothesis, we raised a new phospho-specific antibody against Ser857 phosphorylated SRC-3. Before we could use this antibody for detection of phosphorylated SRC-3 from living cells we needed to test and optimize the new antibody in Western blotting using recombinant purified GST-SRC-3 phosphorylated by MK5 *in vitro*. To accomplish this, we performed a kinase assay, by adding the two proteins: active his-MK5, and the CID region of GST-SRC-3 (aa 841-1080) in a phosphorylation reaction, along with assay buffer and ATP-mix. Then a Western blot assay was conducted using the phosphorylated protein and antibodies from the 3 sheep bleeds with the concentration 1µg/ml (1:300) as recommended by manufacturer, as illustrated in Figure 12.

Figure 12B is showing that antibodies from 2nd bleed do recognize full-length MK5-phosphorylated SRC-3 CID protein as expected at 52 kDa (Long et al, 2012), while in Figure 12C we can see that antibodies from 3rd bleed recognize to some degree full-length GST-SRC-3 CID protein as a faint band is observed at 52 kDa. . No bands were detected using the 1st bleed antibodies as in Figure 12A.

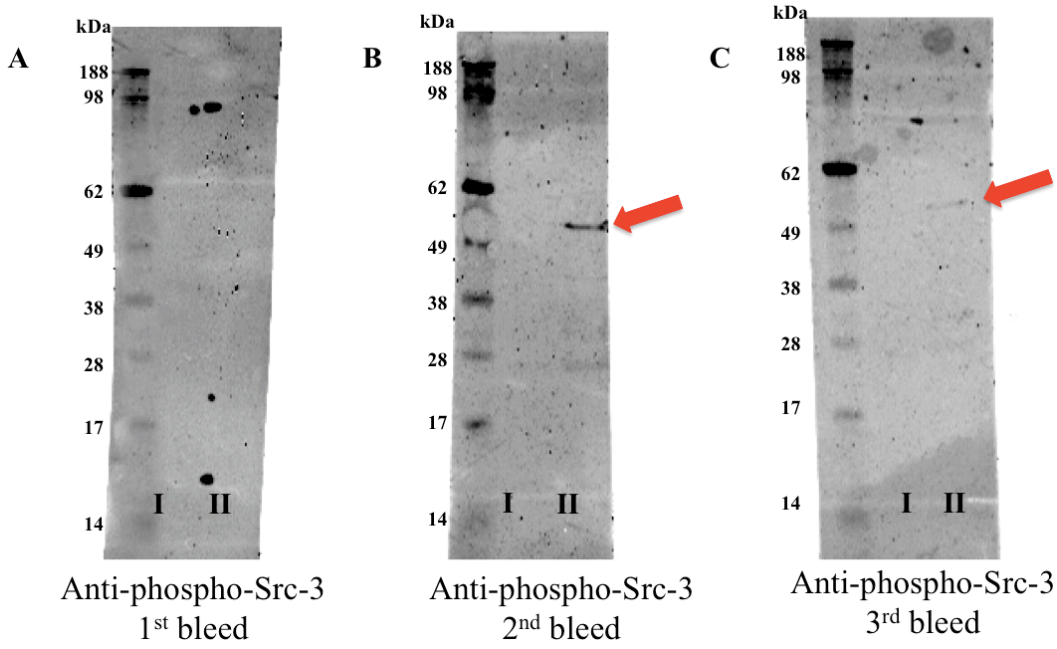


Figure 12: Optimizing anti-phospho-SRC-3 by testing antibodies from 3 different bleeds on a kinase reaction. Kinase assay was conducted on (I) only GST-SRC-3 CID (1 µg) as negative control and (II) active his-MK5 protein (30 ng of 250 U/mg) and GST-SRC-3 CID (1 µg). Kinase reaction cocktail was incubated for 30 minutes at 30°C. Then a Western blot assay was run. A) Blot incubated in 1st bleed antibodies. Red arrows, at 52 kDa in B) and C) when using 2nd and 3rd bleed antibodies, respectively, represent the phosphorylated full-length GST-SRC-3 CID. All primary antibodies were diluted (1:300), and anti-sheep 800 CW donkey secondary antibodies were used (1:10000). SeeBlue® Plus2 Pre-Stained ladder from Invitrogen was used for band identification.

1.3. Testing different conditions and concentrations of primary antibodies

Next, we decided to increase the antibody concentration to 1:250, and at the same time double protein amount in kinase assay. In Figure 13A we have the PageBlue™ stained kinase assay gel, and it confirms that the protein is present. Figure 13B and C is showing the bands for full-length phosphorylated GST-SRC-3 CID at 52 kDa when using antibodies from 2nd and 3rd bleed, respectively. What is worth mentioning here is; GST-SRC-3 CID protein looks fragmented, as bands are spread down over the blots.

We have now much obvious bands of MK5-phosphorylated GST-SRC-3 CID at 52 kDa. Accordingly, we can also conclude that both antibodies from 2nd and 3rd bleed are of acceptable performance at this concentration. So we are proceeding with antibodies from 2nd bleed from here on in this study.

In regard to the fragmented protein, we agree to purify new GST-SRC-3 CID protein, re-do the kinase reaction again without any modifications, and incubate the blot in antibodies from 2nd bleed with different concentrations.

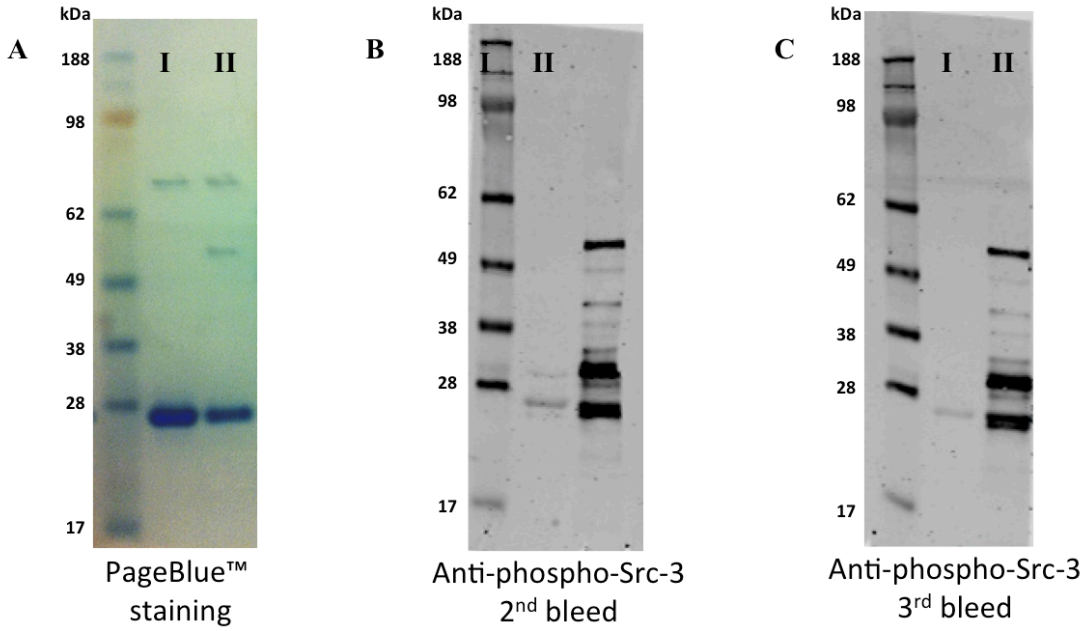


Figure 13: Optimizing anti-phospho-SRC-3 by testing 2 bleed antibodies on a kinase reaction. Kinase assay was conducted on (I) only GST-SRC-3 CID (2 µg) as negative control and (II) active his-MK5 protein (65 ng of 250 U/mg) and GST-SRC-3 CID (2 µg). Kinase reaction cocktail was incubated for 30 minutes at 30°C. Then a Western blot assay was run. The phosphorylated GST-SRC-3 CID is present at 52 kDa in B) 2nd and C) 3rd bleed antibodies blot. All primary antibodies were diluted (1:250), and anti-sheep 800 CW donkey secondary antibodies were used (1:10000). A) PageBlue staining was performed on an SDS-PAGE gel with same kinase reaction components for 1 hour, and then washed extensively. Stained gel confirms the presence of protein. SeeBlue® Plus2 Pre-Stained ladder from Invitrogen was used for band identification.

New GST-SRC-3 CID protein was expressed and purified. Figure 14 shows the PageBue™ staining, of a SDS-PAGE gel with samples from the 8 fractions (lanes 1-8) from GST-tagged protein purification column. The protein of interest is present, at approximately 52 kDa, in only 5 eluted fractions. These fractions (lanes 1-5) were collected and dialyzed before further use.

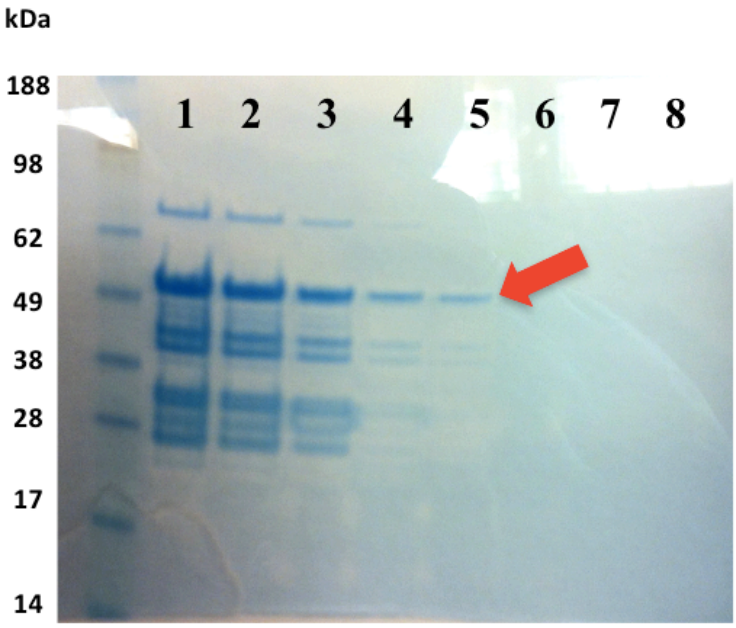


Figure 14: PageBlue staining on SDS-PAGE gel with GST-SRC-3 CID wt protein fractions. 10µl from each fraction of the first 8 eluted fractions (1-8) collected from column as a part of GST-tagged protein purification in 3rd day of the procedure. An SDS-PAGE gel electrophoresis was run for 45 min, and gel was then stained in PageBlue staining dye for 1 hour, and then washed extensively. The first 5 fractions contained protein and therefore were used in dialysis step. The protein bands representing full-length GST-SRC-3 CID are marked with a red arrow at 52 kDa. SeeBlue® Plus2 Pre-Stained ladder from Invitrogen was used for band identification.

After expressing and purifying new GST-SRC-3 CID protein, the previous kinase assay was re-conducted with the same setup. Three different concentrations of 2nd bleed antibodies were tested. Some bands were shown in all three blots at 52 kDa, representing the full-length GST-SRC-3 CID, with good result with antibodies dilution 1:500, and to some degree 1:250, as illustrated in Figure 15.

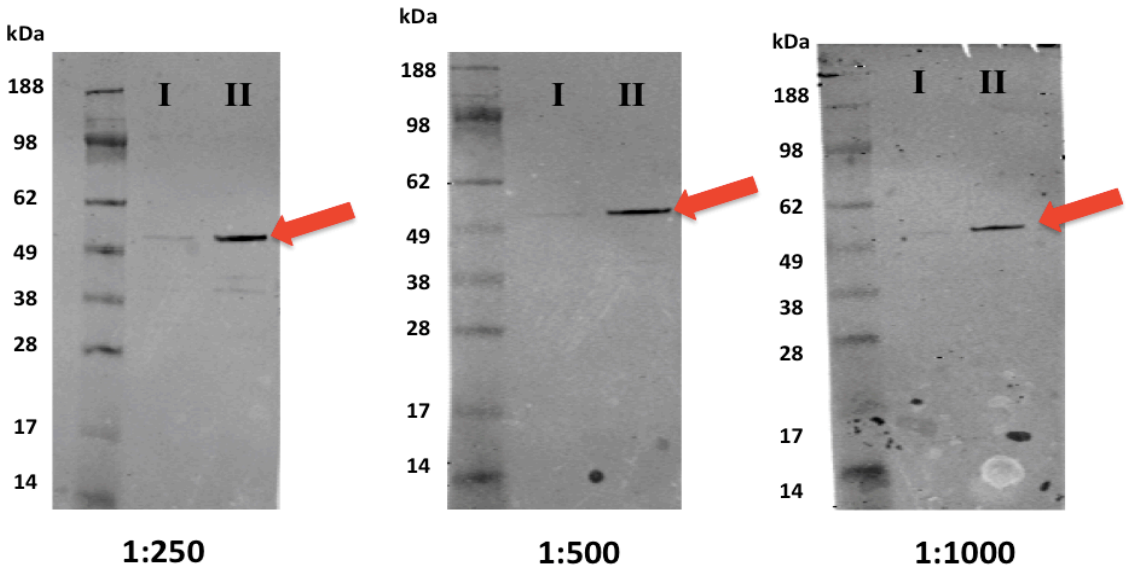


Figure 15: Titration experiment of anti-phospho-SRC-3 antibodies from 2nd bleed. 3 dilutions were tested on the indicated kinase assay. Kinase assay was conducted on (I) only GST-SRC-3 CID (2 µg) as negative control and (II) active his-MK5 protein (65 ng of 250 U/mg) and GST-SRC-3 CID (2 µg). Kinase reaction cocktail was incubated for 30 minutes at 30°C. Then a Western blot assay was run. The phosphorylated full length GST-SRC-3 CID is present at 52 kDa (red arrows) in all dilutions (from the left; 1:250, 1:500, and 1:1000) of 2nd bleed primary antibodies blot, and anti-sheep 800 CW Donkey secondary antibody was used (1:10000). SeeBlue® Plus2 Pre-Stained ladder from Invitrogen was used for band identification.

In order to examine this phosphorylation theory at that specific site further, we expressed and purified the mutant GST-SRC-3 CID S857A protein. A new kinase assay was conducted, so we could compare the phosphorylation activity in mutant and wild type protein using Western blotting.

Figure 16 is illustrating the PageBue™ staining, of a SDS-PAGE gel with purified protein in the 8 fractions (lanes 1-8) collected from GST-tagged protein column. Only first 3 eluted fractions contained the mutant protein of interest, shown just about 52 kDa, and these fractions were collected and dialyzed.

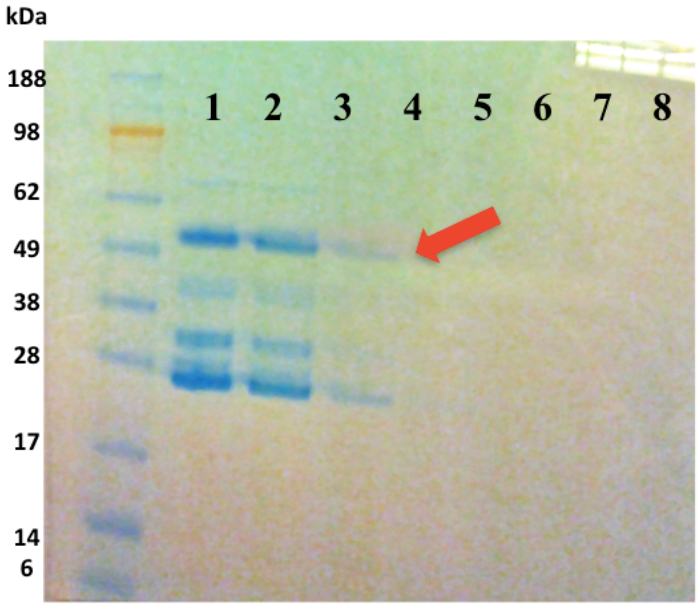
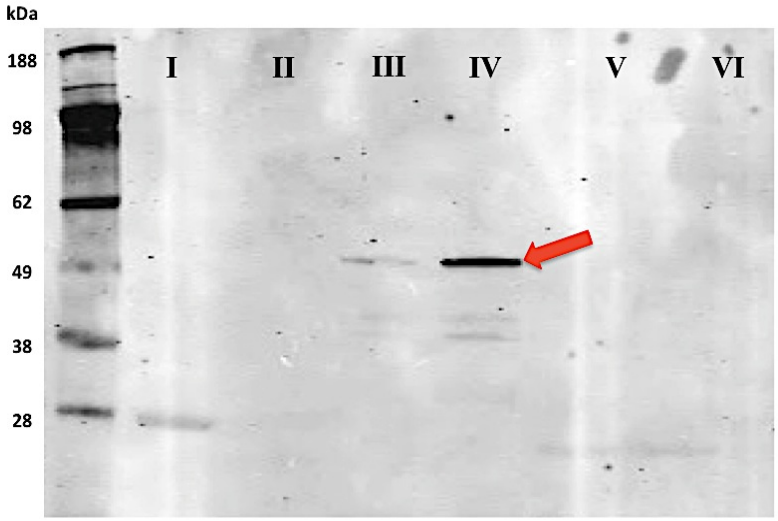


Figure 16: PageBlue staining on SDS-PAGE gel with GST-SRC-3 CID S857A protein fractions. 10µl from each fraction of the first 8 eluted fractions (1-8) collected from column as a part of GST-tagged protein purification in 3rd day of the procedure. An SDS-PAGE gel electrophoresis was run for 45 min, and gel was then stained in PageBlue staining dye for 1 hour, and then washed extensively. The first 3 fractions contained protein and therefore were used in dialysis step. The protein bands representing GST-SRC-3 CID are marked with a red arrow at 52 kDa. SeeBlue® Plus2 Pre-Stained ladder from Invitrogen was used for band identification.

With the new mutant GST-SRC-3 CID S857A protein in place, another kinase assay was performed. The phosphorylated protein was analyzed by Western blotting, using antibodies from the 2nd bleed with the dilution 1:500, as demonstrated in Figure 17.

A clear band appears at 52 kDa, representing the phosphorylated full-length GST-SRC-3 CID, in sample lane with both MK5 and GST-SRC-3 CID wt (Figure 17 IV). No bands were observed in lanes containing GST, MK5, GST-CID wt, GST-CID S857A, or in the combination MK5 and GST-CID S857A.

This finding indicates that MK5 phosphorylates the CID-containing region of SRC-3 at Ser857 residue and can be detected by our new antibodies.



