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The impact of wild type and mutant variants of cellular repressor of E1A-stimulated genes (CREG) on mammary tumour cell invasiveness

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ABSTRACT

Cellular repressor of E1A-stimulated genes (CREG) is a lysosomal glycoprotein whose biological function is still unknown, but it is expected to play a role in cell proliferation, differentiation and development. It is synthesized in the endoplasmatic reticulum and targeted to the lysosomes through the M6P-receptor, which is also responsible for the internalization of secreted CREG. It was shown to be upregulated during EMT (epithelial to mesenchymal transition) and hence possibly contributes to the formation of metastases. Recently orthologues in *Arabidopsis thaliana* and *Drosophila melanogaster* were found, all sharing significant sequence similarities and likewise localisations with the mammalian proteins, indicating a evolutionarily conserved function of the protein.

It has been shown that CREG expression in NMuMG cells results in protection from TGF-β1 induced apoptosis. These experiments were now repeated with CREG-deficient EpRas cells that were reconstituted with wild type and mutant CREG variants. No significant changes in various signal transduction pathways (ERK, p38, AKT, SMAD) were observed upon TGF-β1 treatment of the cells. Nevertheless, first evidence was obtained that the invasiveness of EpRas cells can be reduced by CREG downregulation.

KURZFASSUNG

Obwohl die biologische Funktion des lysosomalen Proteins CREG (cellular repressor of E1A-stimulated genes) noch unbekannt ist, wird vermutet, dass eine Funktion in der Zellentwicklung, -proliferation und –differenzierung innehat. Das Protein wird im Endoplasmatischen Retikulum synthetisiert bevor es über die M6P Rezeptoren zu den Lysosomen transportiert wird. Die Rezeptoren haben neben dem direkten Transport zu den Lysosomen noch die Aufgabe sekretiertes CREG über einen Recycling-Weg wieder in die Zelle zu transportieren. Während EMT (epithelial to mesenchymal transition) wird CREG hochreguliert, was auf eine Beteiligung an der Bildung von Metastasen hinweist. Neben den bereits bekannten CREG Varianten in Mensch und Maus wurden auch Orthologe in *Arabidopsis thaliana* und *Drosophila melanogaster* gefunden, die den Säugetierformen in Sequenz und Lokalisierung ähneln, folglich liegt die Vermutung nahe, dass es sich um ein stark konserviertes Protein handelt.

Da ein Schutz vor TGF-β1 induzierter Apoptose bei CREG Überexpression in NMuMG Zellen kürzlich nachgewiesen wurde, wurden nun mehrere CREG-Mutanten im EpRas Zellsystem analysiert. Obwohl verschiedene Signaltransduktionswege (ERK, p38, AKT, SMAD) untersucht wurden, konnten keine signifikanten Veränderungen aufgrund der Expression von Wildtyp- und mutiertem CREG festgestellt werden. Es konnten aber Hinweise darauf gefunden werden, dass eine Reduktion des CREG-Gehaltes von EpRas-Zellen deren Invasivität – ein Maß für die Malignität von Tumorzellen – vermindert.

LIST OF ABBREVIATIONS

aa	amino acid
ab	antibody
AKT	see PKB
AMPS	2-acrylamido-2-methylpropane sulfonic acid
AtCREG	*Arabidosis thaliana* cellular repressor of E1A stimulated genes
bp	base pair
BSA	bovine serum albumin
CD-MPR	cation dependent mannose-6-phosphate receptor
cDNA	copy desoxyribonucleic acid
CI-MPR	cation-independent mannose-6-phosphate receptor
CREG	cellular repressor of E1A stimulated genes
c_T	cycle threshold
DAPI	4′,6-diamidinö-2-phenylindole
ddH_2O	double distilled water
DMEM	Dulbecco's Modified Eagle Medium
DmCREG	*Drosophila melanogaster* cellular repressor of E1A stimulated genes
DMSO	dimethyl sulfoxide
DNA	desoxyribonucleic acid
dNTP	deoxynucleotidetriphosphate
dpc	days post coitus
dsDNA	doublestrand desoxyribonucleic acid
DTT	dithiothreitol
E1A	early region 1 gene products of adenovirus
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EMT	epithelial to mesenchymal transition
EpH4	mouse mammary epithelial cells
EpRas	EpH4 cells transformed with oncogenic *ras*
EpRas XT	cells retrieved from tumours formed upon injection of EpRas cells
ER	endoplasmatic reticulum
FACS	Fluorescence Activated Cell Sorting
FCS	fetal calf serum
FMN	flavin mononucleotide
FS-293	FreeStyle-293 cells
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GFP	green fluorescent protein
GlcNAc-1-phosphotransferase	UDP-N-acetylglucosamine:lysosomal enzyme N-acetylglucosamine-1-phosphotransferase
GYT	glycerol-yeast extract-tryptone medium
HITASHY	human internal thoracic artery Shenyang cells

HPRT	hypoxanthine phosphoribosyltransferase 1
HRP	horseradish peroxidase
Hsp70	heat shock protein 70
hum	human
humCREG	human cellular repressor of E1A stimulated genes
IF	immunofluorescent
IGF-II	insulin-like growth factor II
IgG	immunoglobulin G
IHC	immunohistochemistry
ILEI	interleukin-like EMT inducer
IMP	Research Institute of Molecular Pathology
LAMP-1	endosomal/lysosomal membrane protein 1
LB	lysogeny broth
LIMP-2	lysosomal integral membrane protein 2
L-M(TK$^-$)	murine thymidine kinase negative L-fibroblasts
LSD	lysosomal storage diseases
M6P	mannose-6-phosphate
MAPK	mitogen-activated protein kinase
MCS	multiple cloning site
MEK-ERK1/2	mitogen-activated protein kinase-extracellular signal-regulated kinase 1/2
ms	mouse
msCREG	murine cellular repressor of E1A stimulated genes
MSCs	bone marrow-derived stem cells
OD600	optical density at 600 nm
p38 MAPK	p38 mitogen activated protein kinase
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFA	paraformaldehyde
PI3K	phosphatidylinositol-3`-kinase
PKB	Protein Kinase B
Plg	plasminogen
puro	puromycin
PVDF	polyvinylidenedifluoride
qrt-PCR	real time quantitative polymerase chain reaction
rb	rabbit
RIPA	buffer for cell lysis
RNA	ribonucleic acid
SDS	sodium dodecylsulfate
shCREG#0	CREG knockdown#0
shCREG#1	CREG knockdown#1
shCREG#2	CREG knockdown#2
shCREG#3	CREG knockdown#3
SMAD	Mad/Sma protein
SOC	super optimal broth + glucose

TAE	Tris/acetate-EDTA buffer
TBP	TATA binding protein
TBST	Tris-sodium chloride-Tween 20
TE	Tris/HCl-EDTA buffer
TEMED	tetramethylethylenediamine
TGF-β1	transforming growth factor β
TNF α	tumour necrosis factor alpha
Tris	Tris(hydroxymethyl)aminomethane
uPAR	urokinase-type plasminogen activator receptor
UV	ultraviolet
VSMCs	vascular smooth muscle cells

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1. Introduction

1.1. The Lysosome – A subcellular compartment

Lysosomes are membrane-enclosed cell organelles only found in mammalian and insect cells, although compartments – called vacuoles – with similar assignments exist also in plant cells (Alberts *et al*, 2002).
These organelles were first discovered 55 years ago by biochemical fractionation of rat liver cells (de Duve *et al*, 1955).
The main purpose of the lysosomes is the degradation and recycling of macromolecules (proteins, lipids, polysaccharides or nucleic acid) which were obtained through endocytosis, autophagy or other cellular trafficking pathways. To fulfil this task more than 60 types of soluble catabolic enzymes are stored within the lysosomal lumen. These enzymes - called acid hydrolases – evolved to maintain their function in the acidic environment (pH 5) of the lysosome, which is achieved by ATP-dependent proton pumps. Lysosomes are also associated with numerous human diseases; most of these 50 monogenetic ailments which are known today are so called lysosomal storage diseases (LSD). There is also proof that lysosomes play an important role in polygenetic diseases such as cancer or arthritis (Lübke *et al*, 2009).

1.2. Sorting of Lysosomal Proteins

A vast number of the newly synthesized lysosomal hydrolases contain M6P (mannose-6-phosphate) residues which are recognized by specific M6P receptors that directs the targeting to the lysosomes. Alternative routes independent of the M6P receptors are also known such as sortilin or the lysosomal integral membrane protein LIMP-2. The trafficking and sorting of lysosomal proteins can be regulated through phosphorylation and lipid modifications (Braulke *et al*, 2009).
An overview of the transport is shown in figure 1. The respective lysosomal hydrolase precursor is recognized in the *cis* Golgi network, the enzyme is modified (glycosylations and phosphorylations) throughout its transport to the *trans* Golgi network where it is separated from other proteins. M6P receptors that are bound by

adaptins to the clathrin coat bind the M6P group of the enzyme. These clathrin coated vesicles bud off the *trans* Golgi network and fuse with the late endosomes. Because of the low pH inside the lumen of the late endosomes the hydrolases are released from the receptor and the phosphate residue is removed. The M6P receptor is recycled to the Golgi network (Alberts *et al*, 2002).

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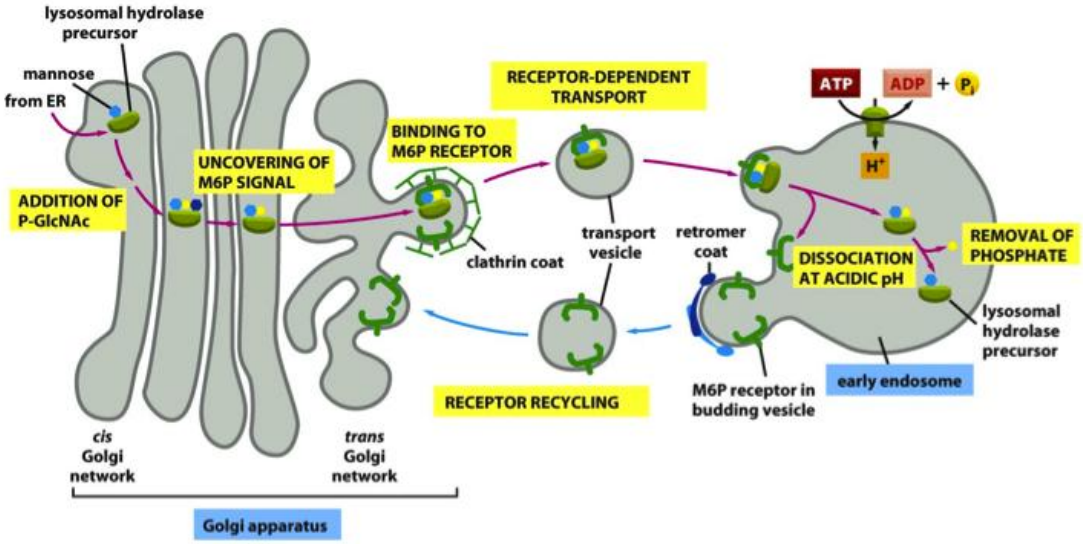


Figure 1: Transport and sorting of newly synthesized hydrolases through the mannose-6-phosphate pathway (Alberts *et al*, 2008).

1.2.1. Posttranslational modifications of lysosomal enzymes

Immediately after entering the lumen of the ER the oligosaccharyl transferase attaches a precursor oligosaccharide (consisting of 14 sugars: *N*-acetylglucosamine, mannose and glucose) *en bloc* to the NH_2 group of selected asparagines residues, which have to be within the sequence Asn-X-Ser/Thr (X can be any amino acid except Pro or Asp) (Alberts *et al*, 2002).

The creation of M6P on *N*-linked oligosaccharides is a two-step process (see figure 2) that takes place during the transport through the endoplasmatic reticulum (ER) and Golgi apparatus. Upon reaching the *cis* Golgi network the first enzyme, called GlcNAc-1-phosphotransferase (UDP-*N*-acetylglucosamine:lysosomal enzyme *N*-acetylglucosamine-1-phosphotransferase, IUBMB accession number EC 2.7.8.17) attaches GlcNAc-1-phosphate to the C6 hydroxyl group of one or more mannose residues. During the second step another enzyme N-acetylglucosamine-1-phosphodiester-α-N-acetylglucosaminidase ("uncovering enzyme", IUBMB

accession number EC 3.1.4.45) cleaves the GlcNAc moiety and generates the phosphomonoester (Dahms *et al*, 2008).

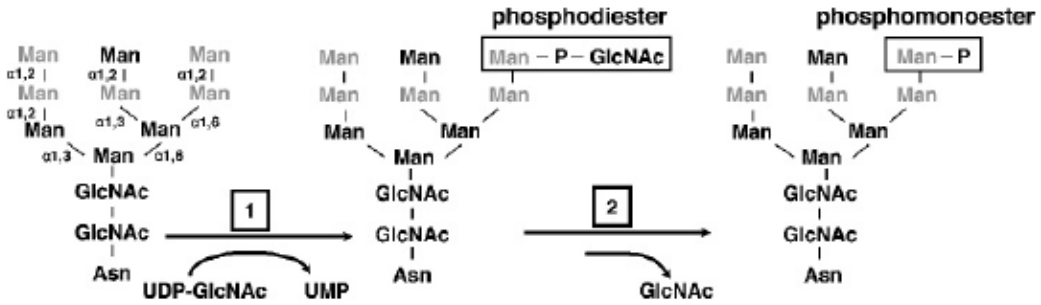


Figure 2: Processing of the oligosaccharide residues: enzyme 1: GlcNAc-1-phosphotransferase; enzyme 2: N-acetylglucosamine-1-phosphodiester-α-N-acetylglucosaminidase (Dahms *et al*, 2008)

1.2.2. GlcNAc-1-phosphotransferase

The most critical step during sorting is the discrimination between lysosomal and secretory proteins. The recognition of the lysosomal enzymes is facilitated by the γ-subunit of GlcNAc-1-phosphotransferase, a hexamer consisting of α_2, β_2 and γ_2 subunits, whereas the α and β subunits carry the catalytic activity (Gelfman *et al*, 2007). Kinetic studies show that the recognition of a specific lysosomal protein named uteroferrin is approximately 163 fold more effective than of a non-lysosomal glycoprotein (ribonuclease B). It is possible to block the transfer of GlcNAc-1-phosphate to cathepsin D by using specific antibodies, thus proposing a protein-protein dependent interaction (Bao *et al*, 1996).

Despite the great number of different lysosomal proteins they all must share a common recognition feature. This conformational determinant (see figure 3) is sought after by various scientists but until now no generally valid recognition patch could be verified.

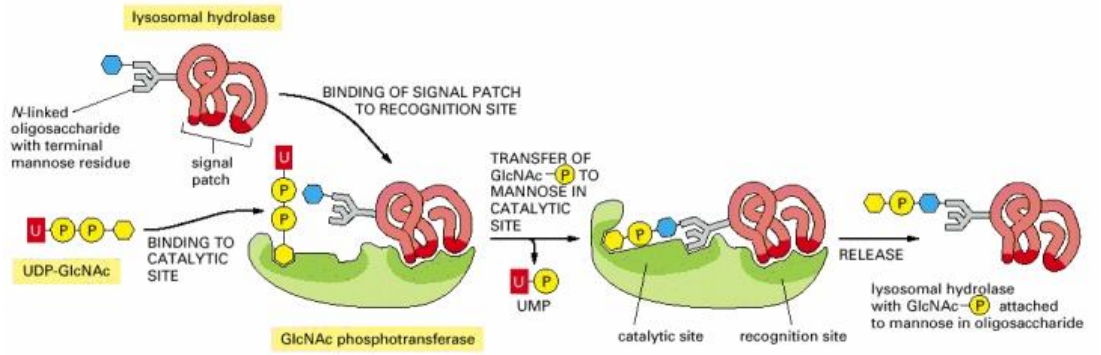


Figure 3: Biosynthesis of the M6P recognition marker (Alberts *et al*, 2002).