Sample	I	II	III	IV	V	VI
GST	2 µg	-	-	-	-	-
Active his-MK5	-	65 ng of 250U/mg	-	65 ng of 250U/mg	-	65 ng of 250U/mg
GST-SRC-3 CID wt	-	-	2 µg	2 µg	-	-
GST-SRC-3 CID S857A	-	-	-	-	2 µg	2 µg

Figure 17: MK5 phosphorylates SRC-3 at S857 *in vitro*. Kinase assay was conducted on GST, active his-MK5, GST-SRC-3 CID wt, GST-SRC-3 CID S857A (I-VI). Kinase reaction with stated protein amounts was incubated for 30 minutes at 30°C with shaking. Then a Western blot assay was run. The red arrow, at 52 kDa in lane IV, highlights the phosphorylated GST-SRC-3 CID wt. Anti-phospho-SRC-3 primary antibodies are used (1:500), and anti-sheep 800 CW Donkey secondary antibody was used (1:10000). SeeBlue® Plus2 Pre-Stained ladder from Invitrogen was used for band identification.

2. The construction of the pGL3-basic MMP2 promoter reporter gene vector

In this study we planned to investigate expression regulation of MMP2 gene indirectly, by measuring the transcriptional activity of its promoter. Therefore, a reporter gene technique is used; a so-called dual luminescence-based reporter gene assay. One of the reporter genes in this assay is the luciferase gene in luciferase vector pGL3-basic, into which we wished to insert the MMP2 promoter. By joining those two into one plasmid, their activity becomes correlated. Thus, by measuring the luciferase activity, an indication of MMP2 promoter activity is provided for us. The second reporter gene is β-galactosidase gene, which was cotransfected into cells as a part of the plasmid pCH110. This gene is used for normalizing the activity of the experimental reporter gene, luciferase gene in this context, to the activity of the internal control, resulting in minimizing the experimental variability caused by differences in cell viability or transfection efficiency.[50]

2.1. Subcloning of the MMP2 promoter into pGL3 Basic

The desired luciferase plasmid pGL3-basic MMP2 promoter was constructed by cutting both insert and vector by restriction enzymes KpnI (5`GGTAC⇧C3`) and XhoI (5´C⇧TCGAG3`) in order to generate sticky and compatible ends, and then DNA fragments were ligated (Figure 18). The construct was then transformed into DH5α competent cells for replication.

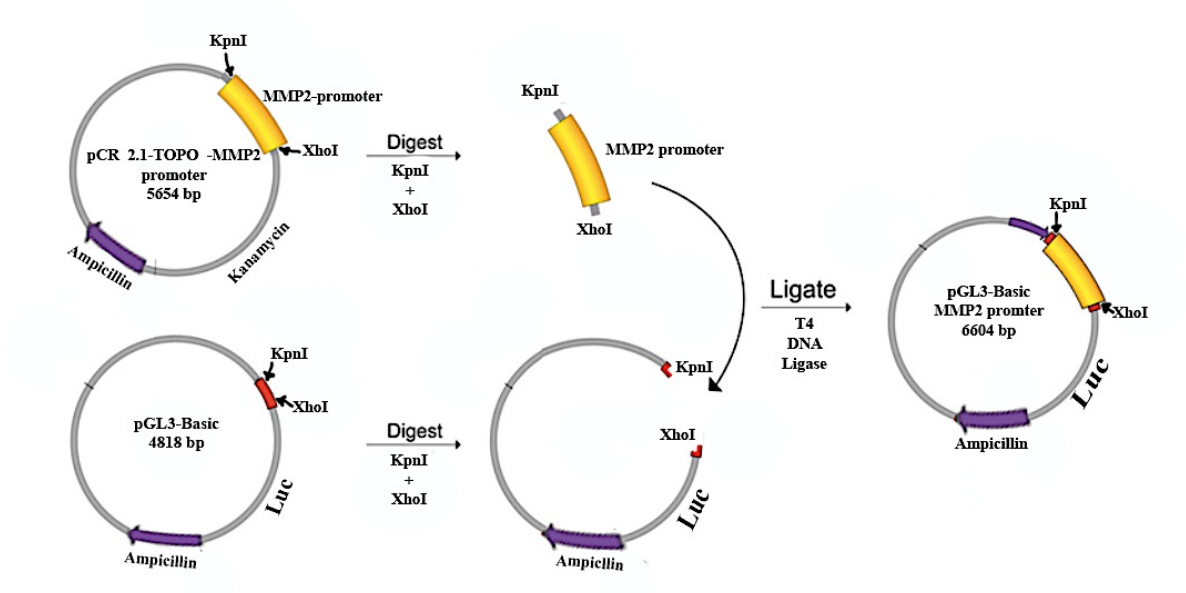


Figure 18: Graphic illustration of pGL3-basic MMP2 promoter subcloning by restriction enzymes. KpnI and XhoI restriction enzymes catalyze the cleavage of both donor (pCR®2.1-TOPO® MMP2-promoter) and recipient plasmid (pGL3-basic), at 37°C for 2 hours, yielding MMP2 promoter insert and cleaved recipient plasmid with sticky ends. The fragments are then linked together by T4 ligase, for 1.5 hour at room temperature. Ligated construct was then transformed into DH5α, with ampicillin as selecting agent.

2.2. Restriction analysis of construct plasmid pGL3-basic MMP2 promoter

In order to confirm that the pGL3-basic MMP2 promoter plasmid was properly constructed and cloned, a DNA restriction analysis using agarose gel electrophoresis was run on 8 purified construct plasmids (wells 1-8) using KpnI and XhoI restriction enzymes. Figure 19 is showing the bands 4791 bp and 1813 bp (Appendix 1) that represent pGL3 basic vector and MMP2 promoter insert, respectively. This verifies the accurate performance of subcloning.

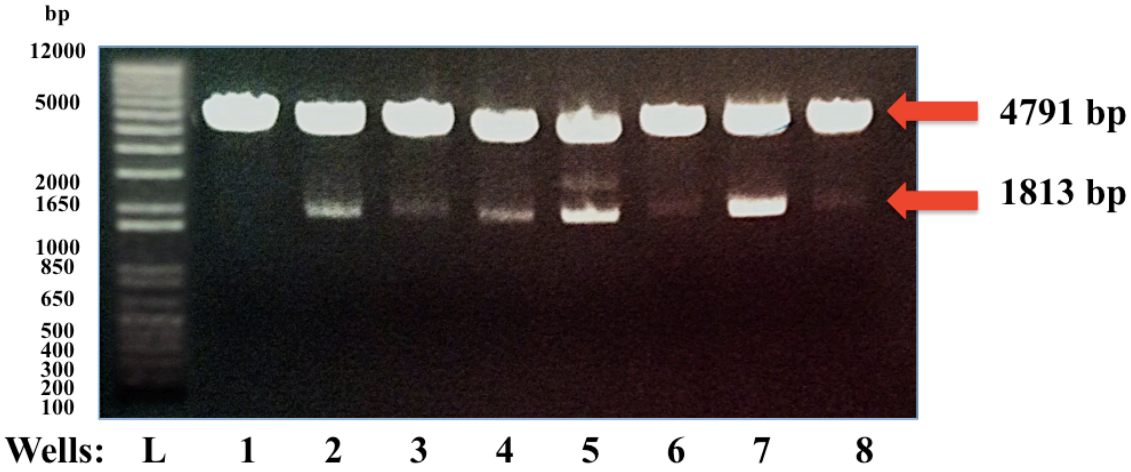


Figure 19: Restriction analysis of subcloned construct pGL3-basic MMP2 promoter. 60 ng of DNA from 8 transformed DH5α colonies (1-8) were digested by KpnI and XhoI restriction enzymes and separated on a 0.7 % agarose gel for 1 hour together with a ladder (1kb plus DNA ladder, Invitrogen). Arrows show the expected restriction fragments of 4791bp and 1813 bp for pGL3-basic MMP2 promoter. L: DNA ladder, bp: base pair

2.3. MMP2 promoter activity enhanced by dexamethasone-stimulated GR

For further confirmation of the cloned pGL3-basic MMP2 promoter construct, a luciferase assay was run. MMP2 promoter contains a glucocorticoid response element (GRE). As a consequence, MMP2 promoter activity will theoretically be enhanced in the presence of the dexamethasone-stimulated glucocorticoid receptor (Associate Prof. I. Mikkola personal communication). In Figure 20 we can see that MMP2 promoter luciferase activity increases drastically when dexamethasone is added and stimulated the cotransfected Glucocorticoid receptor (GR), compared to negative control and only GR.

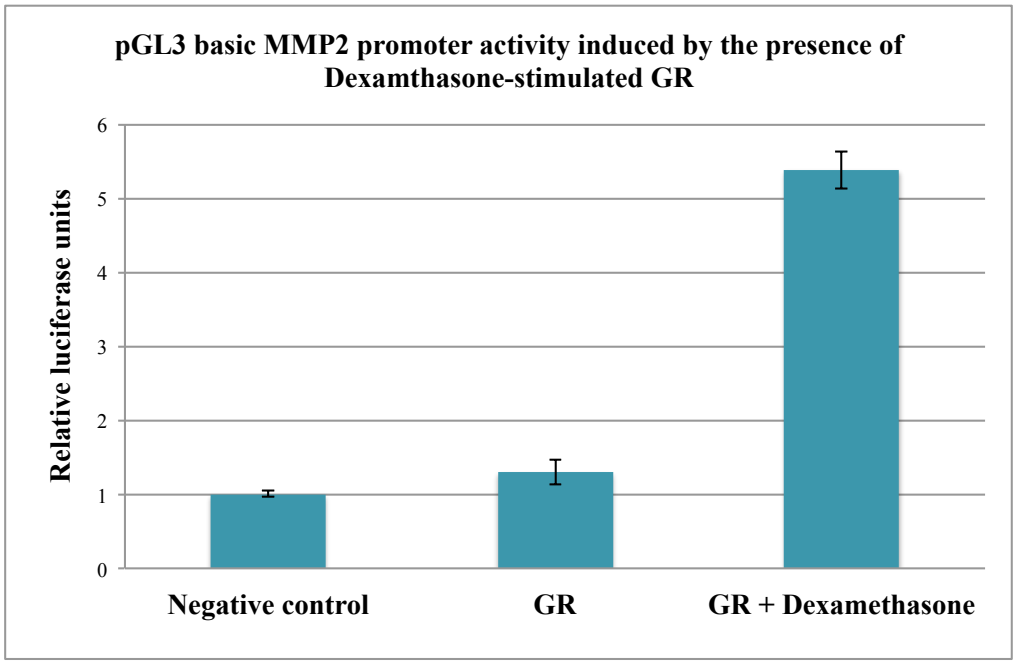


Figure 20: MMP2 promoter activity is increased by dexamethasone-stimulated GR in HeLa S3 cells. 20K cells per well were seeded and cotransfected using Lipofectamine® LTX with Plus™ Reagent, 24 hours after seeding, with pCH110, the newly constructed pGL3-basicMMP2 promoter, and glucocorticoid receptor plasmid (GR), without and with 24 hour Dexamethasone stimulation. Luciferase assay was conducted the next day, and the activity measured using a Luminoscan RT. Luciferase activity was divided by β-galactosidase, and normalized with regard to transfection efficiacy in excel sheet. (Appendix no.7) Relative luciferase units are shown in graph ± coefficient of variation. Sample size: n=3

3. SRC-3 overexpression increases promoter activity of MMP2

Our results so far indicate a connection between MK5 and SRC-3. What is interesting in this this context is the fact that SRC-3 is already declared as bona fide oncogenic protein, working through several pathways facilitating its cancer pathogenesis. One pathway was extensively investigated, for instance in Long et al, 2012, and it involves SRC-3 in the regulation of the expression of matrix metalloproteinase genes. It is thought that SRC-3 coactivates the ETS transcription factor PEA3, leading to overexpression of certain MMP genes. This is significant because it was established that the majority of MMPs have roles in tumorigenesis, invasion, and metastasis as reviewed in Hua et al.[40]

We wanted to verify the connection between SRC-3 overexpression and MMP2 gene activity. So a luciferase assay was carried out, and collected luciferase and β-galactosidase signals data were analyzed and summarized in Figure 21. An increase, up to approximately 20 fold, in MMP2 promoter activity is observed when SRC-3 is overexpressed.

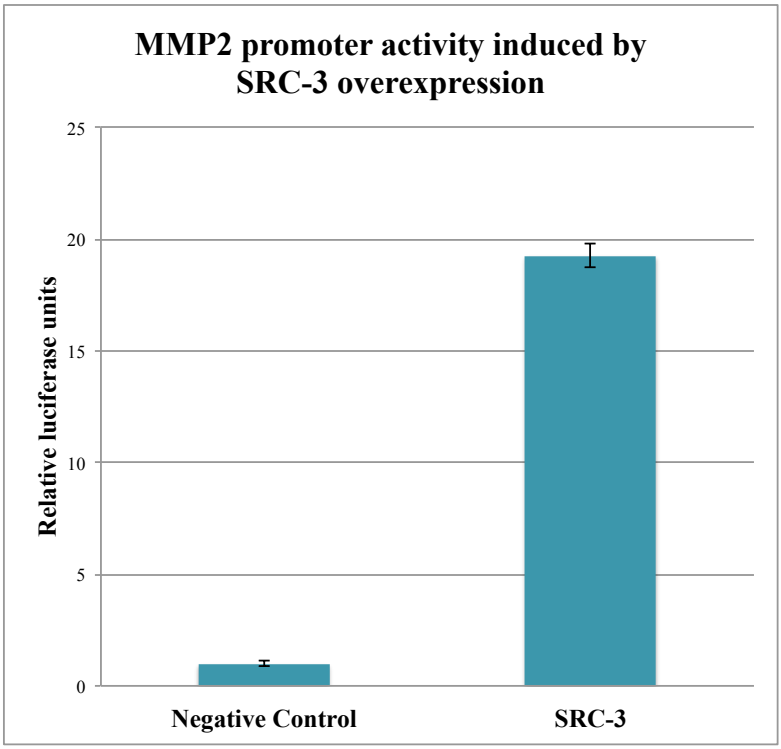


Figure 21: SRC-3 overexpression increases MMP2 promoter activity in HeLa S3 cells. 20K cells per well, in a 24 well plate, were co-transfected using Lipofectamine® LTX with Plus™ Reagent, 24 hours after seeding, with pCH110, pGL3-basicMMP2 promoter, the empty vector pcDNA3, and pcDNA3-SRC-3. Luciferase assay was conducted the following day, and the signals were measured in a Luminiscan RT. Luciferase actvity signal was divided by β-galactosidase for normalizing with regard to transfection efficacy ± coefficient of variation. Sample size: n=3 (Appendix no.8)

4. MK5, ERK3, and SRC-3 overexpression influencing promoter activity of MMP2, MMP9, and MMP10

As postulated, MK5 may phosphorylate SRC-3 at Ser857, thus MK5 might have an influence on the MMP gene expression indirectly. We planned to examine this context thoroughly. In order to accomplish this, a series of luciferase assays were planned to establish whether this MK5-mediated phosphorylation influences the transcription activity of MMP2, MMP9, and MMP10 promoters.

The highest activity of MMP2 promoter was observed when both SRC-3 and ERK3 were co-overexpressed, and SRC-3 seems to increase the activity more than ERK3. On the contrary, MK5 overexpression resulted in lower MMP2 promoter activity (Figure 22).

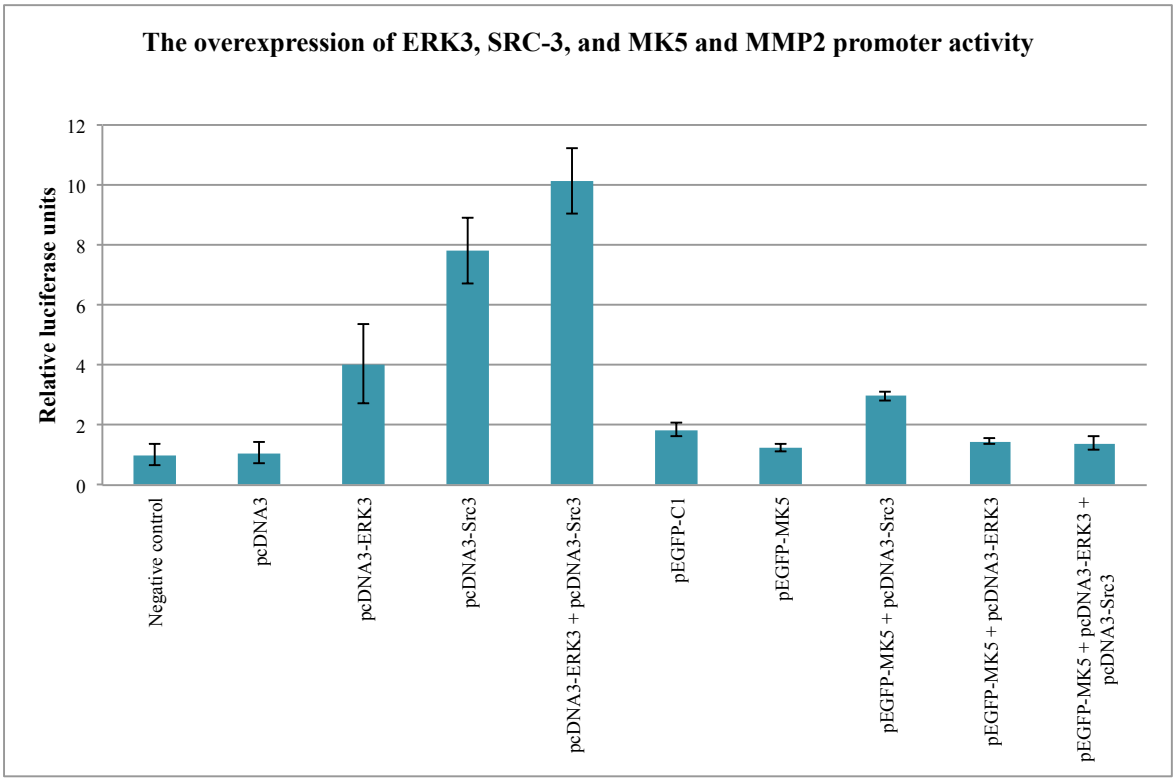


Figure 22: MMP2 promoter activity correlates to SRC-3 expression in A549 cells. 20K cells per well were cotransfected using Lipofectamine® 2000 Reagent, 24 hours after seeding, with pCH110, pGL3-basic MMP2 promoter in each well of the 24 well plate, and control plasmids pcDNA3 and pEGFP-C1. We tested pcDNA3-Src3, pcDNA3-ERK3, and pEGFP-MK5 separately or in combinations as assignated in graph. Luciferase assay was conducted the following day, and the signals were measured in a Luminiscan RT. Luciferase actvity signal was divided by β-galactosidase for normalizing with regard to transfection efficacy in excel sheet. Relative luciferase units are shown in graph ± coefficient of variation. Sample size: n=3 (Appendix no.9)

The exact same luciferase assay setup, as in previous assay, was used for investigating MMP9 promoter activity. The data summarized in Figure 23 indicate that MK5 overexpression is designated for highest activity of MMP9 promoter, even more than the combination of SRC-3 and ERK3. SRC-3 is next in affecting MMP9 activity, and ERK3 was accounted for least effect.

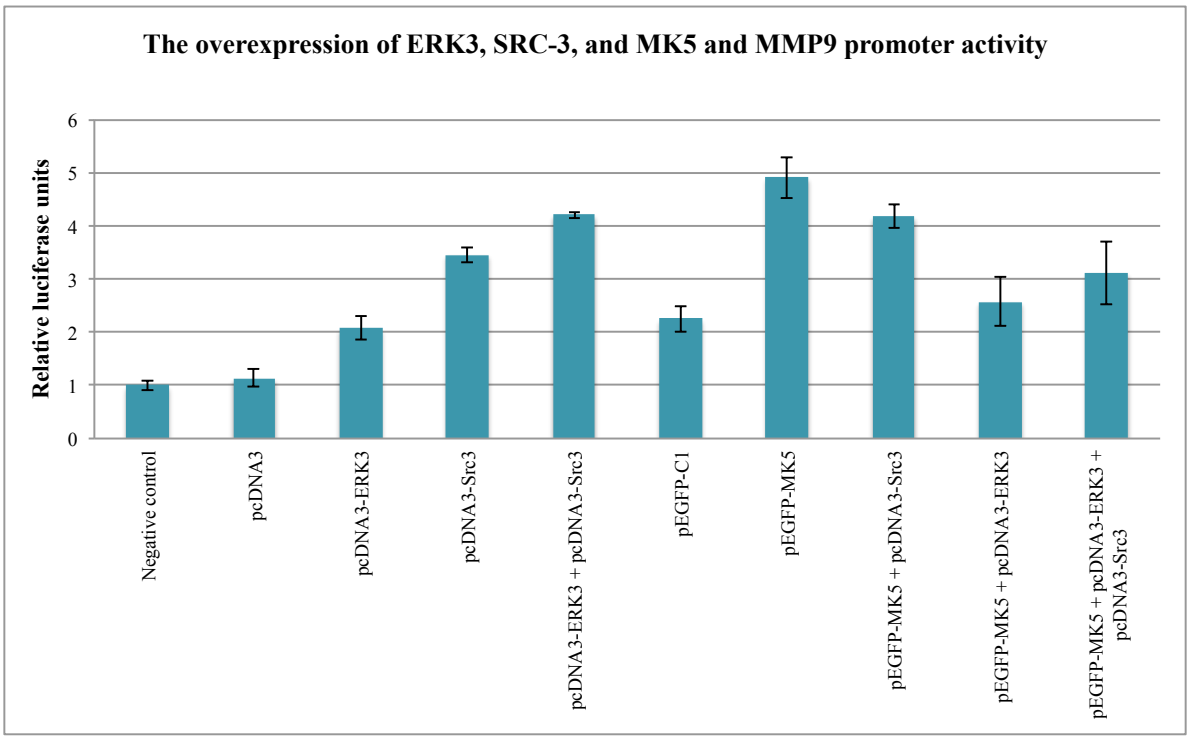


Figure 23: MMP9 promoter activity correlates to SRC-3 expression in A549 cells. 20K cells per well were co-transfected using Lipofectamine® 2000 Reagent, 24 hours after seeding, with pCH110, pGL3-basic MMP9 promoter in each well of the 24 well plate, and control plasmids pcDNA3 and pEGFP-C1. We tested pcDNA3-Src3, pcDNA3-ERK3, and pEGFP-MK5 separately or in combinations as assignated in graph. Luciferase assay was conducted the following day, and the signals were measured in a Luminiscan RT. Luciferase actvity signal was divided by β-galactosidase for normalizing with regard to transfection efficacy ± coefficient of variation. Sample size: n=3 (Appendix no.10)

In this following assay we investigated MMP10 promoter activity, using the same luciferase assay setup as in the two previous assays. The combination of SRC-3 and ERK3 was accounted for the highest MMP promoter activity, as well; SRC-3 alone has increased the activity more than ERK3, as illustrated in Figure 24. As for MK5 overexpression, lower MMP10 promoter activity was observed.

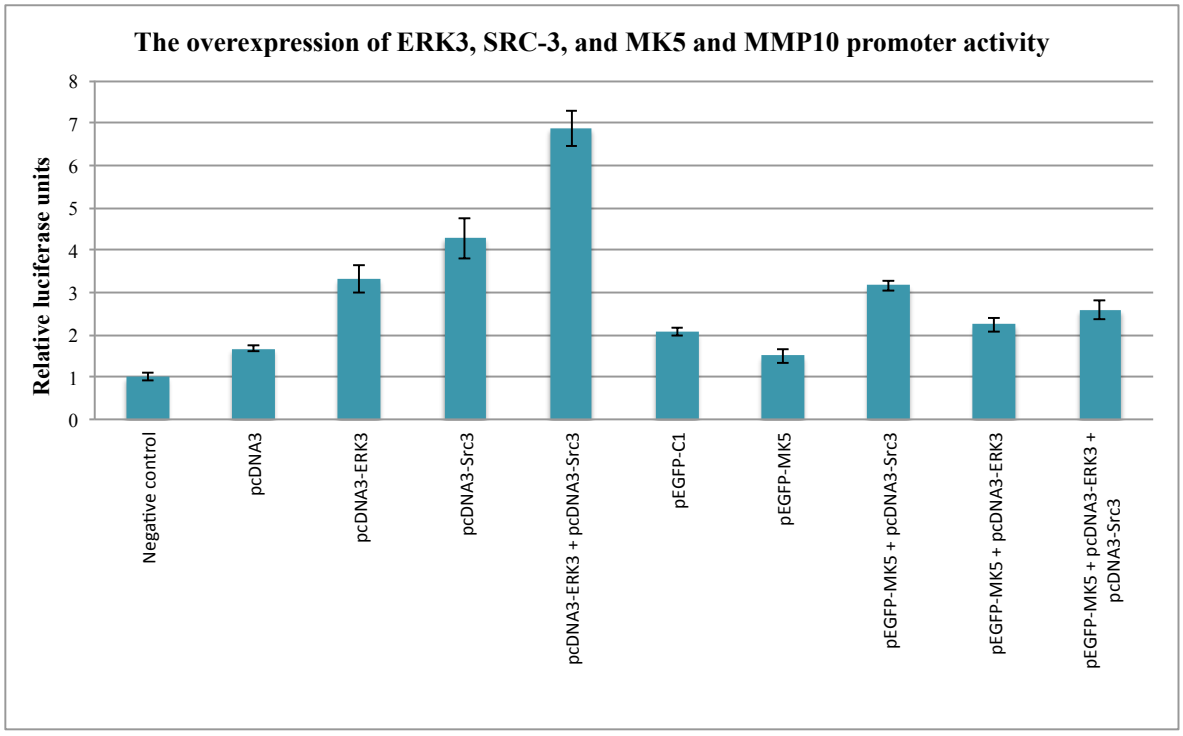


Figure 24: MMP10 promoter activity correlates to SRC-3 expression in A549 cells. 20K cells per well were cotransfected using Lipofectamine® 2000 Reagent, 24 hours after seeding, with pCH110, pGL3-basic MMP10 promoter in each well of the 24 well plate, and control plasmids pcDNA3 and pEGFP-C1. We tested pcDNA3-Src3, pcDNA3-ERK3, and pEGFP-MK5 separately or in combinations as assignated in graph. Luciferase assay was conducted the following day, and the signals were measured in a Luminiscan RT. Luciferase actvity signal was divided by β-galactosidase for normalizing with regard to transfection efficacy ± Coefficient of variation. Sample size: n=3 (Appendix no.11)

5. The siRNA knockdown of MK5 and ERK3 genes decreases the promoter activity of MMP2, MMP9 and MMP10 promoters

For further examining of MK5 and ERK3 and the regulation of MMP promoter activity, we planned new series of luciferase assays measure the activity of MMP2, MMP9, and MMP10 promoters. This time we decided use the siRNA knockdown technique in order to downregulate the expression of MK5 and ERK3. Afterwards, we investigate the influence on the previously mentioned promoters. In the very beginning we wanted to confirm that this downregulation of MK5 and ERK3 is taking place, so we conducted Western blot assays checking for protein expression.

5.1. Confirmation of siRNA knockdown of ERK3 and MK5 in Western blot

Figure 25 is showing that ERK3 protein (at 84 kDa) is absent when treating cells with siERK3 without or with siMK5. ERK3 protein expression is declined when MK5 gene is knocked down as well. ERK3 protein is expressed in non-treated cell and in negative control siRNA cells. In Figure 26, we have the MK5 protein (at 54 kDa) Western blot result. The MK5 protein expression was knocked down in both cells transfected with siMK5 alone, and in combination with siERK3. On the other hand, MK5 protein was still expressed in non-treated cells, siNC cells, and siERK3 treated cells.

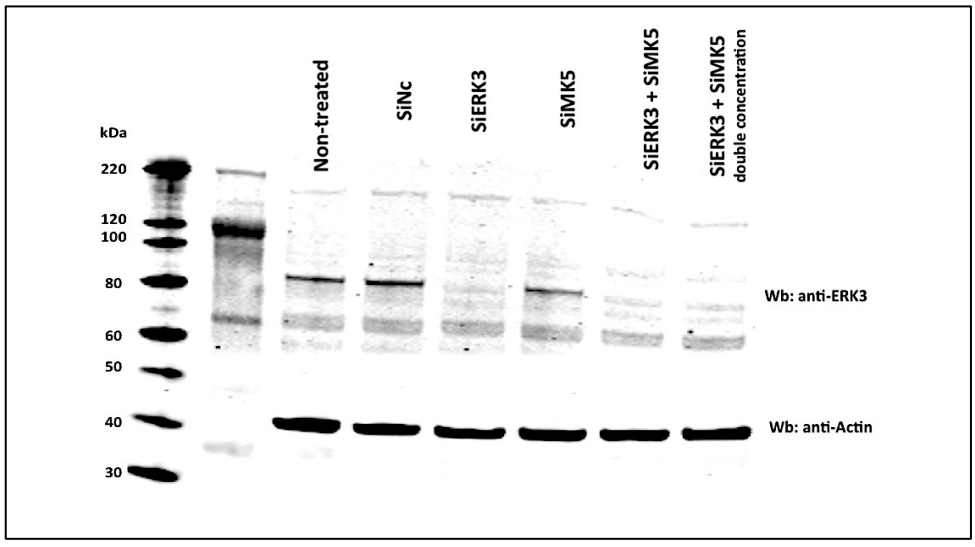


Figure 25: Successful siRNA knockdown of ERK3 in Western blot on A549 cells. 100K cells per well seeded and transfected the next day with 20 nM siRNA of negative control siNC, siERK3, siMK5, and combination siERK3 and siMK5 in two concentrations 10 nM and 20 nM. Cell lysates from each sample was run in SDS-PAGE gel, and the blot was analyzed in Western blotting. Here incubating with anti-ERK3 primary antibodies (1:500), and anti-mouse secondary antibodies Donkey IRDye 800 CW (1:10000). The same blot, in the bottom, was re-used and incubated with anti-actin primary antibodies (1:1000), and anti-rabbit secondary antibodies Donkey IRDye 800 CW (1:10000). MagicMark™ XP Western Protein from Invitrogen was used for band identification.

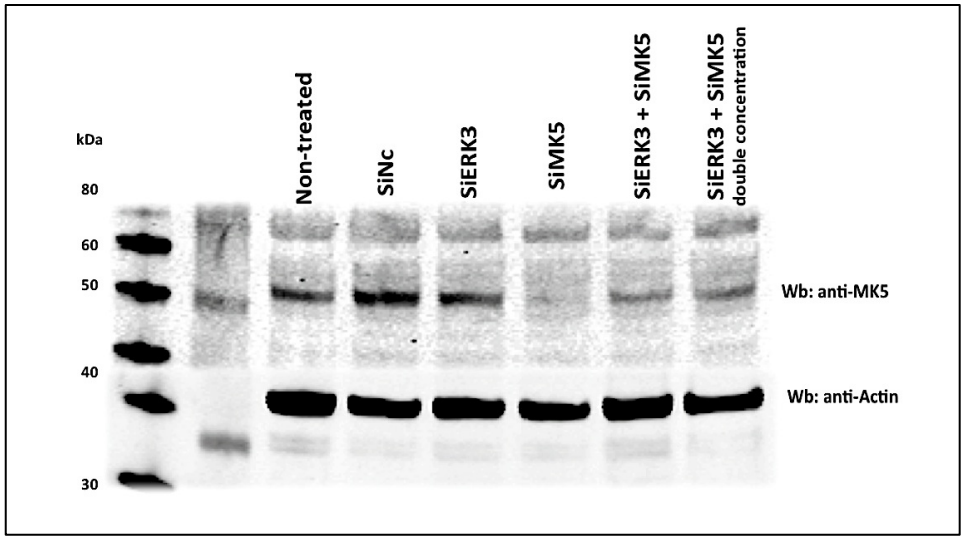


Figure 26: Successful siRNA knockdown of MK5 in Western blot on A549 cells. 100K cells per well seeded and transfected the next day with 20 nM siRNA of negative control siNC, siERK3, siMK5, and combination siERK3 and siMK5 in two concentrations 10 nM and 20 nM. Cell lysates from each sample was run in SDS-PAGE gel, and the blot was analyzed in Western blotting. Here incubating with anti-MK5 A7 primary antibodies (1:500), and anti-mouse secondary antibodies Donkey IRDye 800 CW (1:10000). The same blot, in the bottom, was re-used and incubated with anti-actin primary antibodies (1:1000), and anti-rabbit secondary antibodies Donkey IRDye 800 CW (1:10000). MagicMark™ XP Western Protein from Invitrogen was used for band identification.

5.2. The siRNA-facilitated knockdown of ERK3 and MK5 is influencing MMP promoter activity

In order to examine the effect of the siERK3 and siMK5 on MMP gene promoter activity, a series of luciferase assays were carried out as shown in Figure 27.

The MMP2, MMP9, and MMP10 promoter activity declines in cells treated with siERK3, more than with siMK5. Further decrease was observed when the combination siERK3 and siMK5 was applied.

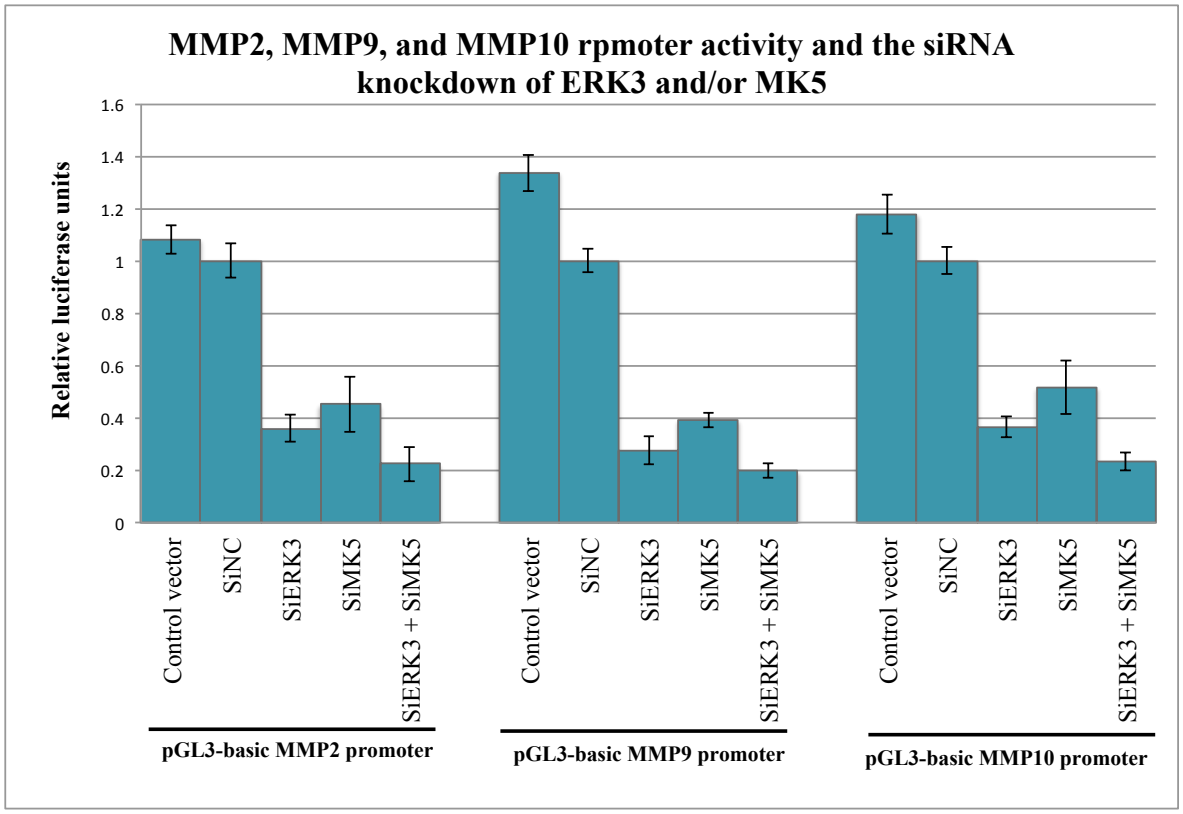


Figure 27: MMP2, MMP9, and MMP10 promoter activity declined with ERK3 and MK5 depletion in A549 cells. 20K cells per well were cotransfected using Lipofectamine® 2000 Reagent, 24 hours after seeding with, pCH110, either pGL3-basic MMP2 promoter, pGL3-basic MMP9 promoter, or pGL3-basic MMP10 promoter in each well of the 24 well plate, and 20nM siRNA of negative control siNC, siERK3, siMK5, and 10nM of combination siERK3 and siMK5 as assignated in graph. Luciferase assay was conducted 48 hours after transfection, and the signals were measured in a Luminiscan RT. Luciferase actvity signal was divided by β-galactosidase for normalizing with regard to transfection efficacy ± coefficient of variation. Sample size: n=3 (Appendix no.12-14)

6. The shRNA knockdown of ERK3 and MK5 in H1299 cells reduces mRNA levels of MMP2 and MMP9

A step further has been taken in order to verify the connection between ERK3, MK5 and MMP genes expression. This time by measuring mRNA level from cells with stable knockdown of ERK3 or MK5. In this assay we used the qRT-PCR technology to screen for ERK3, MK5, MMP2, and MMP9 mRNA expressions in a quantitative manner. Successful knockdown of both MK5 and ERK3 protein expression in H1299 cells was also confirmed by Western blotting as in figure 28B. The results displayed in Figure 28A show that both endogenous MMP2 and MMP9 mRNA expressions have significantly decreased by the knockdown of MK5 and ERK3.