There are studies that suggest that the presence of lysine residues in the recognition structure is indispensable. The mutations of two lysine residues, Lys-203 and Lys-293, on Cathepsin D, a lysosomal protein, by site-directed mutagenesis resulted in an inhibition of the phosphorylation by 70%, and other positively charged amino acids could not substitute for lysine (Cuozzo *et al*, 1998). The substitution of two sequences of Cathepsin D (Lys-203 and amino acids 265-292) with those of glycopepsinogen, which is closely related to Cathepsin D in both amino acid sequence and three-dimensional structure, resulted in a phosphorylation of the secretory protein, thus showing that a 3-dimensional structure was formed that acts as recognition patch (Baranski *et al*, 1990). Later it was shown that introducing only two lysine residues (Lys-203 and Lys-267) into the glycopepsinogen increases the mannose phosphorylation 116-fold (Steet *et al*, 2005). Human lysosomal aspartylglucosaminidase contains three lysine residues (Lys-177 and Lys-183 in the α and Lys-214 in the β-subunit) as well as a tyrosine (Tyr178) that stimulate phosphorylation (Tikkanen *et al*, 1997). Other studies state that a conserved β-hairpin loop acts as recognition site (Lukong *et al*, 1999). Although a substitution of Lys-457 of arylsulfatase A resulted in a reduction of the phosphorylation by 33%, substitutions of Lys-497 and Lys-507 of the highly conserved arylsulfatase B had no effect on phosphorylation. Lys-457 is located in a helix and not an unstructured loop region as seen in other lysosomal enzymes and it is also not strictly conserved among homologous lysosomal sulfatases. Even though arylsulfatases A and B are highly homologous, a conserved recognition patch could not be identified (Yaghootfam *et al*, 2003).

These studies suggest that lysine residues play an important role in the recognition of lysosomal enzymes, but there are still other unknown factors that have an impact on the phosphorylation.

1.2.3. The mannose-6-phosphate receptors

Two distinct M6P receptors – the 300 kDa cation-independent (CI-MPR) and the 46 kDa cation dependent M6P receptor (CD-MPR) - are known in mammalian cells, both are type I transmembrane glycoproteins. The extracyctoplasmic domain of CI-MPR consists of 15 repeating domains that are homologous to the extracytoplasmic domain of CD-MPR (Pohlmann *et al*, 1995). The half-life ($T_{1/2}$ ~ 20h) of both receptors is rather long and optimal ligand binding occurs at pH 6,5. Although either plays a major role in the sorting of lysosomal proteins the CI-MPR can also internalize various exogenous M6P containing enzymes that were not directly sorted to lysosomes. CD-MPRs that are present at the cell surface show a decreased capability to bind M6P at pH 7,4. In total about 10% of all M6P receptors are found at the cell surface (Dahms *et al*, 2008).

The 300 kDa receptor is also known as IGF-II receptor because of his ability not only to bind M6P groups but also insulin-like growth factor II (IGF-II). The dimerization of the receptor is mediated by multivalent ligand binding (York *et al*, 1999). To achieve an optimal binding of M6P a dimeric structure of the receptor is required, whereas the attachment of IGF-II occurs on a single side of each monomer (Hartman *et al*, 2009).

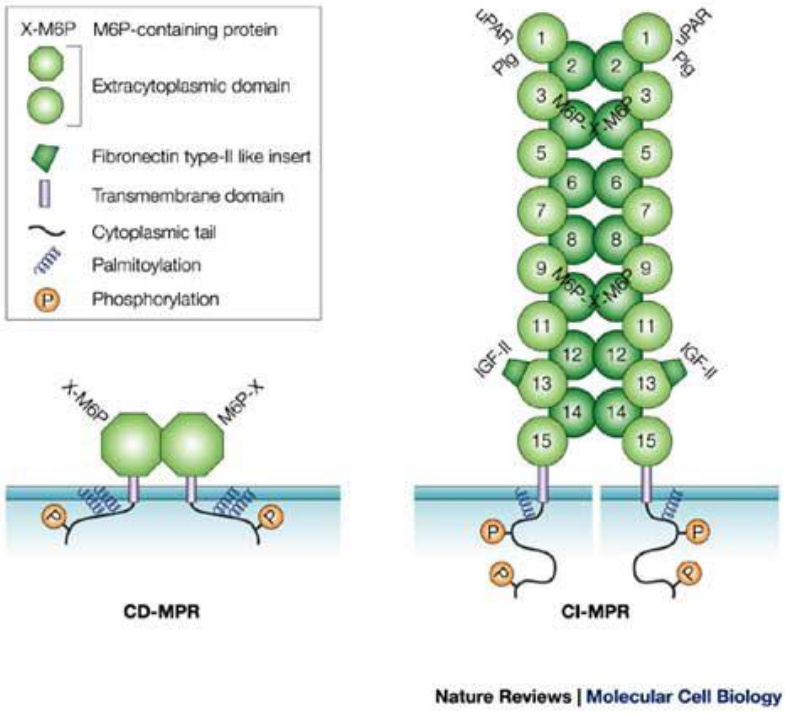


Figure 4: Structure of the M6P receptors: The 46 kDa M6P receptor (CD-MPR) is a homodimer with one M6P binding site, whereas the 300 kDa M6P receptor (CI-MPR) forms a dimer with additional binding sites for

plasminogen (Plg), urokinase-type plasminogen activator receptor (uPAR) and insulin-like growth factor II (IGF-II). Posttranslational modifications (phosphorylation and palmitoylation) are present in the cytoplasmic domains of the receptors (Gosh *et al*, 2003).

It was shown that both receptors are essential for the targeting of lysosomal hydrolases, neither can substitute for the loss of the other indicating that each receptor reacts in vivo with different though overlapping subclasses of lysosomal enzymes (Ludwig *et al*, 1994). To identify the respective subsets of bound hydrolases mice lacking either receptor were created and serum analysis was performed, because the proteins that would have been sorted by the lacking receptor should then be secreted into the blood stream. Several enzymes could be identified as specific targets for each receptor, one of these is CREG (cellular repressor of E1A stimulated genes) whose sorting requires the CD-MPR (Qian *et al*, 2007).

The ability of the M6P/IGF-II receptor to modulate IGF-II levels and to activate TGF-β1 makes it an ideal candidate for a tumour suppressor. The receptor is frequently inactivated during early stages of oncogenesis of liver and breast and analysis of hepatocellular carcinomas shown increased IGF-II levels (Devi *et al*, 1999). More recent studies suggested that the main role of the receptor in tumour cells is to inhibit cell proliferation. Although the reexpression of the CI-MPR in a receptor deficient cell line showed no effects on growth and invasiveness in vitro, the formation of mammary tumours in BALB/c mice was suppressed. The generation of mammary tumours was also inhibited using BALB/c scid mice, thus showing that cytotoxic T cells are not involved in tumour formation (Li *et al*, 2004).

1.3. Lysosomes as targets for Cancer Therapy

Lysosomes gather more and more interest in the field of cancer research because of their ability to trigger an alternative cell death programme by secreting cathepsins upon various death stimuli such as TNF α (tumour necrosis factor alpha), activation of p53 or oxidative stress (Nylansted *et al*, 2004). The cysteine cathepsins B and L and the aspartatyl cathepsin D are suspected to enhance cellular motility, invasion and angiogenesis by degrading the ECM (extracellular matrix). There is experimental evidence to support this theory. Upon pharmacological inhibition of cysteine cathepsins in a pancreatic cancer mouse model the tumour growth could be reduced. When cathepsin B and matrix metalloprotease-9 were genetically

inactivated in a mouse glioblastoma model tumour growth was inhibited as well (Fehrenbacher and Jäättelä, 2005).

The increased activity of phosphatidylinositol-3`-kinase, responsible for lysosome maturation, size and activity, is another characteristic of many tumours (Mousavi *et al*, 2003). Therefore pharmaceuticals that inhibit the phosphatidylinositol-3`-kinase pathways and secretion of cathepsins B, L and D may be ideal to make tumour cell susceptible to cancer drugs (Fehrenbacher and Jäättelä, 2005).

1.4. Cellular repressor of E1A-stimulated genes

CREG (cellular repressor of E1A-stimulated genes) is a 220 aa long glycoprotein whose biological function is still unknown. It has been proposed to play a role in cell proliferation, differentiation and development. It was shown that CREG acts as an antagonist to *ras* and E1A and thus to cellular transformations (Di Bacco *et al*, 2003).

In figure 5 a sequence alignment consisting of the human, mouse, *Drosophila melanogaster* and *Arabidopsis thaliana* CREG aa sequences is shown as well as potential *N*-glycosylation sites (Veal *et al*, 1998).

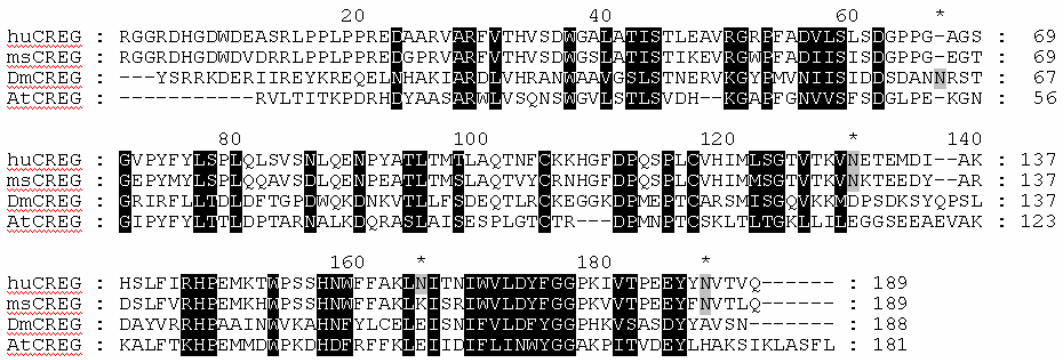


Figure 5: Alignment of homologous CREG sequences: The human and mouse CREG sequences share 77% identity, humCREG is 31% identical to *Drosophila melanogaster* CREG and 29% identical to *Arabidopsis thaliana* CREG. "*" indicates a *N*-glycosylation site.

1.4.1. Structure

The CREG monomer is composed of an antiparallel β-barrel structure whose inner surface consists of hydrophobic residues. The barrel is stabilized due to hydrogen bonds and salt bridges between the β-strand mainchain. In solution and crystal CREG forms a tight dimer. Hydrophobic and polar amino acids as well as twelve water molecules are responsible for the asssociation of the two monomers. The *N*-glycosylation sites are displayed on the surface of the dimer, thus making it to an ideal partner of the IGFII receptor (Sacher *et al*, 2005).

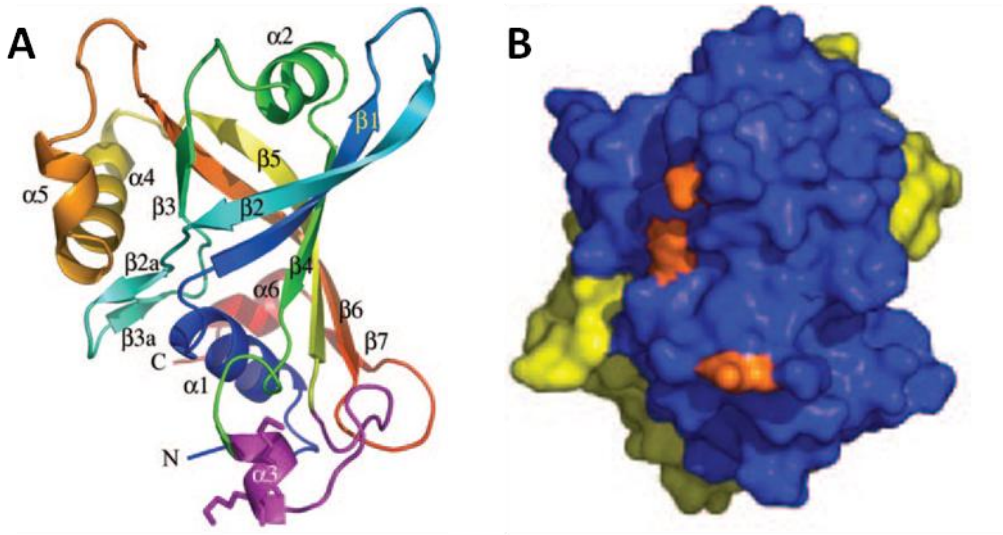


Figure 6: Stuctures of CREG monomer and dimer: (A) A stereoview of the cartoon representation of the CREG monomer. (B) The three potential *N*-glycosylation sites (Asn-160, Asn-193, Asn- 216) are shown in orange. One monomer is coloured yellow the other blue (Sacher *et al*, 2005).

humCREG 2 and msCREG 2 could be identified as two novel members of the CREG family. Both proteins are expressed in a tissue-specific manner and exclusively found in the brain, mainly the limbic system (Kunita *et al*, 2002).

1.4.2. Function

E1A, an adenovirus protein, is able to increase and inhibit gene expression to induce cell growth and prevent differentiation. CREG shares limited sequence similarities with E1A and is able to bind the general transcription factor TBP (TATA binding protein). It is an antagonist to E1A-mediated transformation and prevents the efficient cooperation of E1A and oncogenic *ras*. It is suggested that CREG plays

a part in the transcriptional control of proliferation and differentiation (Veal *et al*, 1998). Using the NTERA-2 cell line the same research group could show that an overexpression of humCREG results in enhanced differentiation – up-regulation of neural specific A2B5 and down-regulation of embryonic antigen SSEA3. A similar result could be obtained by using tissue culture supernatant of CREG-overexpressing cells as addition to culture medium (Veal *et al*, 2000).

The inhibition of proliferation is concentration-dependent and is coupled with the M6P/IGF-II receptor mediated uptake of recombinant CREG by NIH 3T3 fibroblasts, which were created by transfection of pDS_shCREG vectors resulting in an 80% decrease of CREG levels. The direct interaction of receptor and ligand could be verified using immunofluorescence staining, showing that CREG expression correlates with M6P/IGF-II receptor localization. This suggests CREG to be a functional regulator of the M6P/IGF-II receptor that enables IGF-II binding and trafficking and thus growth regulation (Han *et al*, 2008).

CREG inhibits the migration of vascular smooth muscle cells (VSMCs) and maintains their mature phenotype. The knockdown of CREG in VSCMs results in an increase of phosphoinositide 3-kinase (PI3K) and AKT activity and enhanced secretion of IGF-II and thus enhanced migration. The effect can be attenuated by addition of IGF-II neutralizing antibody or the PI3 kinase inhibitior LY294002 (Han *et al*, 2009).

The putative flavin mononucleotide-binding pocket (FMN) of CREG is blocked by a loop following helix α-3. It could be shown that a mutant lacking this loop loses its growth suppression ability but maintains its structure and dimerization, thus the binding to the M6P/IGF-II receptor is necessary but not sufficient for proliferation. This indicates that the residues 141-144 are required for CREG mediated growth inhibition (Sacher *et al*, 2005).

CREG overexpression shows anti-apoptotic effects in VSMCs of human internal thoracic artery Shenyang cells (HITASHY). Apoptosis in inhibited and the expression of caspase 9 mRNA decreased. An increase in expression and activation of p38 mitogen activated protein kinase (p38 MAPK) could be shown as well. Inhibition of CREG expression results in apoptosis and down-regulation of p38 MAPK, thus showing that CREG controls apoptosis and indicating a participation of p38 MAPK (Han *et al*, 2006).

Not only p38 but also JNK signalling pathways are involved in modulating the apoptosis in VSMCs in vitro and in situ. Furthermore CREG overexpression reduces the activation of caspase 3 induced by balloon injury (Han *et al*, 2010).