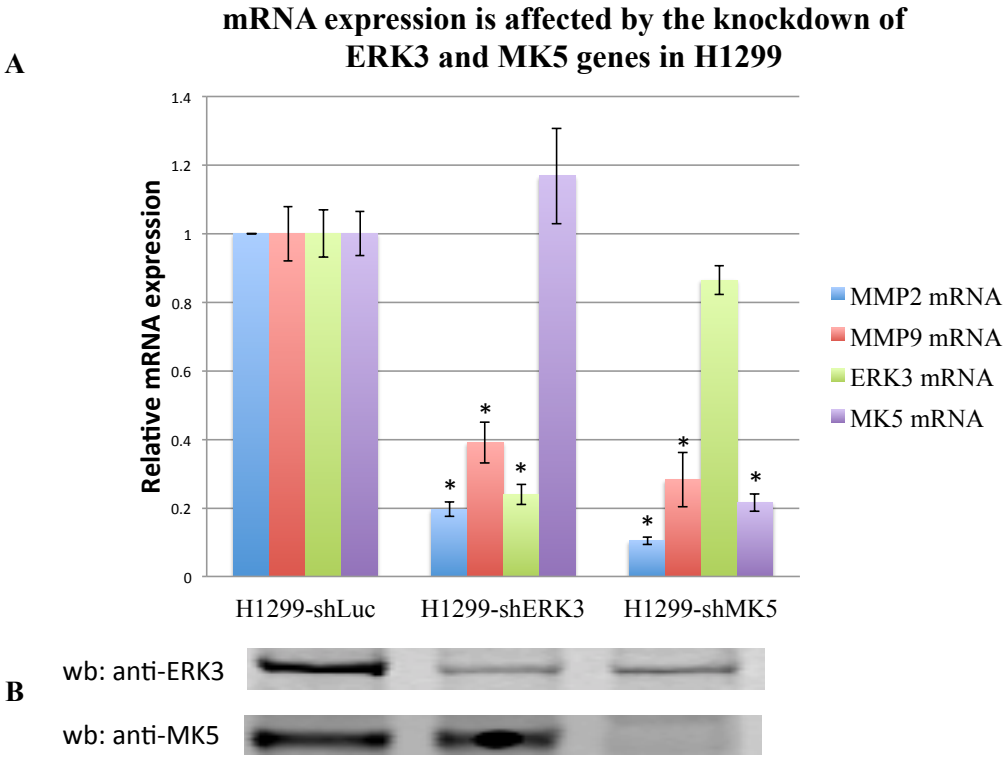


Figure 28: mRNA expression of MMP2 and MMP9 is decreased in qRT-PCR in shERK3 and shMK5 knockdown H1299 cells. A) 150K cells were seeded per well in 6 well plates, and RNA extracted after 24 hours. A reverse transcriptase PCR reaction was run using 100 ng of RNA. Then qPCR reaction was carried out using corresponding primers and 1 µl of cDNA template per 10 µl of total mix volume. The Ct values measured in the Mx3000P qPCR System were exported by MxPro® qPCR Software and analyzed in Excel sheets. The fold change in relative expression is illustrated in the graph ± SEM. (Appendix no. 15). Sample size n=2. * Significantly different from control cell line, $p < 0.05$ (student´s *t*-test).

B) 1500K of H1299-shRNA cells per well were seeded in 9 cm cell culture dishes, and harvested after 24 hours. Protein concentration was measured in each sample, and a standardized protein amount was run in SDS-PAGE gel, and afterwards in Western blotting. The blot on top is incubated anti-ERK3 primary antibodies (1:500), and anti-mouse secondary antibodies Donkey IRDye 800 CW (1:10000). The blot on bottom was incubated in anti-MK5 A7 primary antibodies (1:500), and then anti-mouse secondary antibodies Donkey IRDye 800 CW (1:10000).

7. H1299 cells proliferation ability is affected by the shRNA knockdown of ERK3 and MK5

Many studies have linked the MMPs expression with invasiveness and tumorigenesis. We wished to investigate whether the depletion of ERK3 and MK5 expression has an influence on proliferative abilities of shRNA-mediated knockdown cells, so a colonogenic assay was conducted. This simple and convenient approach was used on various H1299 cell lines, and the results of this assay were visible cells that were counted and analyzed as presented in figure 29. The MK5 knockdown cells proliferation was significantly inhibited compared to wild type cells when seeded 400 cells per well. For this comparison the two-sample student´s *t*-test was used for comparing means. At the same time we find significant differences between cell lines seeded 200 cells or more per well, when differences between and within groups' means are analyzed with one-way ANOVA. Taken all together, the depletion of MK5 in H1299 cells might affect the proliferation of H1299 cells when seeded in higher densities.

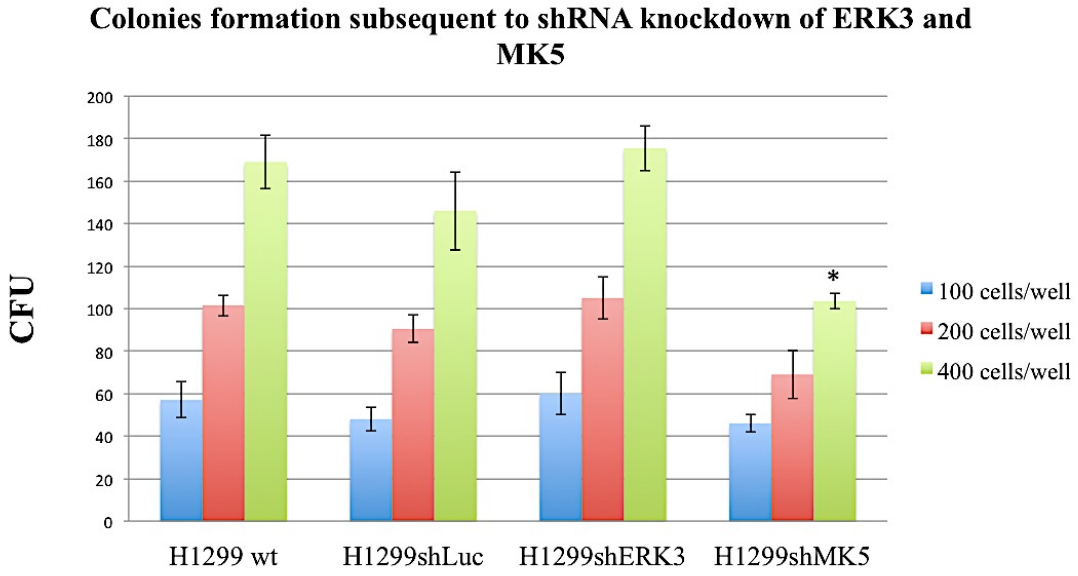


Figure 29: The proliferation ability of H1299 cells has declined when cells are knocked down using shMK5. 100, 200, and 400 cells per well, in two parallels, were seeded in 6 well plates. Plates was cultured with 0.1% or 10 % FBS in media. Cells were grown for 10 days, with medium replacement every three days. After 10 days, cells were washed once with PBS, and then stained with 350µl of colonogenic reagent, and visible cells colonies were counted ± SEM. (Appendix 16-17). Sample size n=2. * Significantly different from control wild type cell line, $p < 0.01$ (student´s *t*-test). CFU: Colony forming units.

8. The migration ability of H1299 cells and the knockdown of MK5 and ERK3

The aim of this assay is to investigate whether MK5 has an additional effect on carcinogenic features in H1299 cells. In this context, the migration ability has been qualitatively measured by using scratch (wound healing) assay. Cells are seeded in high density, so they are confluent when wound is made. The wound healing is then followed in a predetermined time term.

Results from this assay consist of baseline pictures and pictures taken 28 hours later at the same spot, as shown in Figures 30-31. It was challenging trying to catch overall tendencies of migration behavior in H1299-shRNA cells when addressing the pictures. In Figure 30, all cell lines seem to have migrated equally towards closing the wound. While in Figure 31, there are indications pointing out the knockdown of MK5 decreased H1299 cell migration compared to ERK3 knockdown and wild type cells.

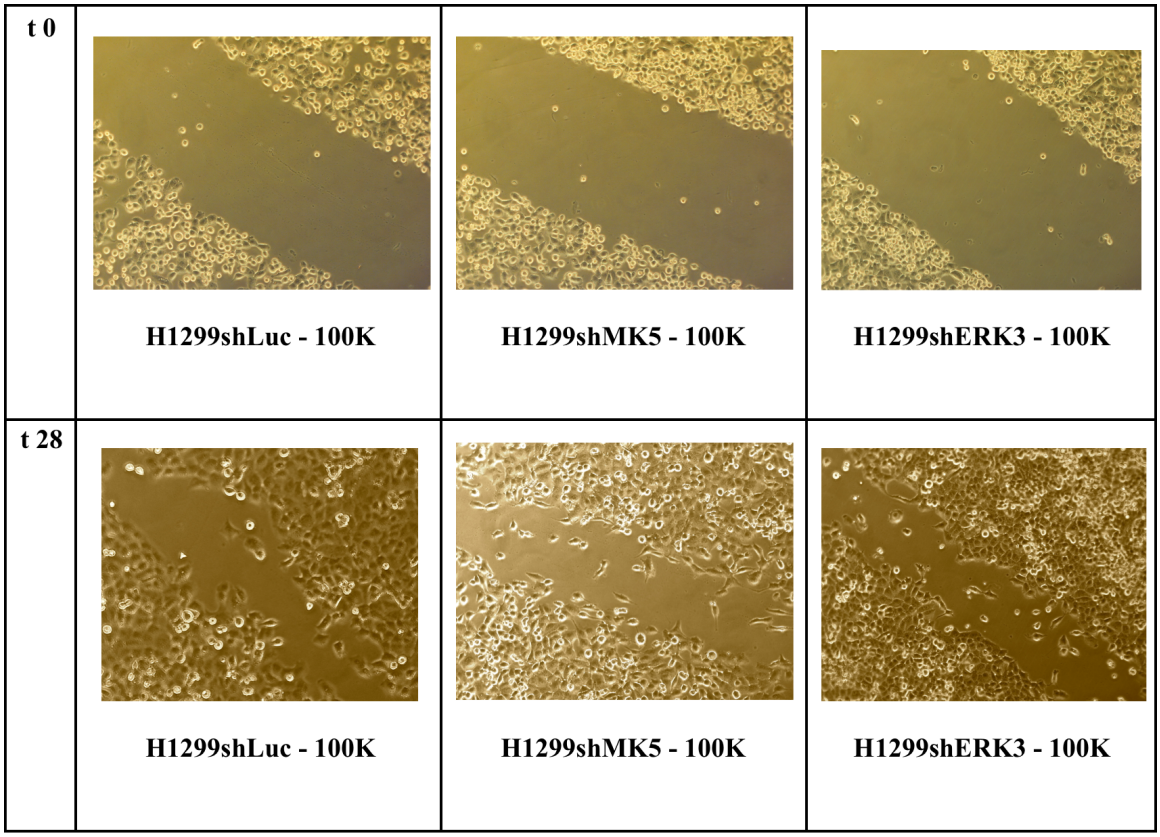


Figure 30: The migration ability of H1299 cells is reduced by shMK5 knockdown. 100K of H1299-shRNA cells were seeded in a 6 well plate for 22 hours, before a scratch was created in the middle of the well, and photos were taken (t 0). After 28 hours new photos were taken (t 28) using Zeiss AxioVert S 100, with DS-L1 Nikon camera.

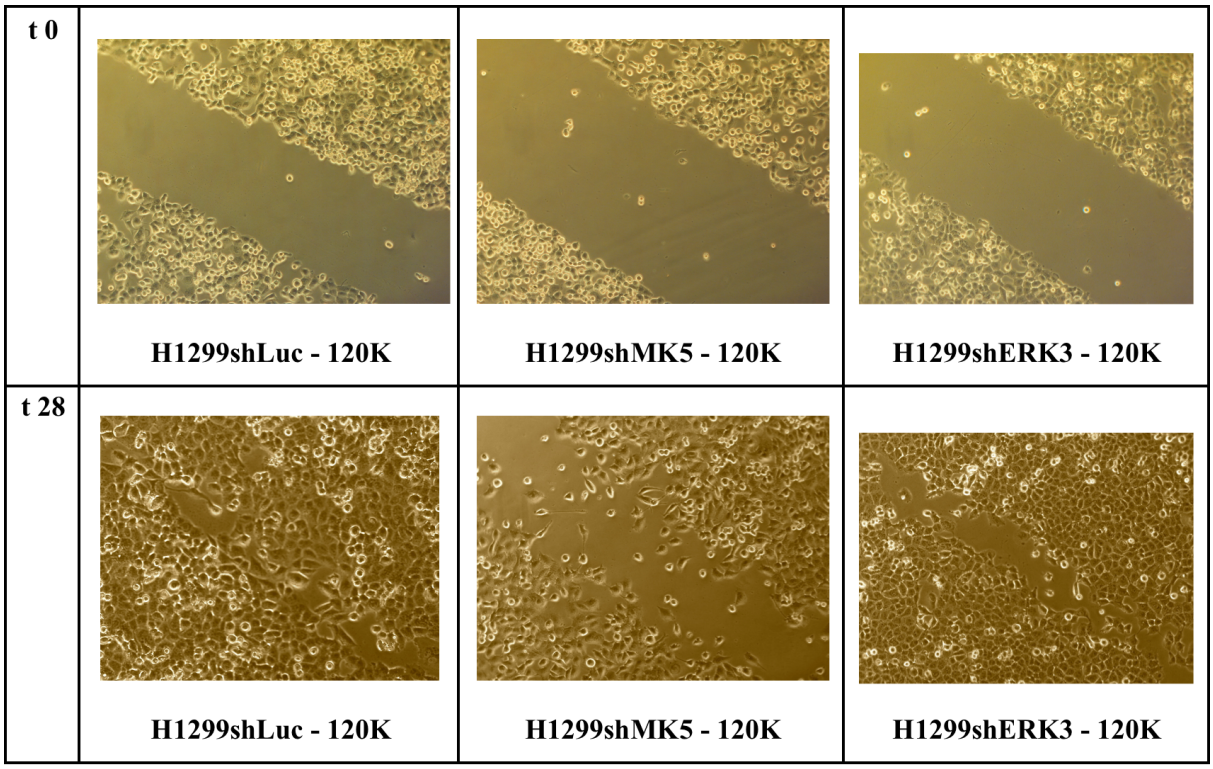


Figure 31: The migration ability of H1299 cells is reduced by shMK5 knockdown. 120K of H1299-shRNA cells were seeded in a 6 well plate for 22 hours, before a scratch was created in the middle of the well, and photos were taken (t 0). After 28 hours new photos were taken (t 28) using Zeiss AxioVert S 100, with DS-L1 Nikon camera.

DISCUSSION

SRC-3 is an oncogenic protein overexpressed in diverse human cancers. This nuclear receptor coactivator has been found to be involved in cellular aspects by acting through many pathways and mechanisms. For instance, it has been reported that SRC-3 has a role as cell cycle regulator, and when overexpressed, SRC-3 promotes cell proliferation. Additionally, SRC-3 is thought to regulate the expression of MMP genes through the coactivation of the transcription factors AP-1 and PEA3. It is established that MMPs help the breakdown of the extracellular matrix, facilitating tumor cell invasion into stromal compartment. SRC-3 has been linked to the activation of focal adhesion kinase, enabling cell motility. Furthermore, SRC-3 has been characterized in many studies as an anti-apoptotic protein. It is also speculated that SRC-3 has roles in inflammation and angiogenesis. Overall, molecular and clinical studies are suggesting that SRC-3 might have prognostic roles that awaken researchers attention. [36]

It is confirmed that SRC-3 undergoes post-translational modifications (PTM) that acquires SRC-3 several functions. The PTMs arise subsequent to extracellular stimuli, such as hormones, growth factors, and cytokines. These stimuli induce signaling pathways, which result in SRC-3 acetylation, dephosphorylation, methylation, phosphorylation, ubiquitinylation, or SUMOylation. So far, up to eight specific and functionally important phosphorylation sites in SRC-3 have been identified.

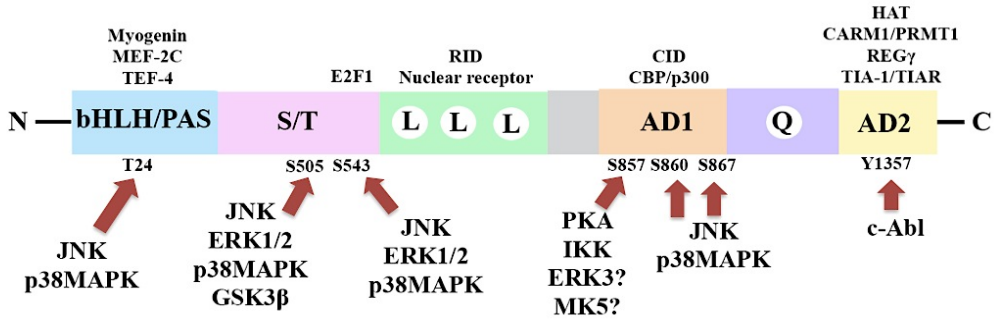


Figure 32: Structural domains and phosphorylation sites of SRC-3. There are seven Ser/Thr phosphorylation sites: Thr24, Ser 505, Ser 543, Ser 601 (not shown in figure), Ser 857, Ser 860, and Ser 867, and one Tyr phosphorylation site: Tyr 1357. Designated phosphor-donors are illustrated. AD1/2: Activation domains. CID: CBP/p300 interacting domain. S/T: Ser/Thr-rich region. bHLH: Basic helix-loop-helix domain. L: LXLL α-helix motif. PAS: Per/ARNT/Sim homologous domain. Q: Gln-rich domain. RID: Receptor interacting domain. Figure modified from Tien and Xu, 2012

There are several kinases phosphorylating these sites such as MAPKs, IKK, GSK3a, GSK3b, and CK1d. SRC-3 functions as a substrate for ABL tyrosine kinase, which can be activated by estrogen and growth factors. ABL tyrosine kinase phosphorylates SRC-3 at Tyr1357,

resulting in increased SRC-3 binding to p300 and transcription factors, hence mediating ER, PR, and NF-kB-dependent transcription activities.[36] SRC-3 phosphorylation at Tyr1357 is observed after treatment with insulin-like growth factor 1, epidermal growth factor, or estrogen in breast cancer cells, and seems to function as a molecular on/off switch facilitating the cross talk between hormone, growth factor, and intracellular kinase signaling pathways in cancer. [55] It was suggested that SRC-3 is phosphorylated at Ser857 by IKK (affecting apoptosis) and by PKA. [56] Long et al (2012) has reported that SRC-3 is phosphorylated by ERK3 at Ser857 promoting the interaction of SRC-3 with PEA3, which in turn induces the upregulation of MMP gene expression and invasion in lung cancer cells.

The significance of SRC-3 phosphorylation lies in its conversion from an inactive protein into a potent transcriptional coactivator, influencing different gene expressions and cellular physiology and causing diseases such as cancer. [36]

1. MK5 phosphorylates SRC-3 at Ser857 residue

We have demonstrated that SRC-3 is a good *in vitro* phosphorylation target for MK5 phosphorylation at Ser857. In addition, and as mentioned above, it was proposed by Long et al, 2012, that ERK3 is kinase responsible for SRC-3 in the same residue.

Taken into account the nature of ERK3-MK5 relationship, we find many interesting aspects. ERK3, with its 30 min half-life, [57] is regulated by protein stability and MK5-chaperoning. Hence, MK5 is engaged in ERK3 stability and regulation. At the same time MK5 has been defined as a downstream substrate for ERK3 as demonstrated by Seternes et al.[42] Later on, Perander et al verified that MK5 and ERK3 mutually phosphorylate each other.[29]

Based on an observation suggesting that the residues neighboring Ser857 within the CID region of SRC-3, it is indicated that this phosphorylation site might be a MK5 and not a MAP kinase site.

Taken all together, the findings above provide a comprehensive theory stating that MK5 phosphorylates SRC-3 at Ser857 directly, on the other hand, ERK3 seemed to phosphorylate SRC-3, but this may actually occur through MK5 activation (Figure 33).

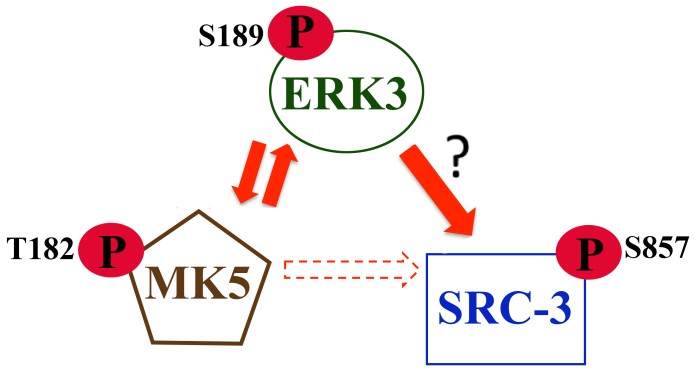


Figure 33: Suggested phosphorylation pathways involving ERK3, MK5, and SRC-3. It is suggested in this study that Src-3 is phosphorylated by MK5. However ERK3 is binding and activating MK5 directly, and this account for further SRC-3 phosphorylation by ERK3 via MK5. We stand unconvinced by the suggested direct phosphorylation of SRC-3 by ERK3

To determine which region of SRC-3 is phosphorylated by MK5, recombinant fusion protein fragments of GST-SRC-3 CID can be expressed and purified as series, which tested with GST as control against active MK5 and MK5 kinase dead proteins in a kinase assay detected by autoradiography. This will help verifying that kinase active MK5 phosphorylates SRC-3 at which region. Afterwards, the *in vitro* phosphorylation of SRC-3 by MK5 needs to be taken a step ahead, by performing a kinase assay in cells to verify whether the same reaction takes place in living systems.

Further research concerning the possible interaction between MK5 and SRC-3 needs to be addressed by using techniques such as immunoprecipitation-Western blot analysis, and protein-protein pull-down assay

2. Testing different conditions and concentrations of primary antibodies

When we tested the antibodies in Western blot for the second time, we got the results shown is Figure 13B and C. It was a good sign that a band around 52 kDa appeared, nevertheless, some other bands raised our concern. After investigating the membrane blot, and the nature of our GST-tagged protein; GST-SRC-3 CID, we thought that these bands represent protein fragments.

Taking into consideration that the purified GST-SRC-3 CID protein is phosphorylated at Ser857, this phosphorylation site is thought to be localized at the most central part of whole protein structure, as illustrated in Figure 34. This means that any fragmentation will probably result in many detectable fragments that contain the phosphorylated Ser857.

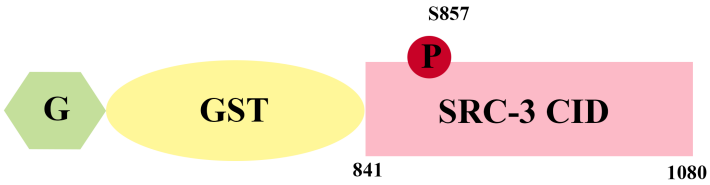


Figure 34: Illustration over GST-SRC3 CID protein structure. The phosphorylation site Ser857 of SRC-3 CID is shown, as well as the proportional GST protein. G: Glutathione unit.

In order to verify this point, we suggest incubating the same membrane with anti-GST antibodies after washing secondary antibodies. This will reveal if GST-containing fragments are present in the blot.

3. SRC-3 and ERK3 enhance of MMP promoter-driven luciferase activity

It has been demonstrated; a couple of times in this study, that SRC-3 overexpression induced MMP2 promoter activity, as well as MMP9 and MMP10 promoters' activity. These findings are in concordance with luciferase assay results described by Long et al.[35] Furthermore, the co-overexpression of SRC-3 and ERK3 remarkably increased MMP2, MMP9, and MMP10 promoters' activity.

The connection between SRC-3 and some MMP genes has been investigated extensively recently. A study by Yan et al. concluded that SRC-3 coactivates the transcription factors AP-1 and PEA3, by which regulating the transcription of MMP2 and MMP13.[58] The same conclusion was partially confirmed in Long et al,[35] stating that SRC-3 phosphorylation is upregulating the MMP gene expression through influencing the ETS transcription factor PEA3, which in turn increases the proinvasive activity in lung cancer cells.

So, with regard to the proposed phosphorylation above, it is interesting to see what effect overexpression of the proteins ERK3, and MK5 have on MMP genes.

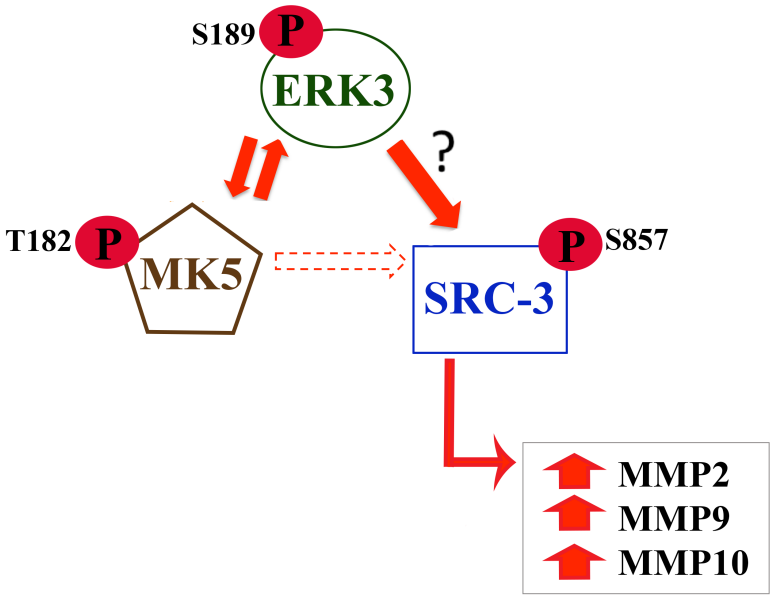


Figure 35: Does ERK3-MK5-SRC-3 cascade have an impact on MMPs gene expression? Given the importance of MMPs in cancer progression and development, any factor upregulating them might influence carcinogenesis as well. Further research in this context remains to be done.

In our experiments we repeatedly observed that ERK3 has functioned as an inducer of MMP2, MMP9, and MMP10 promoters. On the contrary, MK5 remarkably enhanced only MMP9 promoter, having too modest effect on the other two promoters.

The contradiction in these findings suggests that this approach remains to be optimized in order to use it for comparison in this cell system.

Another interesting finding is that a sign of saturation is occurred when we co-transfect with several plasmids, more than 3 at a time. The saturation might occur in transfection liposome system if plasmid concentrations are too high for the carrier capacity. On the other hand, a room opens for competition on transcription machinery as new DNA materials are introduced to cells. As a consequence, some plasmids are transcribed more than other plasmids.

From another point of view, there are many signaling pathways, transcriptional factors, and gene networking affecting a single gene expression endogenously. At the same time a gene undergoes many regulatory processes, for instance under its transcription, post-transcriptional, and when its product protein is translated. While a luciferase system is representing a substitution system, measuring only transcriptional activity that doesn´t necessarily take place in living cells.

4. The siRNA knockdown of MK5 and ERK3 genes reduces the promoter activity of MMP2, MMP9 and MMP10 promoters in A549 cells

In line with Long et al, the siRNA-mediated depletion of ERK3 decreased the activity of the three MMP promoters. Interestingly, the MK5 depletion has also affected the promoters activity negatively, but not in the same manner as ERK3 knockdown.

Further decrease was observed when the combination siERK3 and siMK5 was applied. On the other hand, and in the accompanying Western blot pictures we can see that when this combination is applied ERK3 protein expression totally diminished, while MK5 protein is still detectable, leading to some traces of MK5 activity with unknown effect on MMP genes.

5. The shRNA knockdown of MK5 and ERK3 decreased MMP2 and MMP9 mRNA expression in H1299 cells

What is interesting here is that mRNA level of MK5 in H1299-shMK5 cells is decreased even more that ERK3 mRNA in H1299-shERK3. This correlates with protein expression findings in Western blot as illustrated in Figure 28B. We see that MK5 protein is totally absent in H1299-shMK5 cell lysate; at the same time ERK3 protein has just decreased in H1299-shERK3 cell lysate.

The results suggest that MK5 is involved in the regulation of MMP genes in a more extensive manner than ERK3 at the first sight. However, when we take closer look at the Western blots, we see that MK5 protein expression has totally diminished, while ERK3 protein expression has just declined after shRNA knockdown treatment. Having some ERK3 activity left in the H1299-shERK3 cells might influence the results of this experiment. Therefore, the comparison between some ERK3 presence and the absence of MK5 can´t allow us to deduce a general conclusion from this assay. Nevertheless, by confirming the absence of MK5, we could state that this has significantly reduced mRNA level of MMP2 and MMP9.

This ERK3/MK5 phenomenon is interesting, as it has been previously discussed that MK5 stabilizes ERK3 (first described in Seternes et al[42] and in Schumacher et al [30]). Accordingly, when ERK3 is knocked out, there will be still some basal MK5 activity. On the contrary, MK5 knockdown leads to distinguished decrease in ERK3 expression.

Nonetheless, it is also possible that the retroviral transduction knockdown by facilitating shRNA of MK5 was not as efficient as shRNA knockdown of ERK3.

The MMPs have substantial roles in carcinogenesis promoting angiogenesis, migration, invasion and metastasis. Accordingly, decreased MMPs expression suppresses carcinogenesis.

In this context, the speculated MMP expression diminishing subsequent to MK5 knockdown signifies the clinical importance of MK5, and suggests that MK5 should be considered for therapeutic targeting.

6. MK5 and ERK3 affecting cancer cell proliferation

The MK5 influence on cell proliferation was discussed in a previous study, concluding that MK5 gene knockout in MEF cells results in cell cycle arrest and proliferation inhibition.[59]

As it goes for our colonogenic assay, the number of parallels per cell line was not enough to empower the conclusion statistically. Therefore, we recommend seeding more wells of each parallel.

Interestingly, by utilizing the one-way ANOVA, we get an indication of differences between and within groups, and in this context there are significant differences in cell proliferation when cells are seeded in densities higher than 200 cells per well. In order to have more accurate outcomes, student´s *t*-tests were run for multiple comparisons, yielding that MK5 shRNA knockdown cells seeded as 400 cells per well was assigned for significant inhibition of proliferation compared to wild type H1299 cells. While no significant differences were obtained when compared to H1299-shLuc control cells.

From Western blot results above, Figure 28B, it was confirmed that MK5 protein expression has totally diminished, while ERK3 protein expression has just declined after shRNA knockdown treatment. So by confirming the absence of MK5, we could state that shRNA-mediated knockdown of MK5 significantly reduced H1299 cell proliferation.

7. MK5 and ERK3 in cancer cell migration

Our results indicate that the knockdown of MK5 has a greater impact on H1299 lung cancer cell migration than the knockdown of ERK3.

However, more pictures should be taken manually between the baseline pictures and pictures taken 28 hours after wound creation, or by utilizing automatized picturing technology approaches (such as IncuCyte ZOOM by Essen BioScience®) that enable the quantification of migration.

Nevertheless, our finding suggests that MK5 depleted cells migrate the least towards closing the wound when seeded in high density (120 K cell per well) compared to ERK3 shRNA knockdown cells. Moreover, from the Western blot results (Figure 28B) we can state that shRNA-targeting MK5 decreases H1299 cell migration compared to control cells.

Tak et al have studied MK5´s effect on migration and reported that MK5 promotes HeLa cell migration by regulating actin cytoskeletal organization.[60] A recent study suggested that activated MK5 is involved in the mediating cell migration toward tumors, and the regulation, phosphorylation, and activation of focal adhesion kinase (FAK) and cytoskeletal reorganization. [61] Another suggested mechanism addressing MK5-mediated cell migration is F-actin rearrangement. [62]

Many migration and proliferation assays can be used with regard to studying MK5 knockdown in cells extensively. Two-chamber transwell migration assay is an alternative for migration studies in this system, while Thymidine incorporation assay can be used for quantifying cell proliferation ability.

8. MMPs overexpression and proinvasion in cancer cells

The critical functions MMPs have in invasion emphasize the importance of elucidating MK5 impact on MMPs expression. For example utilizing MK5 knockdown cell pools and transwell Matrigel cell invasion system could clarify the essence of MK5-MMPs connection with relevance to invasion.

In order to investigate whether MK5 has effect in invasion and colonization in larger living systems, an *in vivo* cell invasion assay in xenograft mouse model can be used with GFP-labeled and shRNA stably expressing cells.

CONCLUSION

This study shows very interesting outcomes. The main finding is that MK5, rather than ERK3, seems to phosphorylate SRC-3 *in vitro* at the Ser857. In addition, the study shows that MMP2, MMP9, and MMP10 promoters' activity diminishes by siRNA-facilitated knockdown of ERK3 and MK5 in A549 lung cancer cells. Moreover, MMP2 and MMP9 mRNA expressions decrease significantly when endogenous ERK3 and MK5 expressions are knocked down in H1299 cell lines that are stably expressing shRNAs. There are indications suggesting that MK5 might be influencing H1299 cell´s proliferation and migration.

FUTURE PERSPECTIVES

Comprehensive research is warranted to clarify the connection between MK5, ERK3, and SRC-3 in the phosphorylation cascade context, and to elucidate the effect this cascade might have on the MMPs and their expression.

Yet, much more work remains to be carried out for determining whether SRC-3 is a good downstream target of MK5 in cells and larger living systems. Another important question that needs to be addressed is, if there is a clinical significance in this interaction between SRC-3 and MK5 that potentiates the pathway as a therapeutic target for treatment.

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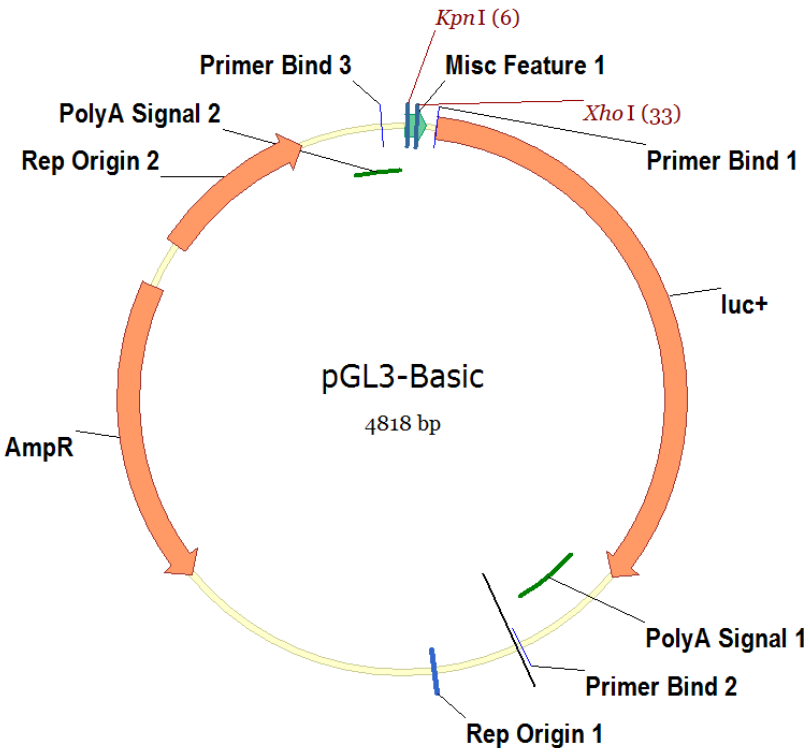
APPENDIX