Addition of the PI3/AKT inhibitor LY294002 to CREG overexpressing rat bone marrow-derived stem cells (MSCs), which are stressed by hypoxic conditions or serum deprivation neutralizes the anti-apoptotic effect of CREG. In the absence of the inhibitor the mitochondrial apoptotic pathway is inactivated, the phosphorylation of AKT increased and p53 levels decreased. p53, whose expression is enhanced in the presence of LY294002, attenuates the anti-apoptotic effect of CREG (Deng *et al*, 2010).

It could be shown that overexpression of humCREG in mice shields murine hearts from fibrosis and hypertrophy in vivo. To induce cardiac hypertrophy aortic banding and injection of angiotensin II in CREG transgenic mice and a control group was used. The overexpression weakened the hypertrophic effect due to attenuation of the mitogen-activated protein kinase (MAPK)-extracellular signal-regulated kinase 1 (MEK-ERK1)/2-dependent signalling cascade. Smad 2/3 activation was blocked due to the inactivation of MEK-ERK1/2 resulting in reduced collagen synthesis both in vitro and in vivo (Bian et al, 2009).

1.4.3. Localisation

In adult mice CREG is expressed in brain, heart, lung, liver, intestines and kidneys. In mouse embryos CREG expression can first be detected in brain, heart, intestines and kidneys at 4,5 dpc (days post coitus) and the levels increase until 18,5 dpc. At 9,5 dpc it can be shown in endothelial cells of blood vessels after the formation of the vascular lumen. Embryonic vascular structures that derive from vascular smooth muscle cells express the highest CREG levels at 15,5 that decrease at 18,5. This expression pattern suggests higher expression during embryogenesis and an involvement in cell differentiation (Yang *et al*, 2011).

Although previous studies suggested msCREG to be a secreted enzyme (Di Bacco *et al*, 2003) it was recently confirmed to be an intracellular protein. It`s two putative *N*-glycosylation sites – both modified with M6P residues – indicate a lysosomal localisation – a suspicion that could be substantiated. Both M6P receptors are required for efficient CREG retention and it`s intracellular trafficking. To analyze the subcellular localisation fluorescent microscopy was used revealing co-localisation of CREG and LAMP-1 (endosomal/lysosomal membrane protein). Proteolytic processing during CREG`s biosynthesis could be confirmed (Schähs *et al*, 2008).

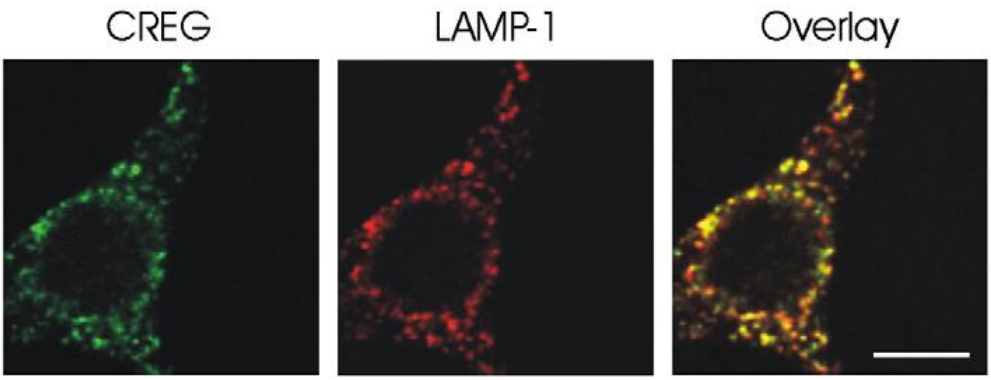


Figure 7: Colocalisation of CREG and LAMP-1: L-M(TK$^-$) fibroblasts were fixed, permeabilised and incubated with antibodies to recombinant CREG and LAMP-1 (Schähs *et al*, 2008).

Although both msCREG *N*-glycosylation sites can be targets for M6P residues the second position (Asn-216) is preferred when expressed in FS293 cells. No M6P residues could be detected on DmCREG whether on the wildtype nor on DmCREG and AtCREG mutants carrying an Asn residue at the position corresponding to Asn216 in msCREG (Schönbacher, 2010).

1.5. Epithelial to Mesenchymal Transition (EMT)

EMT is a process that does not only occur during tumour progression but was first described as an event that takes place during embryonic development. Epithelial cells lose the adherent and tight junctions which maintain their polarity. The morphology is changed towards a mesenchymal phenotype allowing the cells to move because of much weaker adhesions. Mesenchymal cells tend to form structures that are much more diverse in shape than epithelial ones. The transition is characterised by changes in morphology, migration capacity, adhesion and cellular architecture – easily demonstrated by EMT markers that decrease (e.g. E-Cadherin and Cytokeratin) or increase (e.g. Vimentin, Fibronectin and N-Cadherin) (Acloque *et al*, 2008, Lee *et al*, 2006, Guarino *et al*, 2007).

Upon triggering EMT E-Cadherin is tagged by ubiquitin and targeted to the lysosomes instead of following its usual recycling route to the lateral plasma membrane (Palacios *et al*, 2005). This event occurs during the early stages of EMT whereas during the late stages E-Cadherin is downregulated via regulation of transcription (Janda et al, 2006).

Alongside CREG, which was found to be upregulated 8,6 fold during EMT (Jechlinger *et al*, 2003), ILEI (interleukin-like EMT inducer) was found to be an interesting upregulated protein. When overexpressed in EpH4 (mouse mammary epithelial cells) and EpRas (EpH4 transformed with oncogenic *ras*) it induced EMT, tumour growth and metastasis – an effect which was independent of the Tgf-β1 pathway (Waerner *et al*, 2006).

1.5.1. EMT during tumour progression

The vast majority of human cancers are of epithelial origin. The most common cause of death (>80%) in cancer patients is the formation of metastases – an event that requires cells to change from an epithelial phenotype to a more invasive fibroblastoid phenotype (Jechlinger *et al*, 2002).

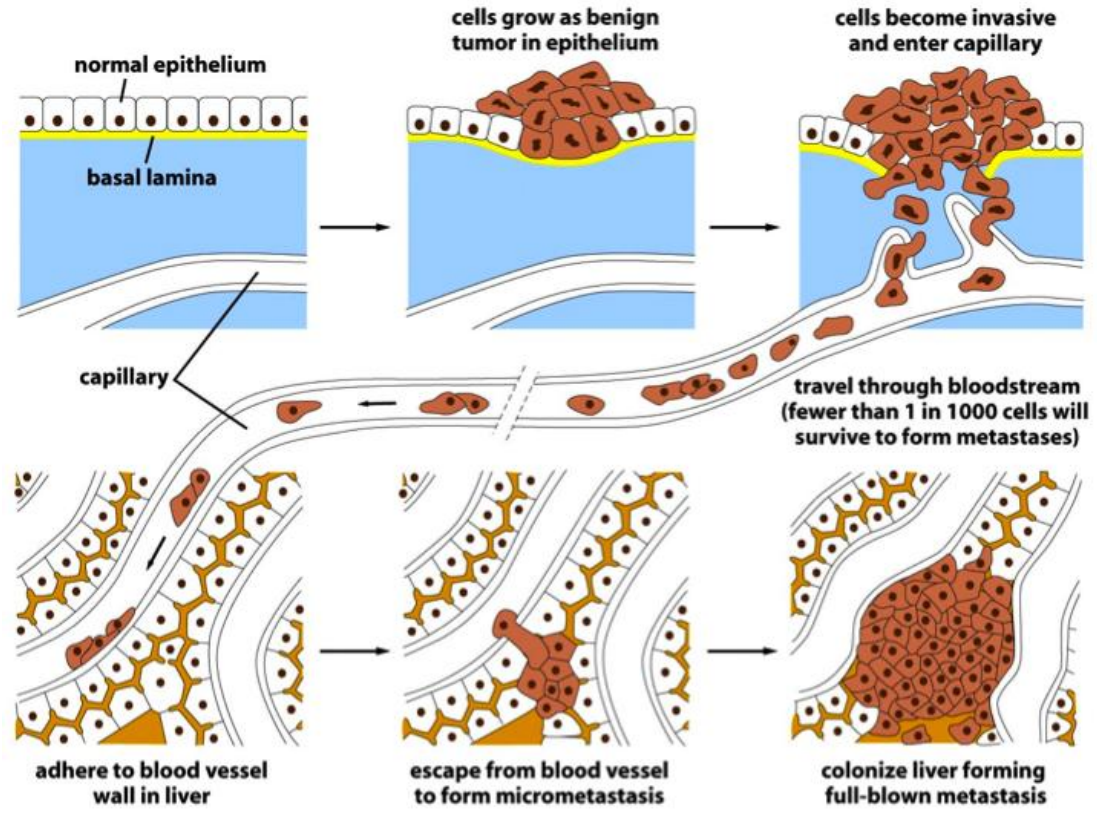


Figure 8: Steps during metastasis. During EMT cells become invasive and travel through the blood- or lymph-stream (not shown), upon escaping they colonize the surrounding tissue and form metastases (Alberts *et al*, 2008).

It has been shown that to induce EMT the activation of *ras* and the transforming growth factor β (TGF-β1) pathway are of importance (Gotzmann *et al*, 2004). The

transition is stabilized through an autocrine TGF-β1-loop that inhibits proliferation of epithelial but not mesenchymal EpRas cells (Oft *et al*, 1996).

The transforming growth factor beta controls various processes during development and carcinogenesis. During early stages of tumorigenesis it acts as a tumour suppressor which explains why the treatment of non-tumorigenic cells results in apoptosis. However, it functions as a tumour promoter in later stages because of an autocrine TGF-β1 loop (Chaudhury et al, 2009).
The apoptotic effect cannot be observed once cells have undergone EMT.

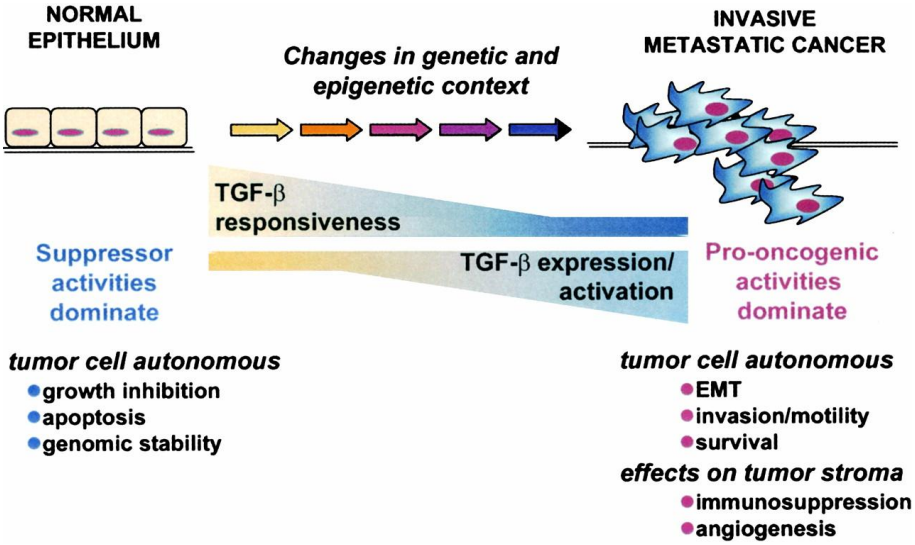


Figure 9: Effects of TGF-β1 during different stages of tumorigenesis.

2. Project Proposal

Little is known about the role of CREG (cellular repressor of E1A-stimulated genes) in tumour invasion and metastasis. Because of the highly conserved sequence (humCREG is 77% similar to msCREG) the function is best studied in mammalian systems. The main aim of this diploma thesis was to characterise CREG in the EpRas cell system. To accomplish this goal various CREG constructs were created and used to produce several cell lines. These were tested to determine differences in signal transduction, behaviour upon TGF-β1 treatment and the ability to invade the microenvironment in vitro.

3. Materials and Methods

3.1. Molecular Biology Methods

3.1.1. Generation of DNA Constructs

3.1.1.1. pWPI_ML+ Vector

pWPI, a bicistronic vector that allows tracking of transduced cells due to the simultaneous expression of GFP and the transgene, is an indispensable tool in the production of lentiviral particles. These particles are used to transfer the transgene into the target cells. pWPI_ML+ was designed at the IMP (Institute of Molecular Pathology, Vienna) and contains an altered form of the multiple cloning site (see Appendix for vector map).

3.1.1.2. Primers

Special Primers, containing specific restriction sites for cloning into pWPI_ML+, had to be created. The Primers were dissolved in ddH_2O to a concentration of 100 µM.

3.1.1.3. Polymerase Chain Reaction

The PCR is a very effective and sensitive method to amplify a selected region of a genome. The only requirement is the knowledge of the DNA sequence. The PCR starts with the separation of the doublestrand DNA by heat treatment. After the detachment complimentary primers hybridize to the DNA. In the next step the DNA polymerase and the four deoxyribonucleoside triphosphates synthesize the new complementary strand. This cycle - the separation of the dsDNA, annealing of the primers and synthesizing of the new DNA strand – is repeated several times resulting in a large number of copies of the selected sequence.

Procedure

	qualitative	quantitative
template	x µL [100 ng]	x µL [100 ng]
primer (forward)	0,5 µL [20 µM]	0,5 µL [10 µM]
primer (reverse)	0,5 µL [20 µM]	0,5 µL [10 µM]
10x buffer	5 µL	2,5 µL
dNTP	1 µL	0,5 µL
polymerase	1 µL (pfu Turbo Polymerase)	0,1 µL (AmpliTaq Polymerase)
$MgCL_2$	–	1,5 µL
DMSO	–	1 µL
ddH_2O	y µL	y µL
final volume	50 µL	25 µL

Table 1: reaction mixtures for PCR reactions

	qualitative		quantitative	
1. initial denaturation	94 °C	5 min	94 °C	5 min
2. denaturation	94 °C	30 sec	94 °C	30 sec
3. annealing	55 °C	30 sec	55 °C	30 sec
4. polymerisation	68 °C	90sec	72 °C	90sec
repeat step 2-4	29 times		29 times	
5. final polymerisation	68 °C	5 min	72 °C	5 min
6. final temperature	4 °C		4 °C	

Table 2: programmes for PCR reactions

The PCR reactions were performed in a BioRad Thermocycler.

3.1.1.4. Agarose Gel Electrophoresis

Agarose Gel Electrophoresis is a commonly used analytical technique to separate DNA fragments according to their size. An electric field forces the DNA to migrate through the gel. Due to their negatively charged phosphate backbone the fragments move towards the anode. Smaller fragments move significantly faster through the gel than larger ones resulting in a separation according to size. The DNA can be visualized using ethidium bromide, a DNA intercalating fluorescent dye. To

determine the size of the various fragments DNA ladders (GeneRuler Fermentas) are used.

Procedure

The used agarose concentration depends on the expected size of the DNA fragments. Most of the time 1% gels were used. 0,8g agarose were suspended in 80 mL 1xTAE buffer and dissolved using a microwave. After dissolving and cooling down 1 µL ethidium bromide (10mg/mL, BioRad) was added, the liquid was cast into a tray and a sample comb was added. After solidification the tray was put in a gel unit filled with 1xTAE buffer. The samples were prepared by mixing with loading buffer containing Orange G and loaded into the sample slots of the gel. The electrophoresis was performed at 110 V until the bands were sufficiently separated. The separation was checked with an UV lamp. If the separation was sufficient the gel was placed on an UV transilluminator, a photograph was taken and the sought-after band removed for further purification using a scalpel.