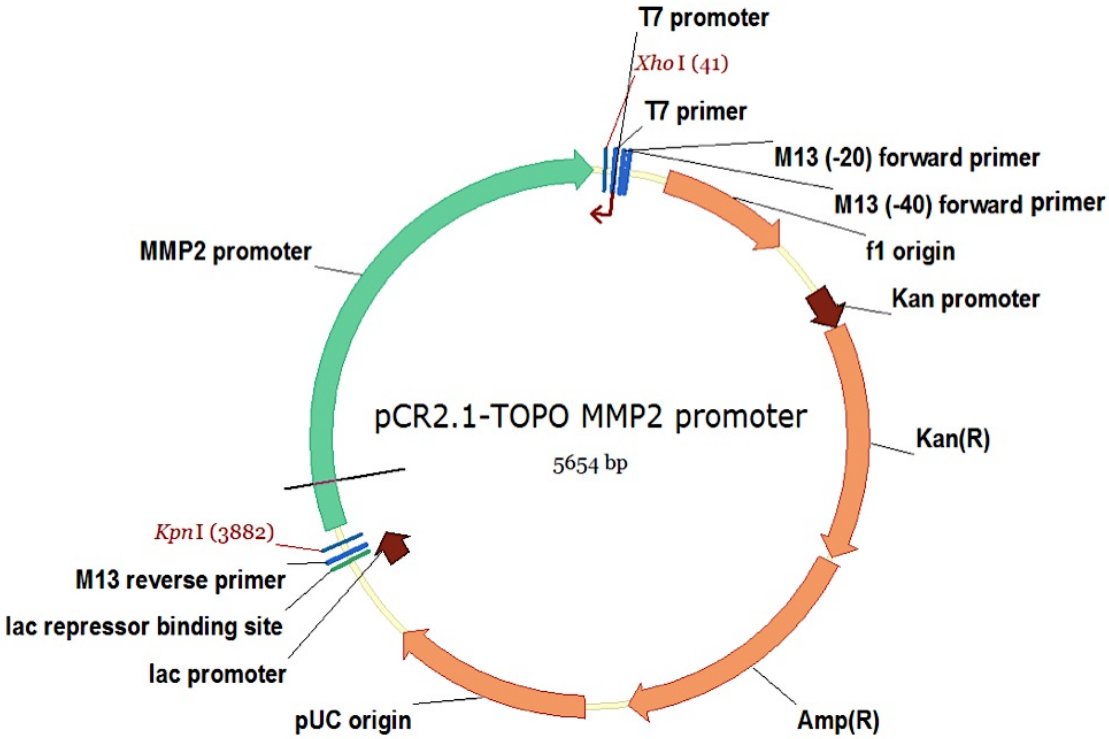
Appendix 1:

pGL3-basic plasmid map with KpnI and XhoI restriction sites on Vector NTI, Pharmacology research group, University of Tromsø



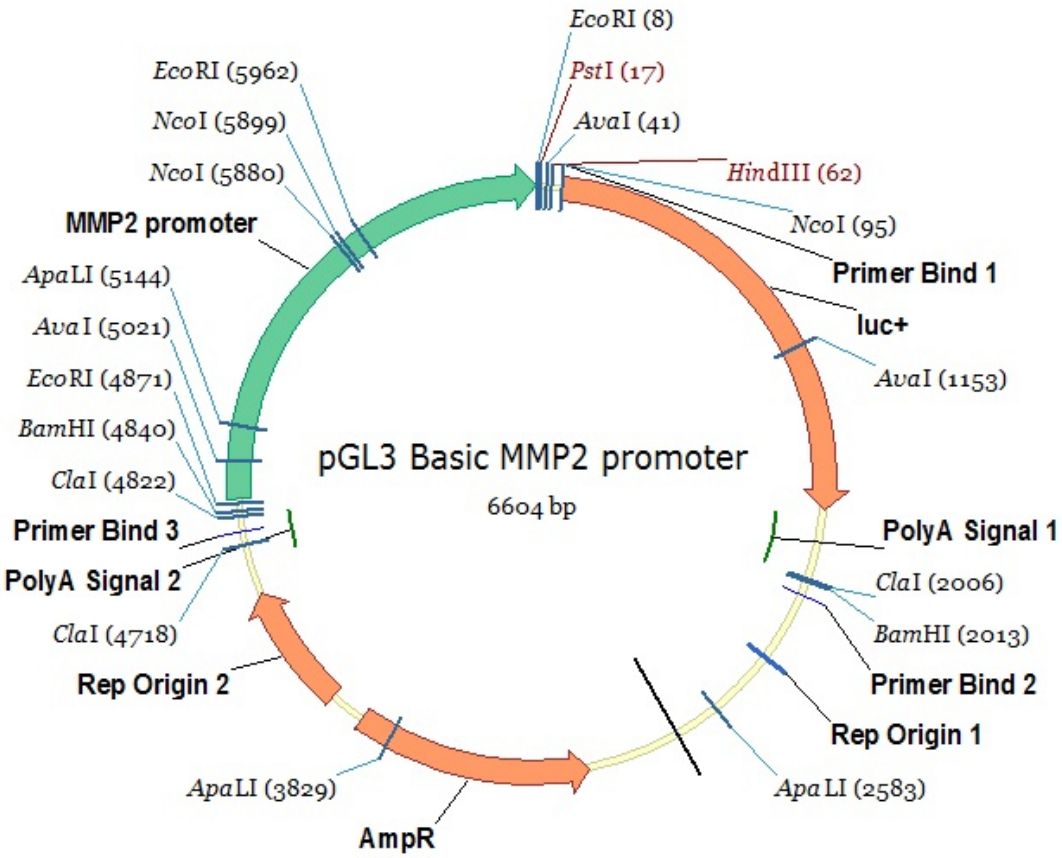
Appendix 2:

pCR®2.1-TOPO® MMP2-promoter plasmid map with KpnI and XhoI restriction sites on Vector NTI, Pharmacology research group, University of Tromsø



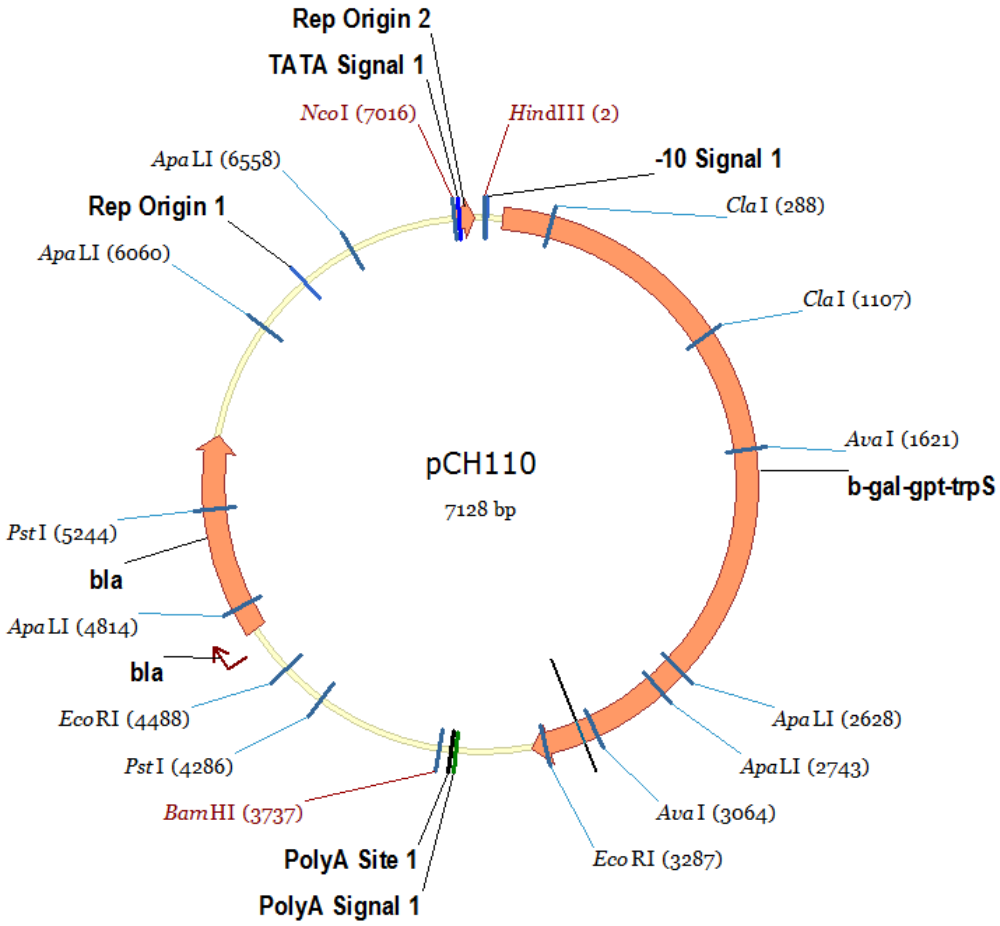
Appendix 3:

The constructed pGL3-basic MMP2 promoter plasmid map with some common restriction sites on Vector NTI, Pharmacology research group, University of Tromsø



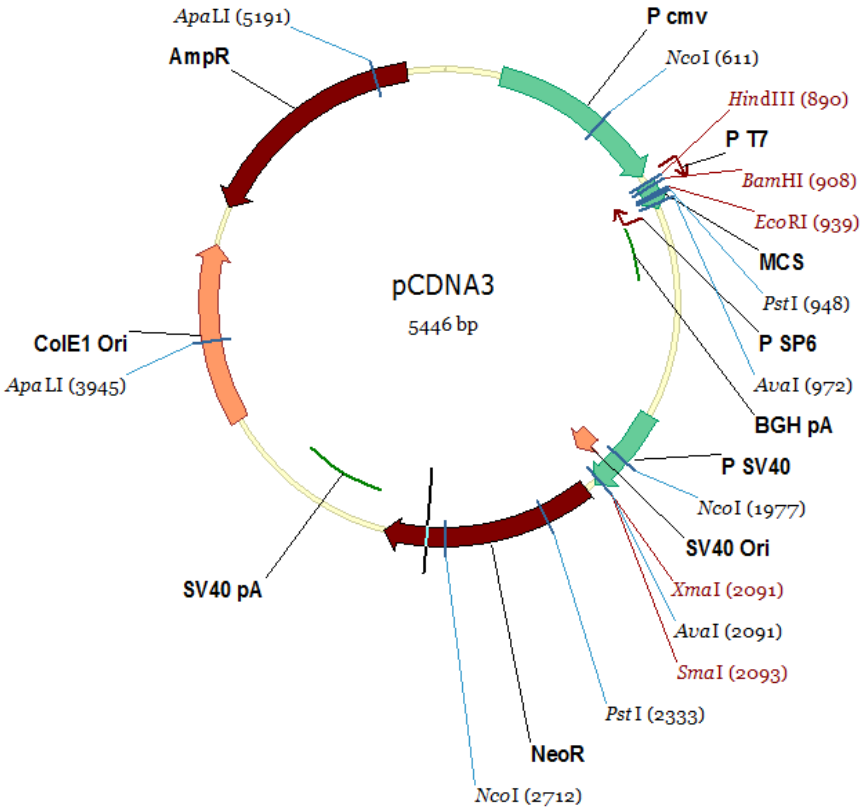
Appendix 4:

pCH110 plasmid map with some common restriction sites on Vector NTI, Pharmacology research group, University of Tromsø



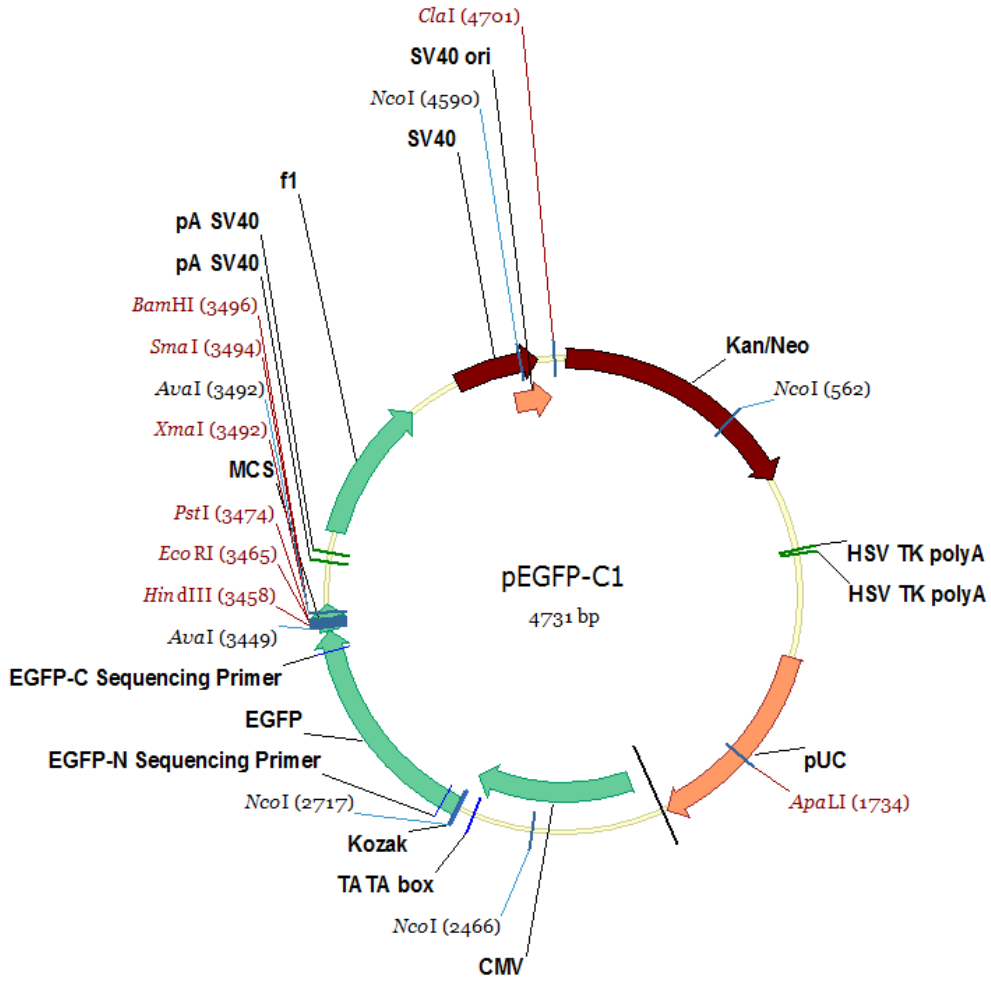
Appendix 5:

pcDNA3 plasmid map with some common restriction sites on Vector NTI, Pharmacology research group, University of Tromsø



Appendix 6:

pEGFP-C1 plasmid map with some common restriction sites on Vector NTI, Pharmacology research group, University of Tromsø



Appendix 7:

pGL3-basic MMP2 promoter activity is induced by the presence of dexamethasone stimulated Glucocorticoid receptor.

Measurement	Plasmids transfected	Parallels		
		1	2	3
Luciferase activity	Non-treated cells	0	0	0
	50 ng pCH110 + 200 ng pGL3-basic MMP2 promoter	18.78	12.53	9.331
	50 ng pCH110 + 200 ng pGL3-basic MMP2 promoter + 400 ng GR	6.264	5.312	7.121
	50 ng pCH110 + 200 ng pGL3-basic MMP2 promoter + 400 ng GR + 1mM dexamethasone	17.3	20.84	21.28
β-galactosidase activity	Non-treated cells	0	0	0
	50 ng pCH110 + 200 ng pGL3-basic MMP2 promoter	370.8	260.8	201.5
	50 ng pCH110 + 200 ng pGL3-basic MMP2 promoter + 400 ng GR	98.98	97.61	100.2
	50 ng pCH110 + 200 ng pGL3-basic MMP2 promoter + 400 ng GR + 1mM dexamethasone	69.49	80.99	77.88

Relative luciferase units	Parallels (Luc/ β-gal)			Average	Standard deviation	Fold change	Coefficient of variation
	1	2	3				
Non-treated cells	0	0	0	0	0	0	0
pCH110 + pGL3-basic MMP2 promoter	0.050647249	0.048044479	0.046307692	0.04833314	0.002184132	1	0.045189119
pCH110 + pGL3-basic MMP2 promoter + GR	0.063285512	0.054420654	0.071067864	0.062924677	0.008329469	1.30189507	0.172334535
pCH110 + pGL3-basic MMP2 promoter + GR + dexamethasone	0.248956684	0.257315718	0.273240883	0.259837762	0.012336981	5.375975198	0.255248911

Appendix 8:

MMP2 promoter activity is induced by the presence of SRC-3 in HeLa S3 cells.

Measurement	Plasmids transfected	Parallels		
		1	2	3
Luciferase activity	Non-treated cells	0	0	0
	50 ng pCH110 + 200 ng pGL3-basic MMP2 promoter + 200 ng pcDNA3	2.785	2.694	2.451
	50 ng pCH110 + 200 ng pGL3-basic MMP2 promoter + 200 ng pcDNA3-SRC-3	34.42	35.42	35.8
β-galactosidase activity	Non-treated cells	0.486	0.527	0.837
	50 ng pCH110 + 200 ng pGL3-basic MMP2 promoter + 200 ng pcDNA3	0.502	0.634	0.493
	50 ng pCH110 + 200 ng pGL3-basic MMP2 promoter + 200 ng pcDNA3-SRC-3	0.374	0.372	0.368

Relative luciferase units	Parallels (Luc/ β-gal)			Average	Standard deviation	Fold change	Coefficient of variation
	1	2	3				
Non-treated cells	0	0	0	0	0	0	0
pCH110 + pGL3-basic MMP2 promoter + pcDNA3	5.547808765	4.249211356	4.971602434	4.922874185	0.650668609	1	0.132172504
pCH110 + pGL3-basic MMP2 promoter + pcDNA3 SRC-3	92.03208556	95.21505376	97.2826087	94.84324934	2.644934251	19.26582841	0.537274395

Appendix 9:

MMP2 promoter activity is affected by the induction of ERK3, MK5, and Src3.