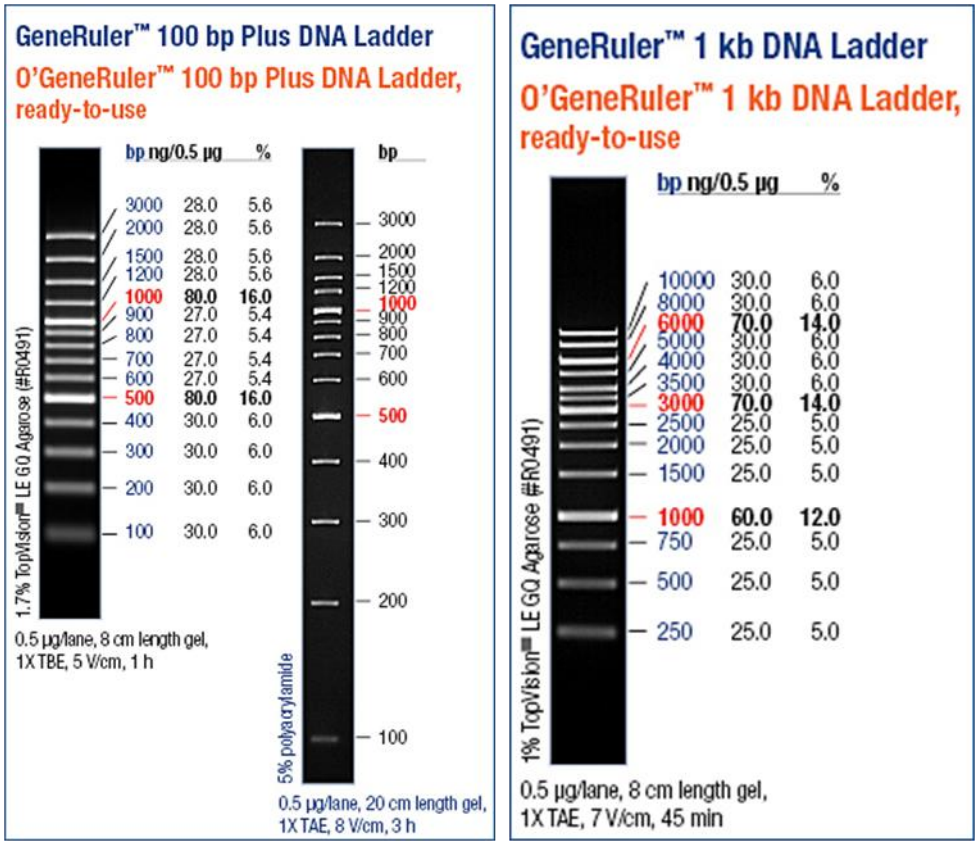


Figure 10: GeneRuler™ DNA size marker (Fermentas)

TAE buffer	*40 mM Tris/Acetate (Roth/Roth); 1 mM EDTA (Applichem); pH 8.2*
Loading buffer containing Orange G (10 mL)	*50 mg Orange G (Sigma); 2,5 g Ficoll T 400; 5 mL 0,2 M EDTA*

3.1.1.5. Purification of DNA Samples

Two different types of purification were performed. The Illustra GFX PCR DNA & Gel Band Purification Kit for DNA bands excised from an agarose gel and an ethanol precipitation method were used to purify the samples after restriction endonuclease digests.

Procedure

Illustra GFX PCR DNA & Gel Band Purification Kit

After the band was cut out from the agarose gel the weight was determined. 10 µL capture buffer type 3 were added for each 10 mg of gel. The tube was incubated at 60 °C for 15-30 minutes until the gel was dissolved. In the meantime the GFX MicroSpin column was placed into a collection tube and the solubilised gel and capture buffer type 3 were transferred onto the column. After a 1 minute incubation the sample was centrifuged at 16.000 x g for 30 seconds (Eppendorf, Microcentrifuge 5424). The flow through was removed and the column washed with 500 µL of washing buffer type 1. After another centrifugation (16.000 x g for 30 seconds, Eppendorf, Microcentrifuge 5424) the column was placed in an Eppendorf tube and incubated with 50 µL ddH_2O for 1 minute. To elute the DNA the tube was centrifuged at 16.000 x g for 30 seconds (Eppendorf, Microcentrifuge 5424).

Ethanol Precipitation

The sample was mixed with 1/10 volume 3M NaAc and 2 ½ volumes ethanol and stored for 20 minutes at -20 °C. Afterwards the tube was spun at 13.200 rpm for 3 minutes (Eppendorf, Microcentrifuge 5424), the supernatant was removed and the pellet resuspended in the desired amount of ddH_2O.

3.1.1.6. Quantification of DNA

To determine the DNA concentration of the sample UV absorption at 260 nm was detected using a photometer – according to the Lambert-Beer`s Law:

$$A = -\log\left(\frac{I}{Io}\right) = \varepsilon * c * l$$

I_o…intensity of the incident light
I…intensity of the incident light after the material
ε…absorbance coefficient at the wavelength λ
l…distance the light travels though the material [cm]
c… concentration

The absorbance coefficient of DNA: ε_{260}=20 mL/mg/cm
To determine the purity of the sample absorption at 280 nm was measured as well. Aromatic amino acids - tyrosine, tryptophan and phenylalanine – are detected at this wavelength. The ratio A_{260}/A_{280} should be between 1,8 and 2,0.

Procedure

Dilutions of the sample (1 µL sample + 199 µL ddH_2O) and a blank (200 µL ddH_2O) were prepared and measured in an Eppendorf Biophotometer 6131. DNA concentrations and purity were calculated automatically.

3.1.1.7. Restriction Enzyme Digest

Bacterial restriction endonucleases are an important tool in molecular biology –three different classes of restriction enzymes, type I, II and III can be distinguished. Although every restriction endonuclease recognize and cleave specific DNA sequences only class II enzymes are used because of their ability to cut at exactly defined positions. Cleavages result either in a sticky (3` or 5` overhang) or blunt end and are the requirements for later ligations.

Procedure

doublestranded DNA sample	x µg
restriction endonuclease	y µL (1 U per µg DNA)
buffer	4 µL
ddH_2O	z µL
final volume	40 µL

Table 3: reaction mixture for restriction endonuclease digest

The reaction mixture was incubated for 1 hour in a water bath at 37 °C and purified using ethanol precipitation.

restriction enzyme	*Bam*H1	*Mlu*1	*Sbf*1
buffer	buffer A	buffer H	buffer 4
supplier	Fermentas	Fermentas	New England Biolabs

Table 4: restriction enzymes and corresponding buffers

3.1.1.8. Alkaline Phosphatase Digest

The vector DNA has to be treated with alkaline phosphatase to remove the 5` phosphate group and thus prevent religation.

Procedure

vector DNA	20 µL
alkaline phosphatase	2,4 µL
alkaline phosphatase buffer	2 µL
final volume	24,4 µL

Table 5: reaction mixture for alkaline phosphatase digest

The mixture was incubated at 37 °C for 30 minutes and inactivated at 65 °C for 10 minutes.

3.1.1.9. Ligation

T4 DNA Ligase catalyzes the forming of a phosphodiester bond between 3`-hydroxy and 5`-phosphate termini of doublestrand DNA. Ligations were performed in the $ratios_{insert:vector}$ 1:1 and 4:1.

Procedure

	negative control	1:1	1:4
T4 DNA Ligase (Fermentas)	1µL	1 µL	1 µL
10x T4 DNA Ligase buffer	3 µL	3 µL	3 µL
insert	–	1 µL	4 µL
vector DNA	1 µL	1 µL	1 µL
ddH_2O	25 µL	24 µL	21 µL
final volume	30 µL	30 µL	30 µL

Table 6: reaction mixture for ligation

The reaction mixture was incubated at least for 4 hours at room temperature. 2 µL were used for transformation.

3.1.2. Transformation

3.1.2.1. Generation of Competent Cells

The efficiency of a transformation can be improved by using specially generated competent cells resulting in up to 10^{10} transformations per µg plasmid DNA when tryptone and yeast extract are added to the transformation medium.
100 mL of LB medium were inoculated with 1 mL of an overnight culture prepared from a frozen stock of *E. coli DH5α* cells and incubated at 200 rpm and 37 °C until the OD600 reached the value of 0,6. The flask was then chilled on ice for 30 minutes. Afterwards the cells were harvested at 4000 x g for 15 minutes at 4 °C, washed twice with 50 mL of ice cold 10% glycerol and resuspended in 2 mL GYT. The cells were stored as 50 µL aliquots at -80 °C.

LB medium	*10 g/L peptone (Roth); 5 g/L yeast extract (Merck); 5 g/L NaCl (Roth); 1 g/L D-glucose (Fluka); pH 7.0*
GYT	*10 % glycerol (Roth); 0,125 % yeast extract (Merck); 0,25 tryptone*

3.1.2.2. Electroporation

Competent cells and the ligation reaction mixture were thawed respectively chilled on ice for approximately 10 minutes. 2µL of the ligation reaction mixture were added to the competent cells and then transferred to prechilled electroporation cuvettes (0,2 cm electrode distance, BioRad). After the electroporation was performed (200 Ω (resistance), 25 µF (capacity), and 2.5 kV (voltage);Gene Pulser, BioRad) the cells were added to 1 mL SOC medium and incubated for 1 hour at 37 °C and 200 rpm. The cells were harvested at 1.200 rcf for 5 minutes, resuspended in 50 µL SOC medium, plated out on LB agar containing 100 µg/mL ampicillin and incubated overnight at 37 °C.

The next day colonies were picked using pipette tips and transferred to 3 mL LB medium + ampicillin. The cell suspension was incubated overnight at 37 °C at 200 rpm. The overnight culture was then used for plasmid preparation.

LB medium + ampicillin	*LB-medium + Ampicillin (final concentration: 100 µg/ml)*
LB agar + ampicillin	*LB-medium + 15 g/L agar (Serva) + Ampicillin (final concentration: 100 µg/ml)*
SOC medium	*20 g/L peptone (Roth); 5 g/L yeast extract (Merck); 10 mM NaCl (Roth); 2.5 mM KCl (Fluka); 10 mM MgCl2 (Merck); 20 mM D-glucose (Fluka)*

3.1.2.3. Plasmid Isolation

3.1.2.3.1. Small Scale Plasmid DNA Preparation ("Miniprep")

The preparation was performed using QIAprep Spin Miniprep Kit (Qiagen). All steps were performed at room temperature and using the same centrifuge (Eppendorf, Microcentrifuge 5424).

Procedure

The 3 mL overnight culture was pelleted for 5 minutes at 5000 rcf in a microcentrifuge. The pellet was resuspended in 250 µL buffer P1 (+ RNase A) and mixed with 250 µL buffer P2. After addition of 350 µL buffer P3 the suspension was mixed immediately and centrifuged for 10 minutes at 13.000 rpm. The supernatant was transferred to the QIAprep spin column and centrifuged for 30 seconds at 13.000 rpm. The column was spun down twice for 1 minute at 13.000 rpm. To elute the DNA 50 µL ddH_2O were added, the column incubated for 1 minute and afterwards centrifuged for 1 minute at 13.000 rpm. The DNA concentration was determined by UV spectrophotometry (Eppendorf, Biophotometer 6131).

3.1.2.3.2. Medium Scale Plasmid DNA Preparation ("Midiprep")

The preparation was performed using QIAGEN Plasmid Midi Kit.

Procedure

25 mL LB medium + ampicillin were inoculated with 50 µL starter culture (obtained from a transformation) and incubated overnight at 37 °C at 200 rpm. The bacterial cells were harvested at 6000 x g for 15 minutes at 4 °C and afterwards resuspended in 4 mL buffer P1 (+ RNase A). The solution was carefully mixed with 4 mL of buffer P2 and incubated for 5 minutes at room temperature. 4 mL of prechilled buffer P3 were added and mixed immediately. After an incubation on ice for 15 minutes the solution was centrifuged for 30 minutes at 3.500 rpm and 4 °C. Afterwards the clear supernatant was carefully decanted, 1/10 volume endotoxin removal buffer (ER) was added and the solution kept on ice for 20 minutes. In the meantime a QIAGEN-tip 100 was equilibrated by applying 4 mL of buffer QBT. Before applying the supernatant to the column the sample was filtered over a prewetted folded filter. Afterwards the column was washed twice using 2 x 10 mL buffer QC. To elute the DNA 5 mL buffer QF were added. The solution was mixed with 3,5 mL isopropanol (Riedl-de-Haën) to precipitate the DNA and centrifuged for 30 minutes at 6.500 rpm using a HS4 rotor. The pellet was washed once in 2 mL 70% ethanol and spun down for 10 minutes at 6.500 rpm. The supernatant was carefully decanted and the pellet air-dried for 10 minutes. Afterwards the DNA was redissolved in a suitable

volume of 1x TE and the concentration determined by UV spectrophotometry (Eppendorf, Biophotometer 6131).

TE buffer	*10 mM Tris/HCl (Roth/Merck); 1 mM EDTA (Applichem); pH 8.0*
LB medium + ampicillin	*see 3.1.2.2.*

3.1.2.3.3. Large Scale Plasmid DNA Preparation ("Maxiprep")

The preparation was performed using QIAGEN Plasmid Maxi Kit.

Procedure

100 mL LB medium + amp were inoculated with 200 µL starter culture and incubated overnight at 37 °C and 200 rpm. The cells were spun down at 6000 x g for 15 minutes at 4 °C and resuspended in buffer 10 mL P1 (+ RNase A). 10 mL of buffer P2 were added and the solution was carefully mixed. After incubating for 5 minutes at room temperature 10 mL of prechilled buffer P3 were pipette to the suspension and mixed immediately. The mixture was kept on ice for 15 minutes and afterwards centrifuged for 30 minutes at 3.500 rpm and 4 °C. The clear supernatant was carefully transferred into a new 50 mL tube (BD Falcon^{TM}), 1/10 volume endotoxin removal buffer (ER) was added and the solution kept on ice for 20 minutes. During this incubation step a QIAGEN-tip 500 was equilibrated by applying 10 mL of buffer QBT. The sample was filtered over a prewetted folded filter before it was transferred to the column. Afterwards the column was washed twice using 2 x 20 mL buffer QC. 15 mL of buffer QF were added on top of the column to elute the bound DNA. 10,5 ml isopropyl alcohol (Riedel-de-Haën) were added to the DNA solution to precipitate the DNA. To pellet the DNA the tube was centrifuged for 30 minutes at 6.500 rpm using a HS4 rotor. Afterwards the pellet was washed once in 5 mL 70% ethanol and spun down for 10 minutes at 6.500 rpm. The DNA pellet was air-dried for 15 minutes after the supernatant was carefully discarded. The DNA was redissolved in a suitable volume of 1x TE and the concentration determined by UV spectrophotometry (Eppendorf, Biophotometer 6131).

TE buffer	*see 3.1.2.3.2.*
LB medium + ampicillin	*see 3.1.2.2.*

3.1.2.4. Sequence Analysis

These analyses were executed by an IMP employee.

3.1.3. Real Time quantitative Polymerase Chain Reaction

qRT-PCR enables the detection and quantification of a specific DNA sequence during the cycles. On the beginning ethidium bromide was used to determine the amount of synthesized DNA (Higuchi et al, 1993). Nowadays SYBR© green I is the most used fluorochrome because the signal:background ratio is improved. SYBR© green I bound to dsDNA absorbs blue (λ=488 nm) and emits green light (λ=522 nm). In addition to the gene of interest it is important to detect housekeeping proteins as well because the amount of DNA at the beginning of the reaction can vary and to detect differences in expression levels one uses the housekeeping genes for normalisation. Preferred housekeeping proteins are GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) or HPRT (hypoxanthine phosphoribosyltransferase 1).
The c_T (cycle threshold) value is the most important parameter when interpreting the results; it describes the number of cycles needed to discriminate between specific and background signals.
The samples are prepared nearly in the same way as samples for the normal PCR, the only difference is the addition of a fluorochrome (SYBR© green I) and the preparation of duplicates. When evaluating the results the mean and the standard deviation of the duplicates are calculated.

3.1.3.1. Preparation of cDNA

3.1.3.1.1. Extraction of RNA

To extract the total RNA Trizol (Invitrogen) was used. It is a toxic solution consisting of phenol and guanidine isothiocyanate that disrupts cells but doesn`t damage RNA or DNA.

Procedure

Cells from a 100 mm cell culture dish (BD Falcon™, BD Bioscience) were harvested (5 minutes, 1.200 rpm, Megafuge 1.0, HERAEUS Sepatech), washed once in 1xPBS (5 minutes, 1.200 rpm, Megafuge 1.0, HERAEUS Sepatech) and resuspended in 1 mL Trizol in a 2 mL Eppendorf tube. After incubating the sample for 5 minutes at room temperature 0,2 mL chloroform were added. The tube was vortexed until the two phases combined, let stand for 3 minutes and centrifuged for 15 minutes at 11.000 rpm at 4 °C (Eppendorf, Microcentrifuge 5424). If the phase separation was not clearly visible the sample had to be vortexed and spun down again. The aqueous phase was transferred to a fresh 2 mL tube and 0,5 mL isopropyl alcohol (Riedel-de-Haën) were added. The solution was incubated for 10 minutes at room temperature and centrifuged for 10 minutes at 11.000 rpm at 4 °C (Eppendorf, Microcentrifuge 5424). The supernatant was carefully removed and the pellet washed once with 1 mL of 75% ethanol and spun down for 5 minutes at 11.000 rpm at 4°C (Eppendorf, Microcentrifuge 5424). The supernatant was discarded and the RNA pellet dried using a Speedvac after sealing the tube with parafilm. The dried pellet was dissolved in DEPC treated water and incubated for 10 minutes at 57 °C using a heating block. Afterwards the RNA concentration was determined using UV spectrophotometry (Eppendorf, Biophotometer 6131).

3.1.3.1.2. Synthesis of cDNA

cDNA is generated from mRNA using reverse transcriptase to catalyze this reaction. Because of the mRNA template cDNA consists only of exons.

Procedure

RNA	5 µg
random hexamers	4 µL
dHH_2O	x µL
final volume	24 µL
The mixture was incubated for 10 minutes at 70 °C and afterwards chilled on ice.	
1^{st} strand buffer	8 µL
0,1 M DTT	4 µL

dNTP	2 µL
The solution was first kept for 10 minutes at room temperature and then for 2 minutes at 42 °C.	
superscript (reverse transcriptase)	2 µL
After addition of the enzyme the mixture was incubated for 50 minutes at 42 °C and 15 minutes at 70 °C.	
RNase H	2 µL
The tube was put in a 37 °C water bath for 30 minutes.	

Table 7: Synthesis of cDNA

3.1.3.2. Execution of qRT-PCR

Because of the great number of samples to be tested a mastermix was prepared:

reaction mix (SYBR© green I, DMSO, buffer, dNTP, polymerase)	11,25 µL
ddH_2O	12 µL

Table 8: mastemix for qRT-PCR

The mastermix was then aliquoted according to the number of cell lines, 0,5 µL of the cDNA solution were added and 23,75 µL of this mixture pipetted into each well of the PCR plate. Before sealing the plate 1,25 µL of the primer mix (forward primer + reverse primer 1:1) were added. The results were analyzed using Excel.

3.2. Biochemical and Cell Biology Methods

3.2.1. SDS Page

The SDS Page as first described by Laemmli (Laemmli, 1970) was used for protein separation.

3.2.1.1. Preparation of samples

Cells were harvested for 5 minutes at 1.200 rpm (Megafuge 1.0, HERAEUS Sepatech) and washed once with 1xPBS (5 minutes at 1.200 rpm 4 °C,

Microcentrifuge 5424 Eppendorf). Afterwards the pellet was resuspended with a sufficient volume of RIPA buffer, stored on ice for 30 minutes and centrifuged for 10 minutes at full speed at 4 °C (Eppendorf, Microcentrifuge 5424). The supernatant was transferred to another Eppendorf tube and the concentration was determined using UV spectrophotometry. The samples were prepared as followed:

	ddH_2O	Bradford solution (BioRad)	BSA/sample
blank	800 µL	200 µL	–
standards	800 µL	200 µL	2,5/5/7,5/10 µL BSA
samples	800 µL	200 µL	1 µL sample

Table 9: mixtures for determination of protein concentration using UV spectrophotometry

Afterwards the samples for loading the gel were prepared.

sample	x µL (40 µg protein)
RIPA buffer	y µL
4x SDS loading buffer	5 µL
final volume	20 µL

Table 10: sample mixtures for SDS-Page

The samples were reduced by incubating for 5 minutes at 95 °C.

RIPA buffer (10 mL)	*2 mL 250 mM Tris-HCl pH 7,5; 2 mL 750 mM NaCl; 2 mL 5% Nonidet© P40 + 2,5% natriumdesoxycholate; 2 mL 0,5% SDS solution; 1 mL ddH_2O; 1x complete™ mini; 100 µL DTT [1 M]; 1 mL 10x PhosStop*
4x SDS loading buffer (20 mL)	*4 mL β-mercaptoethanol; 8,7 mL glycerol; 1,6 g SDS; 0,25 M Tris pH 6,8*
PBS	*8,006 g NaCl (Roth); 0,202 g KCl (Fluka), 1,442 g $Na_2HPO_4x2H_2O$ (Fluka); 0,258 g KH_2PO_4 (Fluka)*

3.2.1.2. Preparation of SDS-Page Gels

10% gels were used for the separation.

	running gel	stacking gel
separation buffer/ stacking buffer	2,41 mL	625 µL
30 % acryl amid	3,22 mL	675 µL
water	2,99 mL	3,675 mL
10% AMPS	55 µL	30 µL
Temed	5,5 µL	12,5 µL

Table 11: composition of running and stacking gels

The separation gel was prepared and poured between two glass plates which were separated by two 1 mm spacers and overlaid with isopropanol (Riedel-de-Haën). After the polymerization the isopropyl alcohol was removed, the stacking gel prepared and poured on top of the separatioin gel. A sample comb was inserted. After solidification the comb was removed and the gel inserted in an electrophoresis chamber which was filled with running buffer. The samples and 5 µL of a protein size ladder (PageRuler™ #SM0671 Fermentas) were loaded and the electrophoresis performed 200 V (Power Pac 300, Bio-Rad) until the blue loading dye reached the end of the gel. The gel was removed from the chamber, the stacking gel cut off and the separation gel used for western blot analysis.

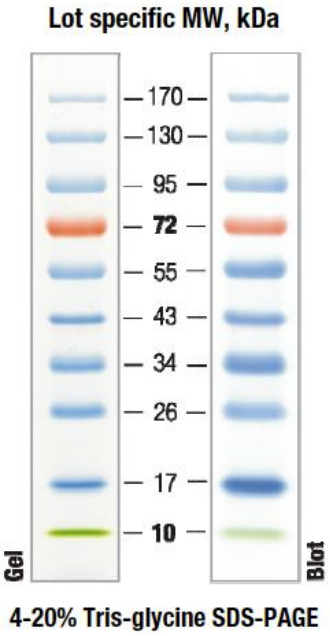


Figure 11: PageRuler™ #SM0671 (Fermentas)

separation gel buffer	*1,5 M Tris pH 8,8; 0,4% SDS*
stacking gel buffer	*0,5 M Tris pH 6,8; 0,4% SDS*
running buffer	*25 mM Tris; 250 mM glycine; 0,1% SDS*

3.2.2. Western Blot

After performing the SDS Page the proteins were transferred to PVDF (polyvinylidenedifluoride) membranes using semidry blotting.
2x3 pieces of blotting paper were equilibrated in semidry blotting buffer and after a short equilibration of the membrane in methanol placed on top of one stack. After removing the stacking gel the running gel was positioned on the membrane and the remaining 3 blotting papers were added. The stack was transferred to the blotting apparatus and bubbles between the layers were carefully removed. Then the lid was closed and pinned down with a weight. The blotting was performed for 2 hours at 60 mA per gel. Afterwards the success of the transfer was controlled using a solution of Ponceau S (3 % trichloroacetic acid [Fluka], 0.1 % Ponceau S [Fluka]) to make protein bands visible. The membrane was washed with ddH_2O until it was completely destained and blocked either in 5% milk buffer or 5% BSA for 1 hour on a shaker. After washing the membrane for 5 minutes with TBST it was incubated for 45-60 minutes on a shaker with the primary antibody which was diluted in 2,5% milk buffer or 2,5% BSA. The blot was washed 3x10 minutes in TBST, except when using antibodies to detect phosphorylated proteins where 5 % milk buffer was used as washing solution. The membrane was incubate for 45 minutes with the secondary antibody (HRP conjugated IgG) that was diluted 1:5000 in 2,5% milk buffer or 2,5% BSA. The blot was again washed 3x10 minutes in TBST. The membrane was overlaid with 1,2 mL ECL (solution A + solution B 1:1, GE Healthcare) or 1,2 mL ECL+ (solution A + solution B 1:40, GE Healthcare) and incubated for 1 minute. The peroxidase catalyzes the oxidation of luminol which results in an enhanced chemiluminescence. Afterwards it was transferred into a film cassette, exposed to an autoradiographic film (GE Healthcare) in a dark room. The exposure time ranged between 5 seconds and 10 minutes and the film was developed using a developer device from Kodak.

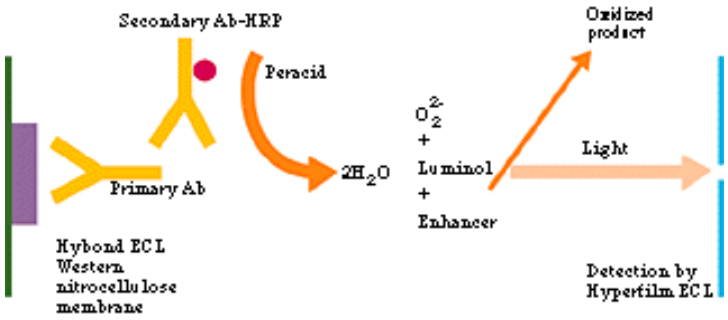


Figure 12: detection of HRP catalyzed oxidation of luminol

primary antibodies	secondary antibodies
rb α β-Actin	α-rb IgG HRP
rb α CREG30	α-ms IgG HRP
rb α CREG92	
rb α pAkt	
rb α / ms α E-Cad	
ms α pERK	
rb α ERK	
rb α pSMAD2	
rb α pp38	
rb α p38	

Table 12: primary and secondary antibodies used for western blot analysis

semidry blotting buffer (2L)	*5,84 g glycine; 11,62 g Tris ; 0,74 g SDS; 400 mL methanol*
5% milk buffer	*5% milk powder in TBST*
2,5% milk buffer	*2,5% milk powder in TBST*
5% BSA	*5% BSA in TBST*
2,5% BSA	*2,5% BSA in TBST*
TBST	*0,1 M Tris HCl pH 8; 0,2 M NaCl; 0,1% Tween 20*

3.2.2.1. Stripping of the membrane

To remove antibodies from the membrane the blot was placed in a cup filled with stripping buffer, sealed and put in a shaking water bath at 55 °C for 15 minutes. Afterwards the solution was discarded and the membrane washed 3x10 minutes in TBST before it was blocked again for 30 minutes.

stripping buffer	*100 mM NaOH; 2% SDS; 0,5% DTT*

3.2.3. Determination of Cell Number

10 µL of the cell suspension were pipette in each half of the Fuchs-Rosenthal counting chamber (Assistant). Each half consists of 4 large squares which are divided in 16 smaller squares. The cells were counted at 100-fold magnification using a brightfield microscope (Wilovert microscope). To determine the cell number 4 large squares were counted and the mean x was calculated.

$$cells\ per\ mL = x * 10^5$$

3.2.4. Freezing and Thawing of Cells

3.2.4.1. Freezing of Cells

Cells were grown on 100 or 200 mm cell culture dishes (BD Falcon™, BD Bioscience) until they formed a confluent monolayer. The cells were trypsinised, the reaction stopped after detachment with FCS containing medium and pelleted for 5 minutes at 1.200 rpm (Megafuge 1.0, HERAEUS Sepatech). In the meantime the freezing medium (DMSO (Sigma) + FCS 1:9) was prepared. DMSO is added to prevent the formation of ice crystals and thus the damaging of cells. The supernatant was removed and the cells reuspended carefully in freezing medium – the volume depending on the size of the pellet. Aliquots were transferred to cryovials (Greiner, bio-one) and put in small polystyrene boxes to ensure a slow cooling rate of approximately 1 °C per minute. The boxes were afterwards placed in a -80 °C freezer. After 24 hours the vials were removed from the polystyrene boxes and placed in storage boxes at the -80 °C freezer.

3.2.4.2. Thawing of cells

The cryovials were removed from the -80 °C freezer and put in a 37 °C water bath. The vials were removed when ice was still visible. The suspension was then transferred into a 15 mL tube (BD Falcon™) containing 9 mL medium. The cells

were spun down for 5 minutes at 1.200 rpm (Megafuge 1.0, HERAEUS Sepatech) and the supernatant discarded. The pellet was resuspended in 10 mL medium and transferred to a 100 mm cell culture dish (BD Falcon™, BD Bioscience).

3.2.5. Lentiviral Transfection of EpRas Cells

3.2.5.1. EpRas

EpRas cells derive from the parental mammary cell line EpH4 that was originally created by infection of spontaneously immortalized Ep1 cells with the empty retroviral vector pHMW. After subcloning in the presence of hygromycin B clones were chosen which form monolayers and express apical and basal epithelial marker proteins (Fialka et al, 1996).
These EpH4 cells were infected with a helper-free retroviral vector that expresses oncogenic v-Ha-Ras. This cell line is capable to form rapidly growing tumours in mice. The cells change their epithelial appearance under TGF-β1 (transforming growth factor) treatment. They appear spindle-like, lose their epithelial surface markers and gain mesenchymal surface proteins. After the transformation the morphology is stabilized through an autocrine TGF-β1 loop (Oft et al, 1996).

3.2.5.2. Cell Trypsinization

To loosen the cell-cell interactions and attachment to the surface of the tissue culture plate trypsin is added. The amount of trypsin depends on the size of the culture dish.

Procedure

After the medium from a 100 mm dish was removed and kept as stopping solution in a 15 mL tube (BD Falcon™) 1 mL of trypsin/EDTA ([2x], Gibco) in 1xPBS was used to overlay the cells. After an incubation for several minutes at 37 °C (Labotec) the detachment was determined using an inverted microscope and the reaction stopped with the stopping solution. Afterwards the cells were pelleted (5 minutes at 1.200 rpm, Megafuge 1.0, HERAEUS Sepatech), resuspended in fresh medium and used for further experiments.

3.2.5.3. Production of Lentiviral Particles

Lentiviral particles are replication incompetent which means they lack the genetic information to replicate after infection. Either a packaging cell line must provide the crucial information or a co-transfection with helper vectors is necessary. The produced particles carry the transgene and are able to infect the target cells but are incapable to produce any progeny.

Procedure

The lentivirus producing cell line was 293-FT (Invitrogen).
One day before transfection $0,7x10^6$ cells were seeded on 60 mm cell culture dishes (BD Falcon™, BD Bioscience). The next day the following solutions were prepared:

solution 1	
plasmid with construct X	1,3 µg
plasmid psPAX2 (expresses capsid proteins)	1 µg
plasmid pMD.2 (expresses envelope proteins)	0,4 µg
DMEM + L-Glu (no FCS and antibiotics)	300 µL
solution 2	
DMEM + L-Glu (no FCS and antibiotics)	300 µL
Lipofectamine 2000 (Invitrogen)	12 µL

Table 13: solutions for the production of lentiviral particles

The solutions were incubated for 15 minutes at room temperature, mixed and incubated again for 15 minutes. During the second incubation step, the medium from the cells was removed, the cells washed with 1xPBS and 5 mL of DMEM + L-Glu (no FCS and antibiotics) were added. After the incubation the solution mixture was added to the cells. It is very important to distribute the mixture evenly over the cells. Afterwards the cells were incubated at 37 °C for 3-4 hours. The medium was removed and replaced with 6 mL fresh medium (DMEM + L-Glu + 10%FCS + Pen/Strep).
After 48 hours, from the time of medium exchange, the medium was harvested by transferring the supernatant into a 15 mL Falcon tube and spinning at 15.000 rpm for 15 minutes (Megafuge 1.0, HERAEUS Sepatech). The supernatant was aliquoted and stored at -80 °C.