Measurement	Plasmids transfected	Parallels		
		1	2	3
Luciferase activity	50 ng pCH110 + 100 ng pGL3-basic MMP2 promoter	2.462	2.843	1.896
	50 ng pCH110 + 100 ng pGL3-basic MMP2 promoter + 100 ng pcDNA3	0.926	0.86	0.763
	50 ng pCH110 + 100 ng pGL3-basic MMP2 promoter + 100 ng pcDNA3-ERK3	5.405	3.278	4.296
	50 ng pCH110 + 100 ng pGL3-basic MMP2 promoter + 100 ng pcDNA3-SRC-3	5.278	5.558	4.146
	50 ng pCH110 + 100 ng pGL3-basic MMP2 promoter + 100 ng pcDNA3-ERK3 + 100 ng pcDNA3-SRC-3	5.605	6.158	5.82
	50 ng pCH110 + 100 ng pGL3-basic MMP2 promoter + 100 ng pEGFP-C1	2.021	2.228	1.975
	50 ng pCH110 + 100 ng pGL3-basic MMP2 promoter + 100 ng pEGFP-MK5	1.375	1.265	1.081
	50 ng pCH110 + 100 ng pGL3-basic MMP2 promoter + 100 ng pEGFP-MK5 + 100 ng pcDNA3-SRC-3	2.897	2.779	2.861
	50 ng pCH110 + 100 ng pGL3-basic MMP2 promoter + 100 ng pEGFP-MK5 + 100 ng pcDNA3-ERK3	1.384	1.575	1.4
	50 ng pCH110 + 100 ng pGL3-basic MMP2 promoter + 100 ng pEGFP-MK5 + 100 ng pcDNA3-ERK3 + 100 ng pcDNA3-SRC-3	1.325	1.371	1.613
β-galactosidase activity	50 ng pCH110 + 100 ng pGL3-basic MMP2 promoter	3.378	5.594	5.454
	50 ng pCH110 + 100 ng pGL3-basic MMP2 promoter + 100 ng pcDNA3	1.174	1.649	1.878
	50 ng pCH110 + 100 ng pGL3-basic MMP2 promoter + 100 ng pcDNA3-ERK3	1.841	1.97	2.365
	50 ng pCH110 + 100 ng pGL3-basic MMP2 promoter + 100 ng pcDNA3-SRC-3	1.42	1.162	1.068
	50 ng pCH110 + 100 ng pGL3-basic MMP2 promoter + 100 ng pcDNA3-ERK3 + 100 ng pcDNA3-SRC-3	1.059	1.275	0.974
	50 ng pCH110 + 100 ng pGL3-basic MMP2 promoter + 100 ng pEGFP-C1	1.842	2.26	2.29
	50 ng pCH110 + 100 ng pGL3-basic MMP2 promoter + 100 ng pEGFP-MK5	1.952	1.815	1.845
	50 ng pCH110 + 100 ng pGL3-basic MMP2 promoter + 100 ng pEGFP-MK5 + 100 ng pcDNA3-SRC-3	1.887	1.835	1.729
	50 ng pCH110 + 100 ng pGL3-basic MMP2 promoter + 100 ng pEGFP-MK5 + 100 ng pcDNA3-ERK3	1.835	1.871	1.936
	50 ng pCH110 + 100 ng pGL3-basic MMP2 promoter + 100 ng pEGFP-MK5 + 100 ng pcDNA3-ERK3 + 100 ng pcDNA3-SRC-3	1.997	2.026	1.856

Appendix 9 (continued)

Relative luciferase units	Parallels (Luc/ β-gal)			Average	Standard deviation	Fold change	Coefficient of variation
	1	2	3				
pCH110 + pGL3-basic MMP2 promoter	0.728833629	0.508223096	0.347634763	0.528230496	0.191385386	1	0.362314155
pCH110 + pGL3-basic MMP2 promoter + pcDNA3	0.788756388	0.521528199	0.40628328	0.572189289	0.196204814	1.08321896	0.371437878
pCH110 + pGL3-basic MMP2 promoter + pcDNA3-ERK3	2.9359044	1.663959391	1.816490486	2.138784759	0.6945259	4.048961152	1.314815983
pCH110 + pGL3-basic MMP2 promoter + pcDNA3-SRC-3	3.716901408	4.78313253	3.882022472	4.127352137	0.573892137	7.813543833	1.086442643
pCH110 + pGL3-basic MMP2 promoter + pcDNA3-ERK3 + pcDNA3-SRC-3	5.29272899	4.829803922	5.975359343	5.365964085	0.57627844	10.15837617	1.090960185
pCH110 + pGL3-basic MMP2 promoter + pEGFP-C1	1.097176982	0.985840708	0.862445415	0.981821035	0.117417398	1.858698128	0.222284399
pCH110 + pGL3-basic MMP2 promoter + pEGFP-MK5	0.704405738	0.696969697	0.585907859	0.662427765	0.066372401	1.254050588	0.125650453
pCH110 + pGL3-basic MMP2 promoter + pEGFP-MK5 + pcDNA3-SRC3	1.535241123	1.514441417	1.654713707	1.568132083	0.075699671	2.968651173	0.143308029
pCH110 + pGL3-basic MMP2 promoter +pEGFP-MK5 + pcDNA3-ERK3	0.754223433	0.841795831	0.723140496	0.773053253	0.061527992	1.463477135	0.116479438
pCH110 + pGL3-basic MMP2 promoter + pEGFP-MK5 + pcDNA3-ERK3 + pcDNA3-SRC-3	0.663495243	0.676702863	0.869073276	0.736423794	0.115067476	1.39413343	0.217835731

Appendix 10:

MMP9 promoter activity is affected by the induction of ERK3, MK5, and Src3.

Measurement	Plasmids transfected	Parallels		
		1	2	3
Luciferase activity	50 ng pCH110 + 100 ng pGL3-basic MMP9 promoter	0.106	0.116	0.118
	50 ng pCH110 + 100 ng pGL3-basic MMP9 promoter + 100 ng pcDNA3	0.048	0.059	0.064
	50 ng pCH110 + 100 ng pGL3-basic MMP9 promoter + 100 ng pcDNA3-ERK3	0.113	0.129	0.115
	50 ng pCH110 + 100 ng pGL3-basic MMP9 promoter + 100 ng pcDNA3-SRC-3	0.232	0.241	0.228
	50 ng pCH110 + 100 ng pGL3-basic MMP9 promoter + 100 ng pcDNA3-ERK3 + 100 ng pcDNA3-SRC-3	0.332	0.296	0.316
	50 ng pCH110 + 100 ng pGL3-basic MMP9 promoter + 100 ng pEGFP-C1	0.129	0.153	0.149
	50 ng pCH110 + 100 ng pGL3-basic MMP9 promoter + 100 ng pEGFP-MK5	0.279	0.271	0.26
	50 ng pCH110 + 100 ng pGL3-basic MMP9 promoter + 100 ng pEGFP-MK5 + 100 ng pcDNA3-SRC-3	0.26	0.209	0.23
	50 ng pCH110 + 100 ng pGL3-basic MMP9 promoter + 100 ng pEGFP-MK5 + 100 ng pcDNA3-ERK3	0.122	0.14	0.163
	50 ng pCH110 + 100 ng pGL3-basic MMP9 promoter + 100 ng pEGFP-MK5 + 100 ng pcDNA3-ERK3 + 100 ng pcDNA3-SRC-3	0.19	0.166	0.13
β-galactosidase activity	50 ng pCH110 + 100 ng pGL3-basic MMP9 promoter	4.167	3.808	4.192
	50 ng pCH110 + 100 ng pGL3-basic MMP9 promoter + 100 ng pcDNA3	1.789	1.829	1.761
	50 ng pCH110 + 100 ng pGL3-basic MMP9 promoter + 100 ng pcDNA3-ERK3	2.159	1.992	2.027
	50 ng pCH110 + 100 ng pGL3-basic MMP9 promoter + 100 ng pcDNA3-SRC-3	2.452	2.371	2.413
	50 ng pCH110 + 100 ng pGL3-basic MMP9 promoter + 100 ng pcDNA3-ERK3 + 100 ng pcDNA3-SRC-3	2.862	2.509	2.642
	50 ng pCH110 + 100 ng pGL3-basic MMP9 promoter + 100 ng pEGFP-C1	2.348	2.3	2.202
	50 ng pCH110 + 100 ng pGL3-basic MMP9 promoter + 100 ng pEGFP-MK5	1.93	1.902	2.079
	50 ng pCH110 + 100 ng pGL3-basic MMP9 promoter + 100 ng pEGFP-MK5 + 100 ng pcDNA3-SRC-3	2.11	1.879	1.976
	50 ng pCH110 + 100 ng pGL3-basic MMP9 promoter + 100 ng pEGFP-MK5 + 100 ng pcDNA3-ERK3	2.056	1.951	1.909
	50 ng pCH110 + 100 ng pGL3-basic MMP9 promoter + 100 ng pEGFP-MK5 + 100 ng pcDNA3-ERK3 + 100 ng pcDNA3-SRC-3	1.848	1.858	1.866

Appendix 10 (continued)

Relative luciferase units	Parallels (Luc/ β-gal)			Average	Standard deviation	Fold change	Coefficient of variation
	1	2	3				
pCH110 + pGL3-basic MMP9 promoter	0.025437965	0.030462185	0.028148855	0.028016335	0.00251473	1	0.089759425
pCH110 + pGL3-basic MMP9 promoter + pcDNA3	0.026830632	0.032258065	0.036342987	0.031810561	0.004771941	1.135429067	0.170327095
pCH110 + pGL3-basic MMP9 promoter + pcDNA3-ERK3	0.052339046	0.064759036	0.05673409	0.057944057	0.006297782	2.068224034	0.224789639
pCH110 + pGL3-basic MMP9 promoter + pcDNA3-SRC-3	0.094616639	0.101644876	0.094488189	0.096916568	0.004095338	3.459287885	0.146176797
pCH110 + pGL3-basic MMP9 promoter + pcDNA3-ERK3 + pcDNA3-SRC-3	0.116002795	0.117975289	0.119606359	0.117861481	0.001804475	4.206884351	0.064407978
pCH110 + pGL3-basic MMP9 promoter + pEGFP-C1	0.054940375	0.066521739	0.067665758	0.063042624	0.00704003	2.250209539	0.251283061
pCH110 + pGL3-basic MMP9 promoter + pEGFP-MK5	0.144559585	0.142481598	0.125060125	0.137367103	0.010708678	4.903107537	0.3822298
pCH110 + pGL3-basic MMP9 promoter + pEGFP-MK5 + pcDNA3-SRC-3	0.123222749	0.111229377	0.116396761	0.116949629	0.00601577	4.174337199	0.214723656
pCH110 + pGL3-basic MMP9 promoter +pEGFP-MK5 + pcDNA3-ERK3	0.059338521	0.071758073	0.085385018	0.072160538	0.013027912	2.575659439	0.465011279
pCH110 + pGL3-basic MMP9 promoter + pEGFP-MK5 + pcDNA3-ERK3 + pcDNA3-SRC-3	0.102813853	0.08934338	0.069667738	0.08727499	0.01666958	3.115146597	0.594995032

Appendix 11:

MMP10 promoter activity is affected by the induction of ERK3, MK5, and Src3.

Measurement	Plasmids transfected	Parallels		
		1	2	3
Luciferase activity	50 ng pCH110 + 100 ng pGL3-basic MMP10 promoter	2.833	2.331	3.158
	50 ng pCH110 + 100 ng pGL3-basic MMP10 promoter + 100 ng pcDNA3	1.541	1.459	1.53
	50 ng pCH110 + 100 ng pGL3-basic MMP10 promoter + 100 ng pcDNA3-ERK3	8	7.095	6.766
	50 ng pCH110 + 100 ng pGL3-basic MMP10 promoter + 100 ng pcDNA3-SRC-3	8.881	8.479	8.772
	50 ng pCH110 + 100 ng pGL3-basic MMP10 promoter + 100 ng pcDNA3-ERK3 +100 ng pcDNA3-SRC-3	8.113	7.404	7.955
	50 ng pCH110 + 100 ng pGL3-basic MMP10 promoter + 100 ng pEGFP-C1	2.238	2.557	2.182
	50 ng pCH110 + 100 ng pGL3-basic MMP10 promoter + 100 ng pEGFP-MK5	1.205	1.366	1.238
	50 ng pCH110 + 100 ng pGL3-basic MMP10 promoter + 100 ng pEGFP-MK5 + 100 ng pcDNA3-SRC-3	2.873	2.695	2.813
	50 ng pCH110 + 100 ng pGL3-basic MMP10 promoter + 100 ng pEGFP-MK5 + 100 ng pcDNA3-ERK3	2.097	2.211	1.82
	50 ng pCH110 + 100 ng pGL3-basic MMP10 promoter + 100 ng pEGFP-MK5 + 100 ng pcDNA3-ERK3 + 100 ng pcDNA3-SRC-3	2.158	2.476	2.039
β-galactosidase activity	50 ng pCH110 + 100 ng pGL3-basic MMP10 promoter	5.827	4.363	5.536
	50 ng pCH110 + 100 ng pGL3-basic MMP10 promoter + 100 ng pcDNA3	1.727	1.728	1.66
	50 ng pCH110 + 100 ng pGL3-basic MMP10 promoter + 100 ng pcDNA3-ERK3	4.087	4.367	4.027
	50 ng pCH110 + 100 ng pGL3-basic MMP10 promoter + 100 ng pcDNA3-SRC-3	4.197	3.309	4.121
	50 ng pCH110 + 100 ng pGL3-basic MMP10 promoter + 100 ng pcDNA3-ERK3 +100 ng pcDNA3-SRC-3	2.397	1.98	2.101
	50 ng pCH110 + 100 ng pGL3-basic MMP10 promoter + 100 ng pEGFP-C1	2.148	2.357	1.894
	50 ng pCH110 + 100 ng pGL3-basic MMP10 promoter + 100 ng pEGFP-MK5	1.747	1.585	1.495
	50 ng pCH110 + 100 ng pGL3-basic MMP10 promoter + 100 ng pEGFP-MK5 + 100 ng pcDNA3-SRC-3	1.662	1.589	1.762
	50 ng pCH110 + 100 ng pGL3-basic MMP10 promoter + 100 ng pEGFP-MK5 + 100 ng pcDNA3-ERK3	1.808	1.712	1.632
	50 ng pCH110 + 100 ng pGL3-basic MMP10 promoter + 100 ng pEGFP-MK5 + 100 ng pcDNA3-ERK3 + 100 ng pcDNA3-SRC-3	1.715	1.664	1.497

Appendix 11 (continued)

Relative luciferase units	Parallels (Luc/ β-gal)			Average	Standard deviation	Fold change	Coefficient of variation
	1	2	3				
pCH110 + pGL3-basic MMP10 promoter	0.486185001	0.534265414	0.570447977	0.530299464	0.042271253	1	0.079712042
pCH110 + pGL3-basic MMP10 promoter + pcDNA3	0.892298784	0.844328704	0.921686747	0.886104745	0.039049216	1.670951614	0.073636161
pCH110 + pGL3-basic MMP10 promoter + pcDNA3-ERK3	1.957425985	1.624685139	1.680158927	1.754090017	0.178265174	3.307734849	0.336159446
pCH110 + pGL3-basic MMP10 promoter + pcDNA3-SRC-3	2.116035263	2.562405561	2.128609561	2.269016795	0.254159899	4.278746161	0.479276175
pCH110 + pGL3-basic MMP10 promoter + pcDNA3-ERK3 + pcDNA3-SRC-3	3.384647476	3.739393939	3.786292242	3.636777886	0.219606854	6.857970136	0.414118567
pCH110 + pGL3-basic MMP10 promoter + pEGFP-C1	1.041899441	1.084853627	1.152059134	1.092937401	0.055522968	2.060981531	0.104701159
pCH110 + pGL3-basic MMP10 promoter + pEGFP-MK5	0.689753864	0.861829653	0.828093645	0.793225721	0.091183049	1.495807133	0.171946335
pCH110 + pGL3-basic MMP10 promoter + pEGFP-MK5 + pcDNA3-SRC-3	1.728640193	1.696035242	1.596481271	1.673718902	0.068847726	3.15617687	0.129828014
pCH110 + pGL3-basic MMP10 promoter +pEGFP-MK5 + pcDNA3-ERK3	1.159845133	1.291471963	1.115196078	1.188837725	0.091644556	2.241823358	0.17281661
pCH110 + pGL3-basic MMP10 promoter + pEGFP-MK5 + pcDNA3-ERK3 + pcDNA3-SRC-3	1.258309038	1.487980769	1.362057448	1.369449085	0.115014144	2.582407071	0.216885273

Appendix 12:

Downregulation of MMP2 promoter activity with SiRNA knockdown of ERK3 and MK5

Measurement	Plasmids transfected	Parallels		
		1	2	3
Luciferase activity	50 ng pCH110 + 100 ng pGL3-basic MMP2 promoter	0.617	0.619	0.585
	50 ng pCH110 + 100 ng pGL3-basic MMP2 promoter + 20 pmol SiNC	0.578	0.589	0.594
	50 ng pCH110 + 100 ng pGL3-basic MMP2 promoter + 20 pmol SiERK3	0.226	0.194	0.225
	50 ng pCH110 + 100 ng pGL3-basic MMP2 promoter + 20 pmol SiMK5	0.29	0.631	0.268
	50 ng pCH110 + 100 ng pGL3-basic MMP2 promoter + 20 pmol SiERK3 + 20 pmol SiMK5	0.111	0.128	0.149
β-galactosidase activity	50 ng pCH110 + 100 ng pGL3-basic MMP2 promoter	2.358	2.257	2.359
	50 ng pCH110 + 100 ng pGL3-basic MMP2 promoter + 20 pmol SiNC	2.271	2.391	2.655
	50 ng pCH110 + 100 ng pGL3-basic MMP2 promoter + 20 pmol SiERK3	2.55	2.63	2.301
	50 ng pCH110 + 100 ng pGL3-basic MMP2 promoter + 20 pmol SiMK5	2.813	4.63	3.079
	50 ng pCH110 + 100 ng pGL3-basic MMP2 promoter + 20 pmol SiERK3 + 20 pmol SiMK5	2.808	2.486	2.098

Relative luciferase units	Parallels (Luc/ β-gal)			Average	Standard deviation	Fold change	Coefficient of variation
	1	2	3				
pCH110 + pGL3-basic MMP2 promoter	0.261662426	0.274257864	0.247986435	0.261302242	0.013139418	1.081873386	0.054401318
pCH110 + pGL3-basic MMP2 promoter + SiNC	0.25451343	0.246340443	0.223728814	0.241527562	0.015946662	1	0.066024191
pCH110 + pGL3-basic MMP2 promoter + SiERK3	0.088627451	0.073764259	0.097783572	0.086725094	0.012122132	0.359069139	0.050189435
pCH110 + pGL3-basic MMP2 promoter + SiMK5	0.103092784	0.136285097	0.087041247	0.108806376	0.0251142	0.450492585	0.10398068
pCH110 + pGL3-basic MMP2 promoter + SiERK3 + SiMK5	0.039529915	0.051488335	0.071020019	0.054012756	0.015896106	0.223629782	0.065814875

Appendix 13:

Downregulation of MMP9 promoter activity with SiRNA knockdown of ERK3 and MK5.