PBS	*see 3.2.1.1.*

3.2.5.4. Transfection of EpRas

Approximately 200.000 cells were plated in a well of a 24-well plate (24 well plate, Nunc). 800 µL sterile-filtered (0.2 µm, Pall) lentiviral particle containing supernatant were mixed with 8 µg/mL polybrene (H-9268, Sigma) that facilitates virus attachment and added to the cells. After 24 hours the supernatant was replaced by normal cultivation medium.

PBS	*see 3.2.1.1.*

3.2.5.5. FACS Sorting

FACS (FACSCalibur, BD Biosciences) was used for sorting of transduction positive cells. Because of the coexpression of GFP and the gene of interest sorting for GFP positive cells resulted in sorting of transduction positive ones.

Procedure

The cells were trypsinised and washed with the following solutions:

solution 1	2 mL 1xPBS + 16,7 mM EDTA
solution 2	2 mL 1xPBS + 1 mM EDTA
solution 3	2 mL 1xPBS

Table 14: solutions for washing and resuspension steps

After washing steps 1 and 2 the solution was centrifuged for 5 minutes at 1.200 rpm (Megafuge 1.0, HERAEUS Sepatech) and finally resuspended in solution 3. EDTA is added to the washing solutions to break cell-cell interactions. The solution was transferred to a 5 mL FACS tube (BD Falcon™, BD Bioscience) and the sorting was performed by an IMP employee from the Biooptics Facility. Afterwards the cells are spun down, resuspended in growth medium and cultivated.

PBS	*see 3.2.1.1.*

3.2.6. Collagen Gel Assay

Collagen gels provide the cells with an environment for three-dimensional growth. Therefore phenotypical characteristics and changes in the morphology upon treatment are better observable.

3.2.6.1. Preparation of Collagen Gels

Each gel (final volume ~100 µL/gel) consists of 1,6 mg/mL collagen type I (BD Bioscience), 1000 cells and growth medium as dilution solution. 6 gels per cell line were prepared and the sufficient amount of collagen for these gels was placed in Eppendorf tubes on ice to prevent polymerization. Cells were trypsinised, counted and aliquots consisting of 1000 cells + the adequate amount of the dilution solution were prepared. Using a 1000 µL pipette the collagen and cell suspension was carefully mixed and drops were formed in 24-well plates (Nunc) immediately afterwards. The procedure was repeated with the other cell lines and after 10-15 minutes the tissues culture plates were stored in an incubator (Labotec) at 37 °C and 5% CO_2 for 2 hours. After the polymerization 500 µL of the prepared Collagen Gel medium were added to each well.
The medium consists of DMEM/F-12 (1:1) (Gibco) and various solutions and factors added: 4 mM L-glutamine, 20 mM HEPES, 100 U/mL penicillin, 0.1 mg/ml streptomycin (Penicillin-Streptomycin solution, Sigma), 1.4 µg/ml insulin (100 i.E. /ml, actrapid Penfill, Novo Nordisk), 1 µM dexamethasone (Sigma); 1 µM isopropterinol (Sigma), 5 ng/ml TGF α (Sigma) and 13 µg/ml bovine pituitary extract (PromoCell).
After a few days, when visible structures were formed by the cells the TGF-β1 (Peprotech) treatment was started.

3.2.6.2. Stimulation with TGF-β1

Procedure

After several days of incubation when the cells had formed clearly visible three-dimensional structures the TGF-β1 treatment started. To three gels of every cell line TGF-β1 in a final concentration of 5 ng/mL diluted in Collagen Gel medium was

added and changes in morphology were documented using a Leitz Labovert FS microscope and Baumer TWAIN software.

3.2.7. IF Staining of Collagen Gels and Cover Slips

3.2.7.1. Fixation

3.2.7.1.1. Methanol/Acetone Fixation

The medium is removed and the gels and cover slips are washed in 1xPBS. The following fixation and rehydration steps are performed:

solution	incubation time	temperature
methanol/acetone 1:1	8 minutes	-20 °C
ethanol 100%	2 minutes	room temperature
ethanol 75%	2 minutes	room temperature
ethanol 50%	5 minutes	room temperature
ethanol 25%	2 minutes	room temperature
ddH_2O	2 minutes	room temperature

Table 15: solutions for fixation and rehydration of collagen gels

The collagen gels and cover slips can be stored in 1xPBS at 4°C.

3.2.7.1.2. PFA Fixation

Collagen gels and cover slips can also be fixed using 4 % PFA (paraformaldehyde). All steps are performed at room temperature.
The attached cells are washed twice with 1xPBS. The structures were incubated with 4% PFA for 30 minutes at room temperature and afterwards washed 3x10 minutes with 1xPBS:glycine. To permeabilize the cells 0,5% Triton X-100 is added for 5 minutes. The structures are washed 3x10 minutes with TBST. The collagen gels and cover slips can be stored in 1xPBS at 4°C.

PFA 16% (100 mL)	*16 g PFA (Sigma); 0,6 mL 5M NaOH; 3 mL 1M Hepes; 1xPBS*
0,5% Triton X-100 (50 mL)	*0,25 mL Triton X-100; 1xPBS*
TBST	*see 3.2.2*
PBS	*see 3.2.1.1.*
10xPBS:glycine (500 mL)	*38 g NaCl; 9,38 g Na_2HPO_4; 2,07g NaH_2PO_4; 37,5 g glycine; pH 7,4*

3.2.7.2. Staining

Due the light sensitivity of the secondary antibodies the incubation and washing steps following the incubation with the primary antibody were performed in the dark.

3.2.7.2.1. Staining plus additional Postfixation

To prevent unspecific binding of antibodies the cells were incubated with a blocking solution (IHC horse serum blocking solution). After 1 hour the blocking solution was removed and the primary antibody (diluted in blocking solution) added for 1 hour. The structures were washed 3x5 minutes in TBST. The cells were incubated in 4%PFA for 10 minutes, washed 5x5 minutes in TBST and blocked again for 15 minutes at room temperature to postfix the structures. Afterwards the secondary antibody was applied for 1 hour. The structures were washed 3x5 minutes with TBST and mounted on glass slides with Invitrogen Prolong Gold antifade reagent with DAPI. The slides were dried overnight at room temperature and sealed with nail polish.

TBST	*see 3.2.2*
IHC horse serum blocking solution (20 mL)	*400 µL horse serum; 200 µL fish gelatine (10%); 800 µL 2,5% Triton X-100; 400 µL 2,5%Tween 20; 1 mL 0,05% NaN_3; 17,2 mL 1%BSA in1xPBS*

3.2.7.2.2. Staining without additional Postfixation

The structures were incubated for 1 hour in the blocking solution and afterwards the primary antibody (diluted in blocking solution) was added. After a 1 hour incubation

the structures were washed 3x5 minutes in TBST. The secondary antibody (diluted in blocking solution) was applied for 1 hour. Afterwards the cells were washed 3x5 minutes with TBST and mounted on glass slides using Invitrogen Prolong Gold antifade reagent with DAPI. The slides were dried overnight at room temperature and sealed with nail polish.

primary antibodies	postfixation	secondary antibodies
ms α E-Cadherin 1:500	yes	goat α ms (λ=549 nm) 1:1500
rb α Fibronectin 1:500		goat α rb (λ=647 nm) 1:1500
ms α Vimentin 1:500	no	goat α ms (λ=549 nm) 1:1500

Table 16: antibodies used for IF staining

TBST	*see 3.2.2*
IHC horse serum blocking solution	*see 3.2.7.2.1*

3.2.8. Invasion Assay

The ability of tumour cells to invade the surrounding environment is one of the main characteristics of the metastatic phenotype. In vivo invasive cells gain access to the vasculature and lymphatic system upon breaking the membrane boundaries. To mimic this process in vitro Matrigel is used to imitate the microenvironment since it acts as a matrix barrier similar to the ECM (extracellular matrix). Matrigel is a mixture of ECM proteins, primarily laminins and collagen IV. The thin coating Matrigel method was used to create a thin gel barrier on a porous membrane. To stimulate the invasion through the Matrigel a chemoattractant is needed. Usually NIH 3T3 supernatant is used for this purpose.

3.2.8.1. 3T3 Conditioned Medium

A cryovial containing NIH 3T3 fibroblasts was thawed and the cells plated on a 100 mm cell culture dish (BD Falcon™, BD Bioscience) using DMEM containing L-glutamine supplemented with 20 mM HEPES, 100 units/mL penicillin, 0.1 mg/ml streptomycin (Penicillin-Streptomycin solution, Sigma) and 10 % FCS (Foetal Bovine Serum, Gibco). The cells were trypsinised and transferred to a 200 mm cell culture dish (BD Falcon™, BD Bioscience) when they formed a confluent monolayer, which

was later split in the ratio 1:2. After two additional days the supernatant was removed, sterile filtered and stored on ice; the cells were frozen again.

3.2.8.2. Execution of Invasion Assay

On day 0 the transwells (8µM pore, 6,5 mm) were coated with Matrigel and the cells were prepared (split to prevent confluent monolayer). Each transwell was placed in a well of a 24-well plate. The Matrigel was thawed on ice and diluted into cold ddH_2O to a concentration of 0,125 µg/µL using prechilled pipette tips. 40µL of the dilution were added to the transwell, the sides were gently tapped to ensure the completely cover of the bottom and let dry overnight.
The next morning 40 µL DMEM were pipetted onto the Matrigel and the plate incubated at 37 °C for 1 hour (Labtec). In the meantime the cells were prepared. The cells were trypsinised and counted (20.000 cells per transwell and 3 transwells per cell line). After the incubation the medium was removed and 600µL of NIH 3T3 supernatant was added to the wells. 300 µL cell suspension (20.000 cells + DMEM (10% FCS)) were applied to the upper chamber of the transwell. The plate was incubated for 24 hours at 37 °C (Labtec).
To determine the number of invasive cells the Matrigel was removed using a Q-tip and the filter washed twice with 1xPBS. Afterwards 4% PFA were added and incubated at room temperature for 30 minutes. The filter was washed 3x10 minutes with 1xPBS:glycine. After the washing step 0,5% Triton X-100 diluted in 1xPBS was applied for 5 minutes. The filter was washed 3x10 minutes with TBST, removed from the transwell chamber using a scalpel and mounted on a glass slide with Invitrogen Prolong Gold antifade reagent with DAPI. It was dried overnight at room temperature and sealed with nail polish.
The number of invasive cells was determined using a microscope and evaluated using Excel.

PFA 16% (100 mL)	*16 g PFA (Sigma); 0,6 mL 5M NaOH; 3 mL 1M Hepes; 1xPBS*
0,5% Triton X-100 (50 mL)	*0,25 mL Triton X-100; 1xPBS*
TBST	*see 3.2.2*
PBS	*see 3.2.1.1.*
PBS:glycine (500 mL)	*see 3.2.7.1.2.*

4. Results

4.1. Comparison of CREG and IGF-II receptor levels in EpRas and EpRas XT cells

qRT-PCR was performed with samples of EpRas and EpRas XT cDNA to determine differences in CREG and IGF-II receptor expression. The analysis was performed as described above (see 3.1.3.).
EpRas XT is a cell line retrieved from lung tumours, formed upon tail vein injection of EpRas cells in mice.

4.1.1. CREG expression in EpRas and EpRas XT cells

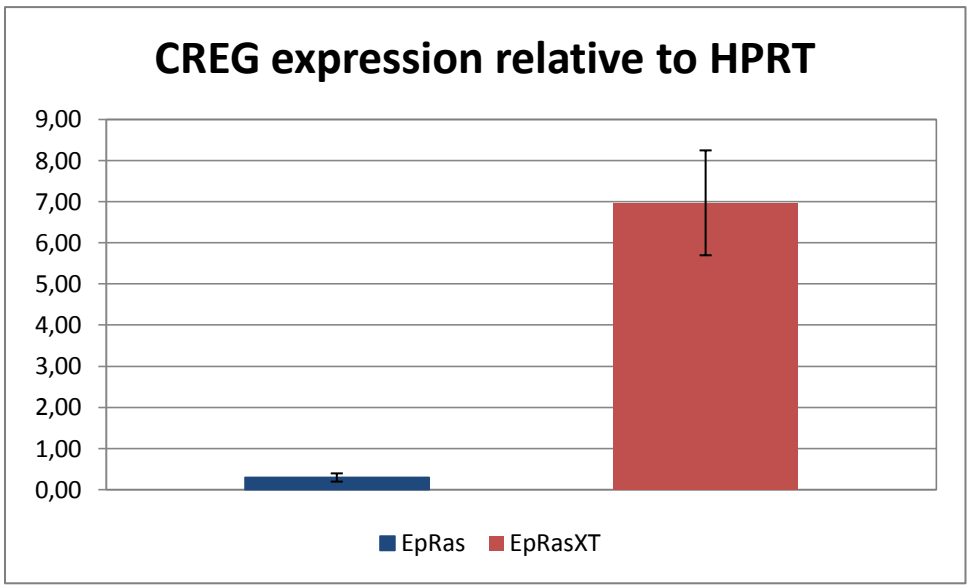


Figure 13: Differences in abundance of CREG cDNA in EpRas and EpRas XT cells. The obtained values were standardized on HPRT cDNA levels. All samples were analysed in duplicates and the experiment was performed once.

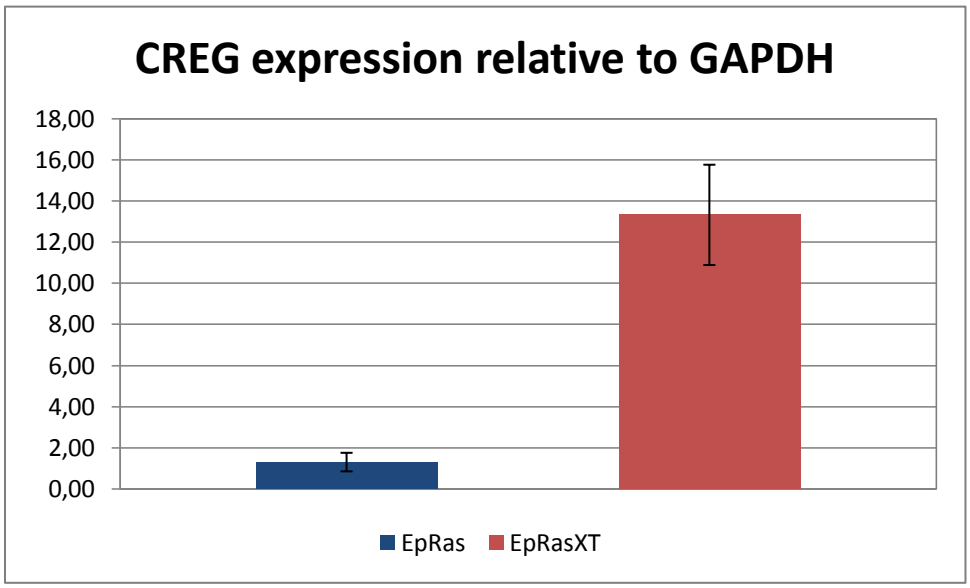


Figure 14: Differences in abundance of CREG cDNA in EpRas and EpRas XT cells. The obtained values were standardized on GAPDH cDNA levels. All samples were analysed in duplicates and the experiment was performed once.

The claim that CREG is upregulated during EMT (Jechlinger *et al*, 2003) could be verified. Using HPRT for normalization CREG expression is upregulated 23,9 times, compared to GAPDH 10,2 times.

4.1.2. IGF-II receptor expression in EpRas and EpRas XT cells

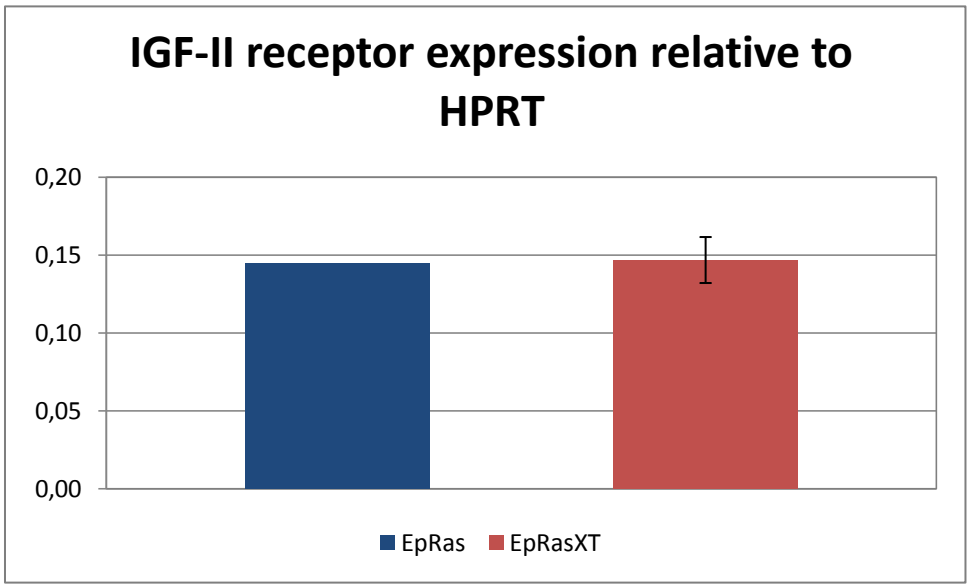


Figure 15: Differences in abundance of IGF-II receptor cDNA in EpRas and EpRas XT cells. The obtained values were standardized on HPRT cDNA levels. All samples were analysed in duplicates and the experiment was performed once.

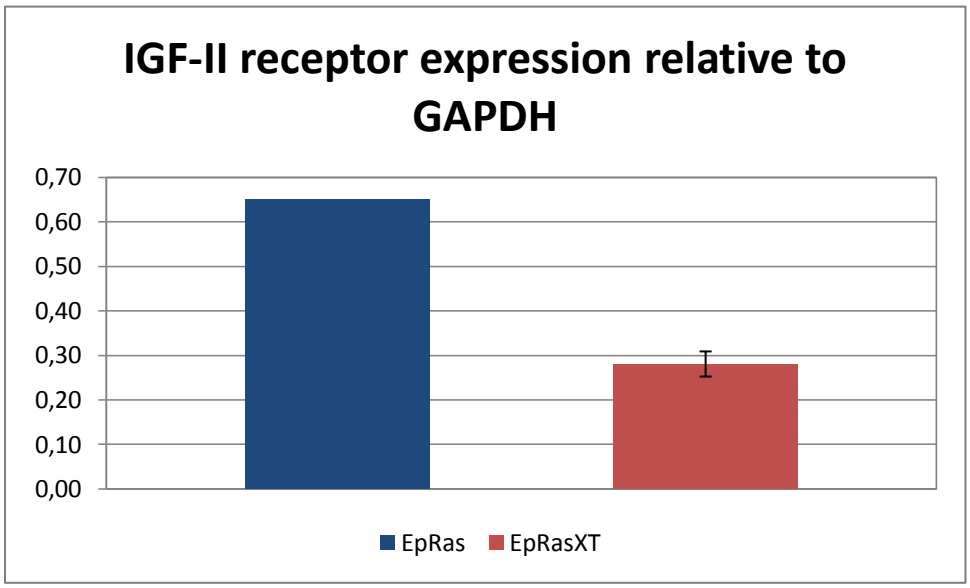


Figure 16: Differences in abundance of IGF-II receptor cDNA in EpRas and EpRas XT cells. The obtained values were standardized on GAPDH cDNA levels. All samples were analysed in duplicates and the experimet was performed once.

The expression of the IGF-II receptor in EpRas and EpRas XT cells is comparable when normalized on HPRT. The receptor levels in EpRas cell are 4,6 times higher when normalised on GAPDH.

4.2. Downregulation of CREG in EpRas cells

4.2.1. Assessment of CREG downregulation

Four different CREG knockdown constructs (shCREG#0, shCREG#1, shCREG#2, shCREG#3) were created. Their efficiency was tested by Western Blotting and #0, #2 and #3 were chosen for further experiments because of the lowest detectable CREG expression.

4.2.2. CREG knockdown with CREG#0 shRNA

4.2.2.1. CREG levels in EpRas shCREG#0 cells

To determine the CREG levels and the effect of short and long term TGF-β1 treatment, EpRas shCREG#0 cells were grown in the presence of TGF-β1 8 and 24 hours respectively. Untreated cells were grown in parallel for 24 hours. The cells were harvested and cell lysates extracted. Protein concentration was determined, samples prepared and loaded on a SDS-PAGE gel. After transfer onto a PVDF membrane the CREG levels were detected using Western Blot technique.

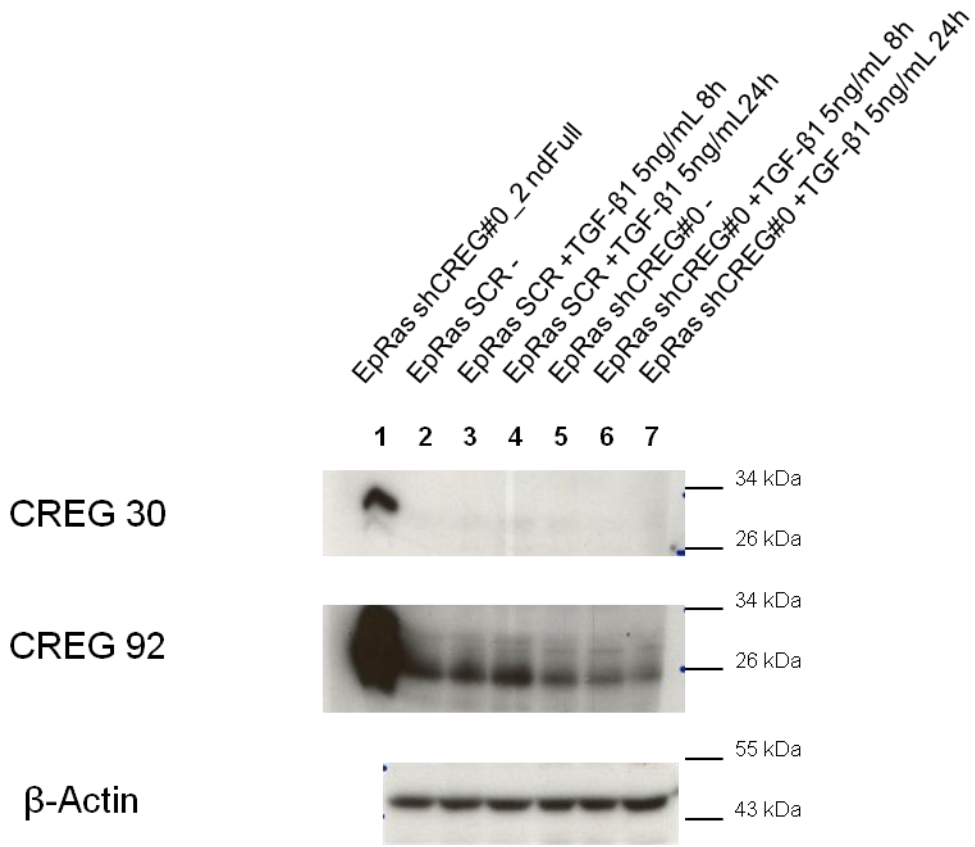


Figure 17: Determination of CREG levels in EpRas shCREG#0 cells using Western Blot analysis. 30 µg of each cell extract were loaded onto the gel. The analysis was performed using rb α CREG 30 and rb α CREG 92 as primary and α rb IgG HRP as secondary antibodies.

CREG expression in EpRas shCREG#0 cells is lower than in EpRas SCR cells and TGF-β1 treatment shows no effects on CREG expression patterns. The reconstitution with msCREG cDNA leads to a strongly increased CREG expression.

4.2.2.2. Collagen Assay of EpRas SCR and EpRas shCRE#0 cells

Three days after casting the gels – when the cells formed tubular structures – the TGF-β1 treatment was started by adding 5ng/mL TGF-β1 to the collagen gel medium.

EMT is characterised in 3D gels by a spiky cell shape and cell structures losing their tight adhesion caused by loss of E-Cadherin.

Differences in behaviour upon TGF-β1 treatment can be seen in Figure 15. EpRas SCR cell structures become spikier whereas EpRas shCREG#0 cells die when growing in the presence of TGF-β1 – indicated by the formation of round structures.

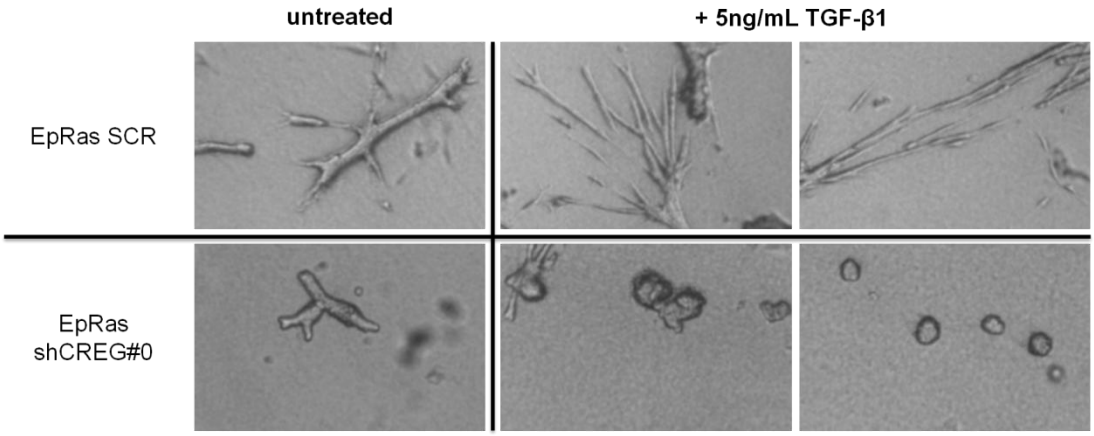


Figure 18: Results of a Collagen Assay of EpRas SCR and EpRas shCREG#0 cells after TGF-β1 treatment. The left picture shows the structures without treatment, the two pictures on the right display cell structures after addition of TGF-β1. The photos were taken 7 days after seeding (seeding density was 1000 cells/gel).

4.2.2.3. Reconstitution of CREG in EpRas shCREG#0 cells

Constructs used to transduce EpRas shCREG#0 cells were already available. The transduction was performed as described in 3.2.5.

4.2.2.4. Differences in Signal Transduction Pathways in EpRas shCREG#0 cells

Cells were grown, harvested and lysed. Samples were prepared and SDS Page and Western Blot performed.

The AKT pathway is regarded as one of the most important signal transduction pathways and takes part in proliferation, motility, growth, glucose homeostasis, survival, and cell death. In 70% of all breast cancers this pathway is mutated, and it is overall the second most frequently mutated pathway in cancers, only overcome by p53. To activate AKT threonine-308 and serine-473 have to be phosphorylated (Wickenden *et al*, 2010).
Extracellular signal-regulated kinases (ERKs) are regulated through phosphorylation cascades that take part in proliferation, differentiation, and cell cycle progression triggered by extracellular stimuli. ERK1/2, phosphorylated and activated by MEK1/2, are overall present in all adult tissues, though the levels differ in tissue types (Pearson et al, 2001).

The EpRas cell lines expressing ΔFMN and dmut CREG show remarkably higher levels of activated AKT than all other cell lines. pERK levels are increased in cells expressing ΔFMN, dmut and full-length FLAG-tagged CREG. Interestingly CREG expression in EpRas shCREG#0 cells is increased by puromycin treatment and approaches almost overexpression levels. Several CREG bands can be observed because of differences in processing between the different CREG constructs used. EpRas shCREG#0 dmut cells show only one band, due to the inability to be trafficked to the lysosomes where processing takes place.

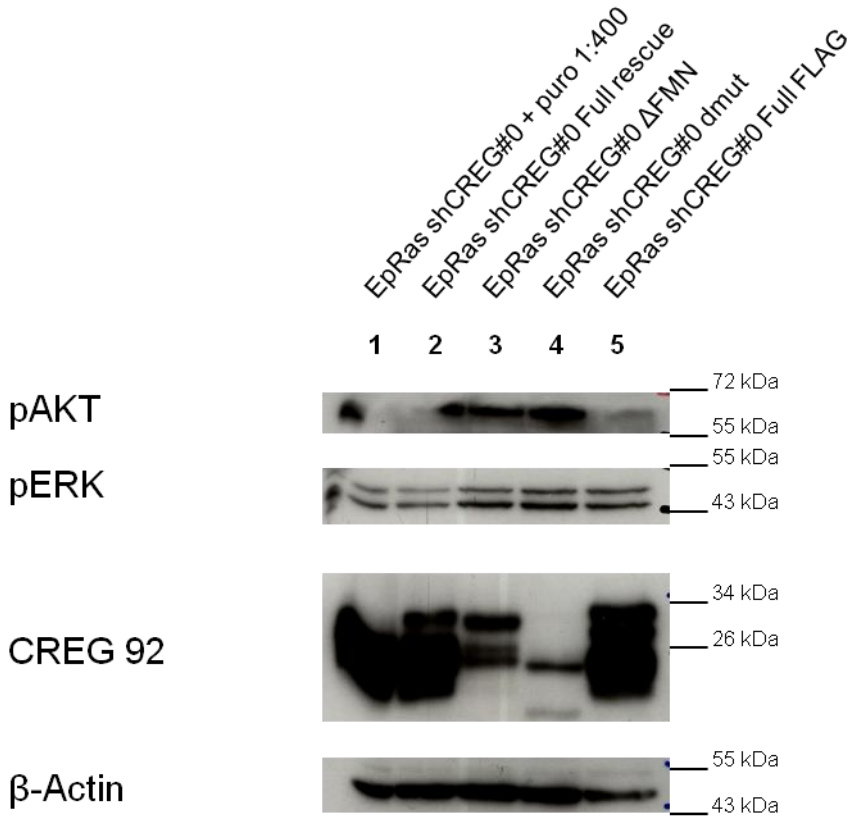


Figure 19: Western Blot analysis of EpRas shCREG#0 cell extracts. Cell extracts (20 µg protein) were loaded and analysed. Prior to addition of the primary antibodies the membrane was cut into several sections to allow simultaneous detection of multiple antigens. The experiment was performed once.

4.2.2.5. Detection of EMT Markers in reconstituted EpRas shCREG#0 cell lines

Cell lines were grown in collagen gels until the formation of tubular cell structures. The cells were fixed and IF staining was performed. Because of the light sensitive secondary antibody all steps following the washing steps after incubation with the primary antibody were performed in the dark. To stain the nuclei Invitrogen Prolong Gold antifade reagent with DAPI was used and after drying over night the microscope slide was sealed with nail polish.

E-Cadherin is the best known epithelial marker and the degradation of this protein is evidence that the cells begin to lose their epithelial phenotype; Fibronectin on the other hand is a mesenchymal marker indicating the shift of the cell towards a mesenchymal phenotype.