Measurement	Plasmids transfected	Parallels		
		1	2	3
Luciferase activity	50 ng pCH110 + 100 ng pGL3-basic MMP9 promoter	0.209	0.211	0.192
	50 ng pCH110 + 100 ng pGL3-basic MMP9 promoter + 20 pmol SiNC	0.169	0.178	0.182
	50 ng pCH110 + 100 ng pGL3-basic MMP9 promoter + 20 pmol SiERK3	0.052	0.04	0.041
	50 ng pCH110 + 100 ng pGL3-basic MMP9 promoter + 20 pmol SiMK5	0.089	0.098	0.091
	50 ng pCH110 + 100 ng pGL3-basic MMP9 promoter + 20 pmol SiERK3 + 20 pmol SiMK5	0.041	0.034	0.038
β-galactosidase activity	50 ng pCH110 + 100 ng pGL3-basic MMP9 promoter	2.432	2.672	2.563
	50 ng pCH110 + 100 ng pGL3-basic MMP9 promoter + 20 pmol SiNC	2.911	3.096	2.844
	50 ng pCH110 + 100 ng pGL3-basic MMP9 promoter + 20 pmol SiERK3	2.432	2.886	2.879
	50 ng pCH110 + 100 ng pGL3-basic MMP9 promoter + 20 pmol SiMK5	4.179	3.807	3.912
	50 ng pCH110 + 100 ng pGL3-basic MMP9 promoter + 20 pmol SiERK3 + 20 pmol SiMK5	3.328	3.605	2.808

Relative luciferase units	Parallels (Luc/ β-gal)			Average	Standard deviation	Fold change	Coefficient of variation
	1	2	3				
pCH110 + pGL3-basic MMP9 promoter	0.0859375	0.078967066	0.074912212	0.079938926	0.005576525	1.33570244	0.069759814
pCH110 + pGL3-basic MMP9 promoter + SiNC	0.058055651	0.05749354	0.063994374	0.059847855	0.003601973	1	0.045059058
pCH110 + pGL3-basic MMP9 promoter + SiERK3	0.021381579	0.013860014	0.014241056	0.016494216	0.004236866	0.275602463	0.053001288
pCH110 + pGL3-basic MMP9 promoter + SiMK5	0.021296961	0.025742054	0.023261759	0.023433591	0.002227523	0.391552734	0.027865309
pCH110 + pGL3-basic MMP9 promoter + SiERK3 + SiMK5	0.012319712	0.009431345	0.013532764	0.011761273	0.002106964	0.196519549	0.026357173

Appendix 14:

Downregulation of MMP10 promoter activity with SiRNA knockdown of ERK3 and MK5.

Measurement	Plasmids transfected	Parallels		
		1	2	3
Luciferase activity	50 ng pCH110 + 100 ng pGL3-basic MMP10 promoter	0.629	0.622	0.669
	50 ng pCH110 + 100 ng pGL3-basic MMP10 promoter + 20 pmol SiNC	0.569	0.573	0.592
	50 ng pCH110 + 100 ng pGL3-basic MMP10 promoter + 20 pmol SiERK3	0.14	0.152	0.141
	50 ng pCH110 + 100 ng pGL3-basic MMP10 promoter + 20 pmol SiMK5	0.666	0.173	0.132
	50 ng pCH110 + 100 ng pGL3-basic MMP10 promoter + 20 pmol SiERK3 + 20 pmol SiMK5	0.083	0.07	0.069
β-galactosidase activity	50 ng pCH110 + 100 ng pGL3-basic MMP10 promoter	5.158	5.36	5.095
	50 ng pCH110 + 100 ng pGL3-basic MMP10 promoter + 20 pmol SiNC	5.79	5.383	5.44
	50 ng pCH110 + 100 ng pGL3-basic MMP10 promoter + 20 pmol SiERK3	4.187	3.72	3.486
	50 ng pCH110 + 100 ng pGL3-basic MMP10 promoter + 20 pmol SiMK5	11.61	2.764	3.137
	50 ng pCH110 + 100 ng pGL3-basic MMP10 promoter + 20 pmol SiERK3 + 20 pmol SiMK5	2.992	2.778	3.362

Relative luciferase units	Parallels (Luc/ β-gal)			Average	Standard deviation	Fold change	Coefficient of variation
	1	2	3				
pCH110 + pGL3-basic MMP10 promoter	0.121946491	0.116044776	0.131305201	0.123098823	0.007695196	1.177818992	0.073628228
pCH110 + pGL3-basic MMP10 promoter + SiNC	0.098272884	0.10644622	0.108823529	0.104514211	0.005534304	1	0.052952646
pCH110 + pGL3-basic MMP10 promoter + SiERK3	0.033436828	0.040860215	0.040447504	0.038248183	0.004171862	0.365961549	0.039916693
pCH110 + pGL3-basic MMP10 promoter + SiMK5	0.057364341	0.062590449	0.042078419	0.05401107	0.010659229	0.516782062	0.101988322
pCH110 + pGL3-basic MMP10 promoter + SiERK3+ SiMK5	0.027740642	0.025197984	0.020523498	0.024487375	0.003660671	0.234297081	0.035025586

Appendix 15:

qPCR assay of ERK3, MK5, MMP2, and MMP9 in shRNA stably transfected A549 and H1299 cells. The values are normalized against B-Act.

Well Name	Assay	Well Type	Ct (dR)	Average	Standard deviation Ct	Δ average Ct (Sample-reference β-Act)	Standard deviation Δ Ct	Normalized against A549shLUC	Fold Change	Standard Deviation
A549-shLuc	mmp2	Unknown	26.24							
A549-shLuc	mmp2	Unknown	26.74	26.49	0.353553391	8.845	0.389165774	0	1	0.273032447
A549-shERK3	mmp2	Unknown	25.4							
A549-shERK3	mmp2	Unknown	25.47	25.435	0.049497475	7.66	0.092195445	-1.185	2.273633946	0.145395521
A549-shMK5	mmp2	Unknown	25.89							
A549-shMK5	mmp2	Unknown	26.22	26.055	0.233345238	8.75	0.238537209	-0.095	1.068065408	0.177401145
NTC	mmp2	NTC	No Ct							
NTC	mmp2	NTC	No Ct							
A549-shLuc	mmp9	Unknown	30.72							
A549-shLuc	mmp9	Unknown	30.64	30.68	0.056568542	13.035	0.172191754	0	1	0.119637806
A549-shERK3	mmp9	Unknown	29.28							
A549-shERK3	mmp9	Unknown	28.85	29.065	0.304055916	11.29	0.313847097	-1.745	3.351948539	0.734955428
A549-shMK5	mmp9	Unknown	30.56							
A549-shMK5	mmp9	Unknown	30.48	30.52	0.056568542	13.215	0.075166482	0.18	0.882702996	0.046010903
NTC	mmp9	NTC	37.7							
NTC	mmp9	NTC	No Ct							
A549-shLuc	ERK3	Unknown	22.81							
A549-shLuc	ERK3	Unknown	22.74	22.775	0.049497475	5.13	0.17	0	1	0.118107902
A549-shERK3	ERK3	Unknown	25.46							
A549-shERK3	ERK3	Unknown	25.55	25.505	0.06363961	7.73	0.100498756	2.6	0.164938489	0.011498981
A549-shMK5	ERK3	Unknown	22.91							
A549-shMK5	ERK3	Unknown	23.21	23.06	0.212132034	5.755	0.217830209	0.625	0.648419777	0.09827628
NTC	ERK3	NTC	33.37							
NTC	ERK3	NTC	33.59							
A549-shLuc	MK5	Unknown	23.84							
A549-shLuc	MK5	Unknown	23.69	23.765	0.106066017	6.12	0.194164878	0	1	0.134991497
A549-shERK3	MK5	Unknown	23.8							
A549-shERK3	MK5	Unknown	23.81	23.805	0.007071068	6.03	0.078102497	-0.09	1.064370182	0.057649453
A549-shMK5	MK5	Unknown	24.02							
A549-shMK5	MK5	Unknown	23.94	23.98	0.056568542	6.675	0.075166482	0.555	0.680657058	0.035479256
NTC	MK5	NTC	No Ct							
NTC	MK5	NTC	No Ct							
A549shLuc	β-Act	Unknown	17.53							
A549shLuc	β-Act	Unknown	17.76	17.645	0.16263456					
A549shERK3	β-Act	Unknown	17.72							
A549shERK3	β-Act	Unknown	17.83	17.775	0.077781746					
A549shMK5	β-Act	Unknown	17.34							
A549shMK5	β-Act	Unknown	17.27	17.305	0.049497475					
NTC	β-Act	NTC	37.21							
NTC	β-Act	NTC	37.23	37.22	0.014142136					

Appendix 15 (continued)

Well Name	Assay	Well Type	Ct (dR)	Average	Standard deviation Ct	Δ average Ct (Sample-reference β-Act)	Standard deviation Δ Ct	Normalised against A549shLUC	Fold Change	Standard Deviation
H1299-shLuc	mmp2	Unknown	27.93	27.865	0.091923882	9.63	0.13	0	1	0
H1299-shLuc	mmp2	Unknown	27.8							
H1299-shERK3	mmp2	Unknown	30.1	30.1	0	11.985	0.16263456	2.355	0.195467411	0.02208168
H1299-shERK3	mmp2	Unknown	30.1							
H1299-shMK5	mmp2	Unknown	31.75	31.64	0.155563492	12.88	0.161245155	3.25	0.105112052	0.011772493
H1299-shMK5	mmp2	Unknown	31.53							
NTC	mmp2	NTC	No Ct							
NTC	mmp2	NTC	No Ct							
H1299-shLuc	mmp9	Unknown	28.7	28.65	0.070710678	10.415	0.115974135	0	1	0.080473751
H1299-shLuc	mmp9	Unknown	28.6							
H1299-shERK3	mmp9	Unknown	29.99	29.885	0.148492424	11.77	0.220227155	1.355	0.390934822	0.059908167
H1299-shERK3	mmp9	Unknown	29.78							
H1299-shMK5	mmp9	Unknown	31.28	31	0.395979797	12.24	0.398246155	1.825	0.282241101	0.078903978
H1299-shMK5	mmp9	Unknown	30.72							
NTC	mmp9	NTC	39.81							
NTC	mmp9	NTC	38.55							
H1299-shLuc	ERK3	Unknown	22.61	22.585	0.035355339	4.35	0.098488578	0	1	0.068320118
H1299-shLuc	ERK3	Unknown	22.56							
H1299-shERK3	ERK3	Unknown	24.57	24.535	0.049497475	6.42	0.17	2.07	0.2381595	0.028128519
H1299-shERK3	ERK3	Unknown	24.5							
H1299-shMK5	ERK3	Unknown	23.36	23.32	0.056568542	4.56	0.070710678	0.21	0.864537231	0.04239045
H1299-shMK5	ERK3	Unknown	23.28							
NTC	ERK3	NTC	30.22							
NTC	ERK3	NTC	32.76							
H1299-shLuc	MK5	Unknown	24.86	24.855	0.007071068	6.62	0.092195445	0	1	0.063948518
H1299-shLuc	MK5	Unknown	24.85							
H1299-shERK3	MK5	Unknown	24.47	24.51	0.056568542	6.395	0.172191754	-0.225	1.168777249	0.139829946
H1299-shERK3	MK5	Unknown	24.55							
H1299-shMK5	MK5	Unknown	27.48	27.595	0.16263456	8.835	0.168077363	2.215	0.21538654	0.02514984
H1299-shMK5	MK5	Unknown	27.71							
NTC	MK5	NTC	No Ct							
NTC	MK5	NTC	36.79							
H1299-shLuc	β-Act	Unknown	18.17	18.235	0.091923882					
H1299-shLuc	β-Act	Unknown	18.3							
H1299-shERK3	β-Act	Unknown	18.23	18.115	0.16263456					
H1299-shERK3	β-Act	Unknown	18							
H1299-shMK5	β-Act	Unknown	18.73	18.76	0.042426407					
H1299-shMK5	β-Act	Unknown	18.79							
NTC	β-Act	NTC	No Ct							
NTC	β-Act	NTC	No Ct							

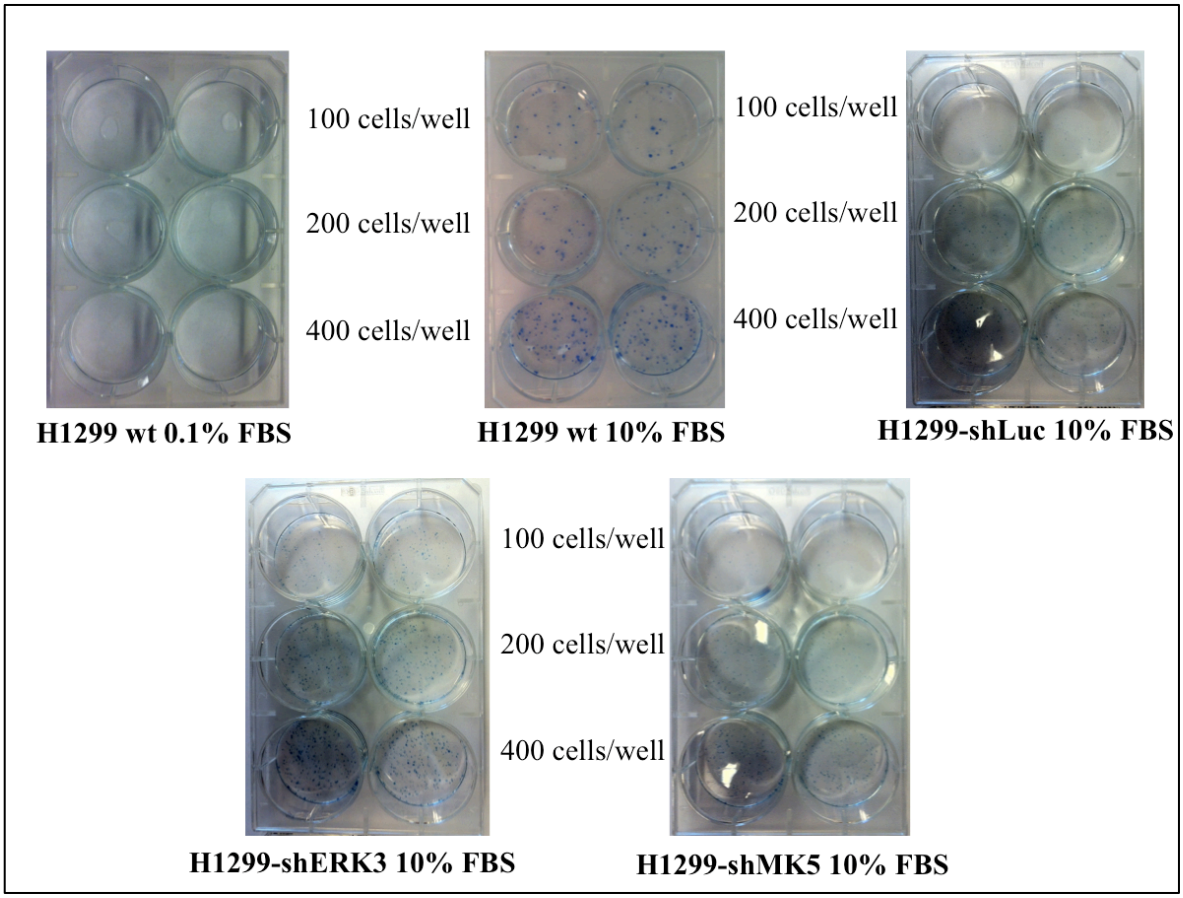
Appendix 16:

Colonogenic assay on shRNA knockdown H1299 cells

Cell line	Cells/well	Parallels		Average	Standard deviation	*p*-values for ANOVA on StatPlus/Excel
		1	2			
H1299 wt						
	100 cells/well	63	51	57	4.242640687	**100 cells/well:** 0.307
	200 cells/well	105	98	101.5	2.474873734	**200 cells/well:** 0.043
	400 cells/well	178	160	169	6.363961031	**400 cells/well:** 0.015
H1299-shLuc		**1**	**2**			
	100 cells/well	44	52	48	2.828427125	
	200 cells/well	95	86	90.5	3.181980515	
	400 cells/well	159	133	146	9.192388155	
H1299-shERK3		**1**	**2**			
	100 cells/well	59	53	56	2.121320344	
	200 cells/well	98	102	100	1.414213562	
	400 cells/well	168	153	160.5	5.303300859	
H1299-shMK5		**1**	**2**			
	100 cells/well	49	43	46	2.121320344	
	200 cells/well	77	61	69	5.656854249	
	400 cells/well	106	101	103.5	1.767766953	

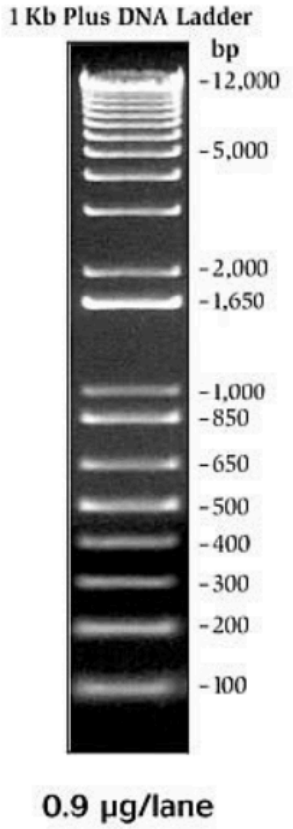
Appendix 17:

Photos taken of colonogenic assay plates



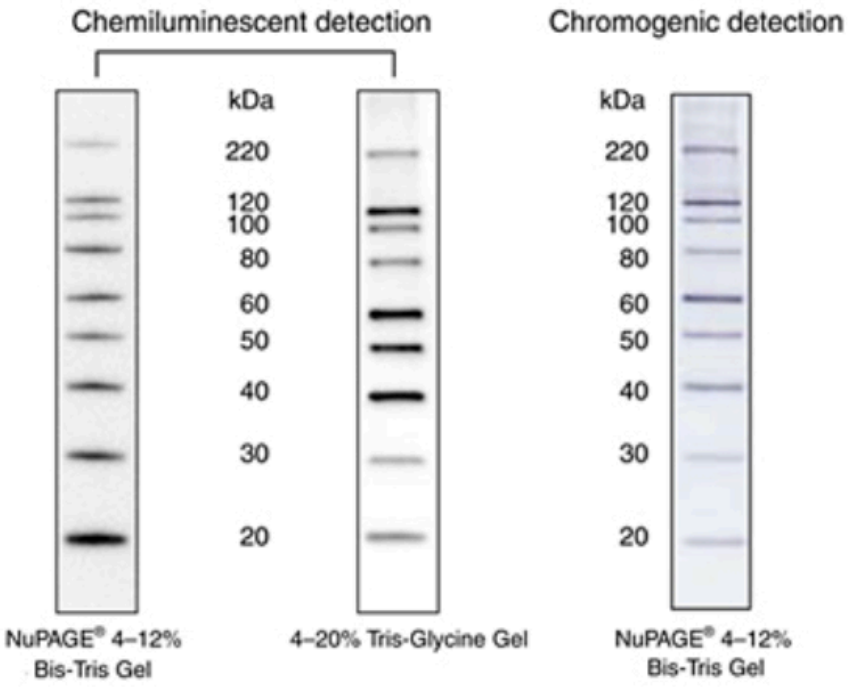
Appendix 18:

1 Kb Plus DNA Ladder, Invitrogen™, Life Technologies™ (www.invitrogen.com)



Appendix 19:

Novex® MagicMark™ XP Western Protein Standard, Invitrogen, Life Technologies™ (www.invitrogen.com)



Appendix 20:

Novex® SeeBlue® Plus2 Pre-Stained Protein Standard 1x, Invitrogen, Life Technologies™ (www.invitrogen.com)