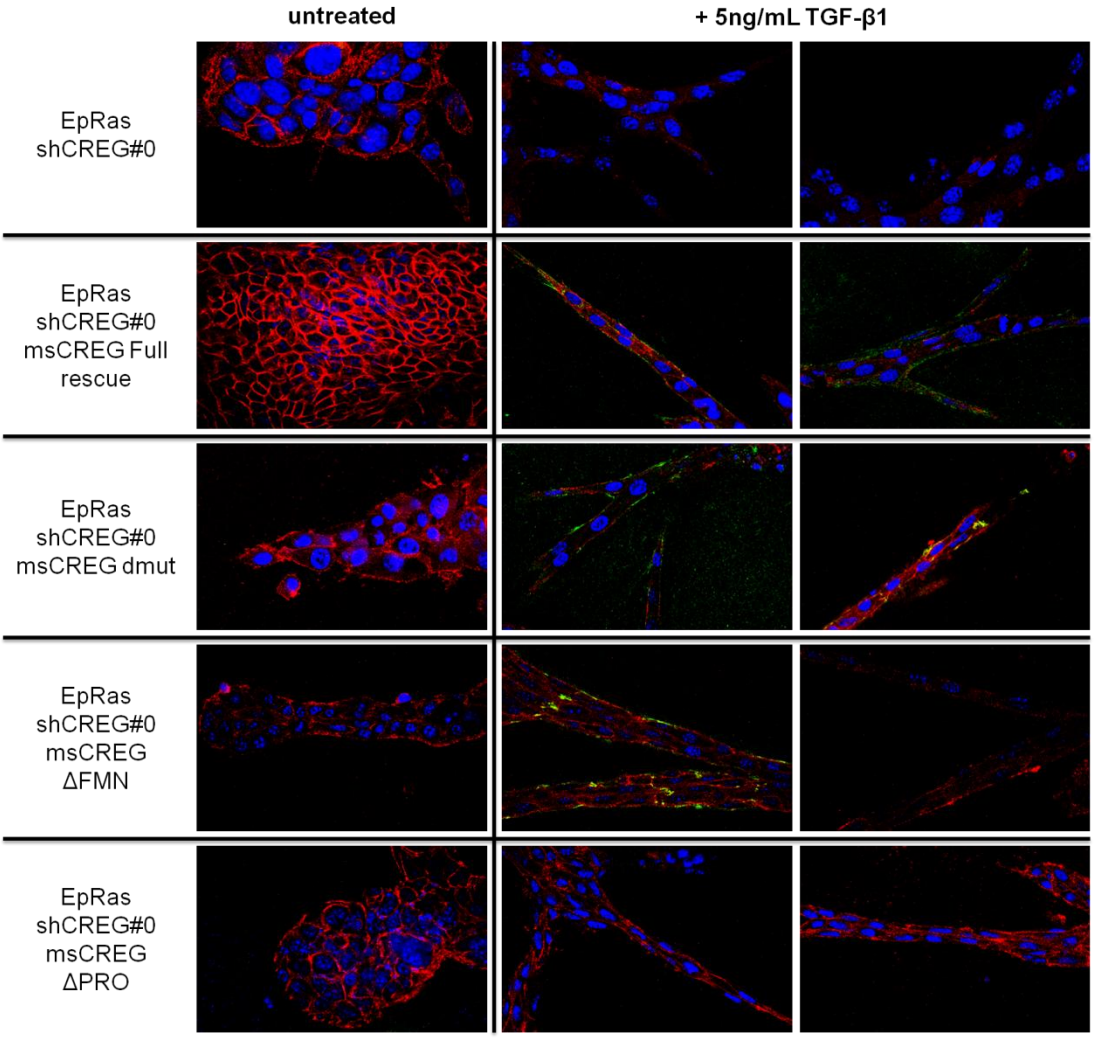


Figure 20: Results of immunofluorescent staining of EpRas shCREG#0 cell lines. The photos on the left show untreated cell structures whereas pictures on the right display the behaviour upon TGF-β1 treatment. E-Cadherin is shown red and Fibronectin is shown green. DAPI (blue) is used to stain the nucleus. The yellow signal indicates an overlapping red and green signal. All cells were grown in collagen gels.

Treatment of EpRas shCREG#0 cells, considered as negative control, with TGF-β1 does not result in changes of the morphology or induction of apoptosis. Although the reconstituted cell lines expressing msCREG Full, msCREG dmut, msCREG ΔFMN and msCREG ΔPRO alter their shape towards a more spiky phenotype, no significant loss of E-Cadherin and gain of Fibronectin can be observed. Fibronectin and E-Cadherin levels should be higher and lower respectively to verify that the cells are undergoing EMT.

4.2.3. CREG knockdown with CREG#2 shRNA

4.2.3.1. CREG levels in EpRas shCREG#2 cells

Cells were grown, harvested and samples prepared as described above (see 4.1.2.1).

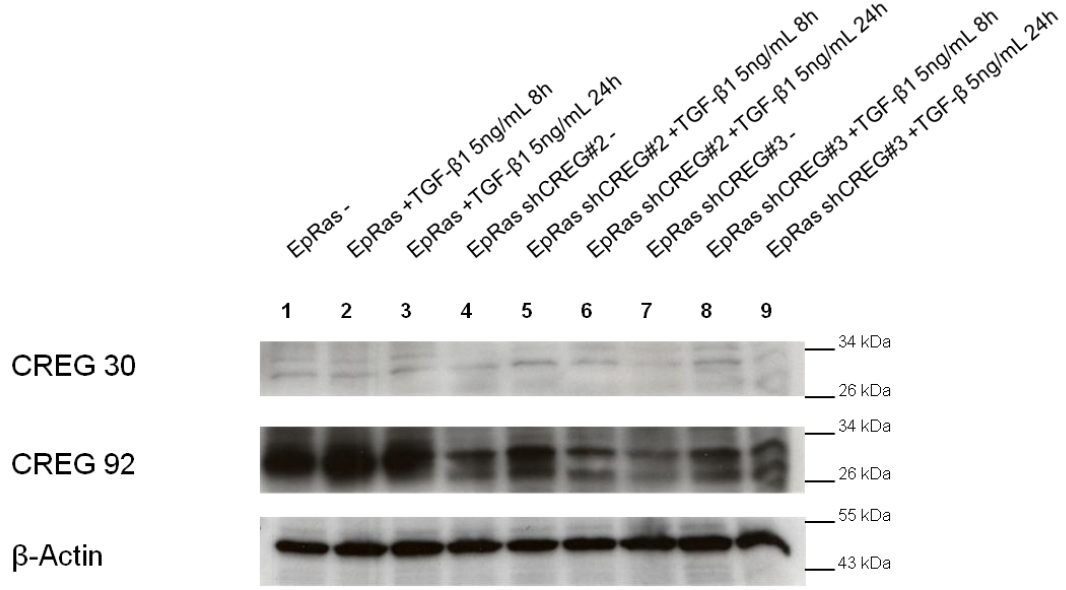


Figure 21: Determination of CREG levels in EpRas shCREG#2 cells using Western Blot analysis. Equal amounts of protein (30 µg) were loaded onto the gel. The analysis was performed using rb α CREG 30 and rb α CREG 92 as primary and α rb IgG HRP as secondary antibodies.

A decrease in CREG levels can be observed when compared to EpRas cells. Short term treatment with TGF-β1 increases the CREG expression of EpRas shCREG#2 cells. Detection of β-Actin shows the uniformity in protein content of the loaded samples. shCREG#2 cell lines show a similar decrease in CREG levels as shCREG#3 cells.

4.2.3.2. Cloning into pWPI_ML+ GFP

Two constructs were created and cloned into the pWPI_ML+GFP vector:

Description	Mutation
msCREG dmut rescue full length msCREG cDNA with mutations in both N-glycosylation sites that is not targeted by shCREG#2	Asn-160 → Gln-160 Thr-218 → Ala-218
msCREG dmut rescue FLAG full length msCREG cDNA with mutations in both N-glycosylation sites plus an FLAG tag at the C-terminus that is not targeted by shCREG#2	Asn-160 → Gln-160 Thr-218 → Ala-218 + FLAG (C-terminus)

Table 17: CREG mutants designed for CREG knockdown #2.

Several other constructs which were used during the experiments were already available.

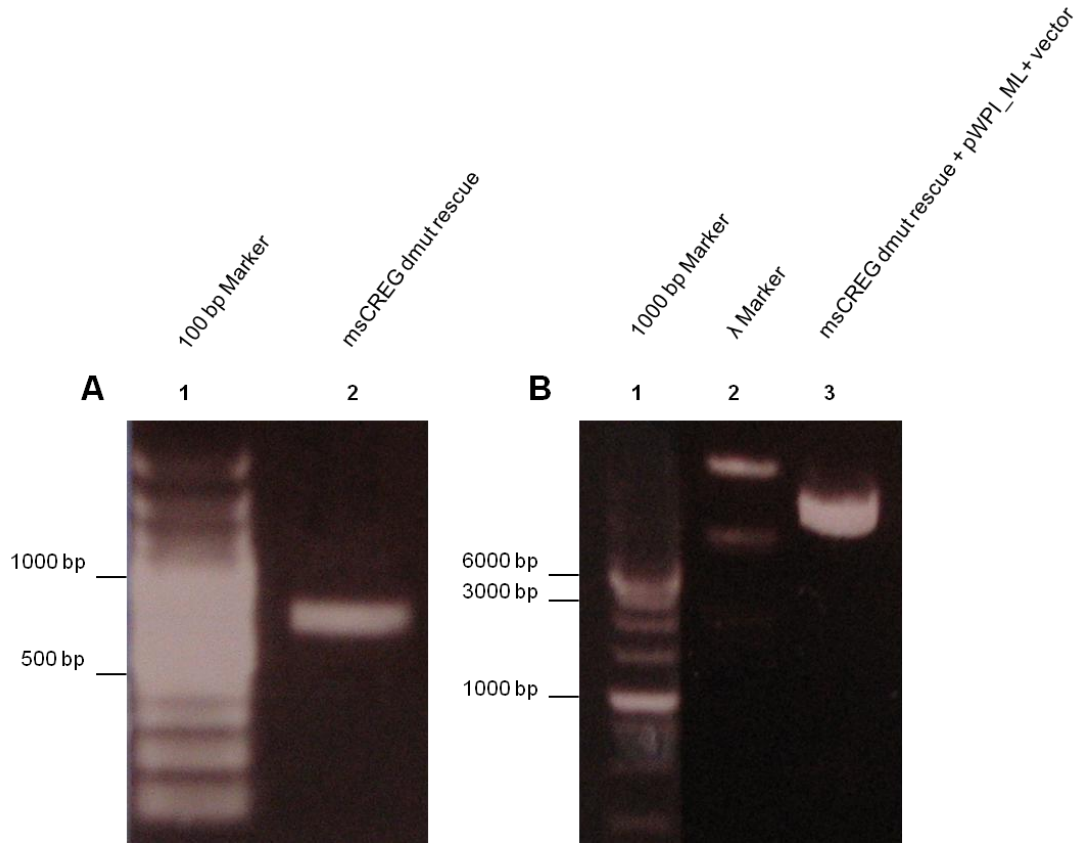


Figure 22: PCR and ligation products of msCREG dmut rescue. (A) shows the PCR product of msCREG dmut (~800 bp) (the entire sample was loaded), (B) displays the construct after ligation with pWPI_ML+ vector (1 µL of the sample was loaded). 10 µL GeneRuler™ 100 bp Plus DNA Ladder (Fermentas) and GeneRuler™ 1000 bp DNA Ladder (Fermentas) respectively were applied to determine the size of the bands, additionally 3µL Lambda Eco/Hind III size marker (provided by IMP service department) was applied.

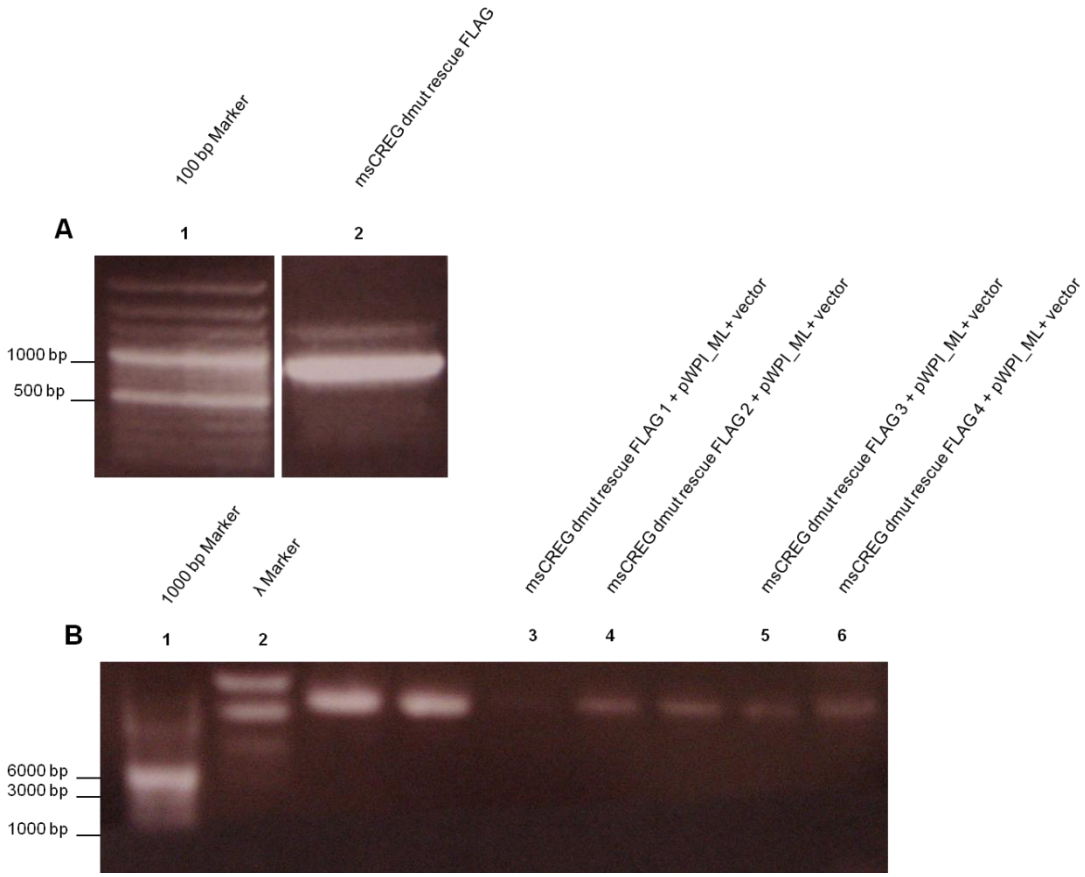


Figure 23: PCR and ligation products of msCREG dmut rescue FLAG. (A) shows the PCR product of msCREG dmut (~800 bp) (the entire sample was loaded), (B) displays the construct after ligation with pWPI_ML+ vector (1 µL of the sample was loaded). The sequence of four samples could be confirmed, one was chosen for Midi Prep. 10 µL GeneRuler™ 100 bp Plus DNA Ladder (Fermentas) and GeneRuler™ 1000 bp DNA Ladder (Fermentas) respectively were applied to determine the size of the bands, additionally 3µL Lambda Eco/Hind III size marker (provided by IMP service department) was applied.

After creating the constructs using PCR the samples were loaded on an agarose gel to control the size of the fragments. Depending on the mutant one (two primers) or two (four primers) PCR reactions per construct were performed. The bands were cut out and purified before digesting with *Mlu1* and *BamH1* or *Sbf1* respectively. Ligation was performed with a precut pWPI_ML+ GFP vector and the ligation mix used for transformation. After plating onto ampicillin agar plates single colonies were picked and the plasmid was isolated using Mini Preps. A sequence analysis was performed to determine the correct sequence.

When the sequence was confirmed to be correct transformation was again executed and larger amounts of plasmid were harvested using Midi Prep technique.

EpRas shCREG#2 cells were transformed by lentiviral transduction and sorted for GFP expression using FACS.

4.2.3.3. Collagen Assay of EpRas SCR and reconstituted EpRas shCREG#2 cell lines

The experiment was executed as described above (see 4.1.2.2.).

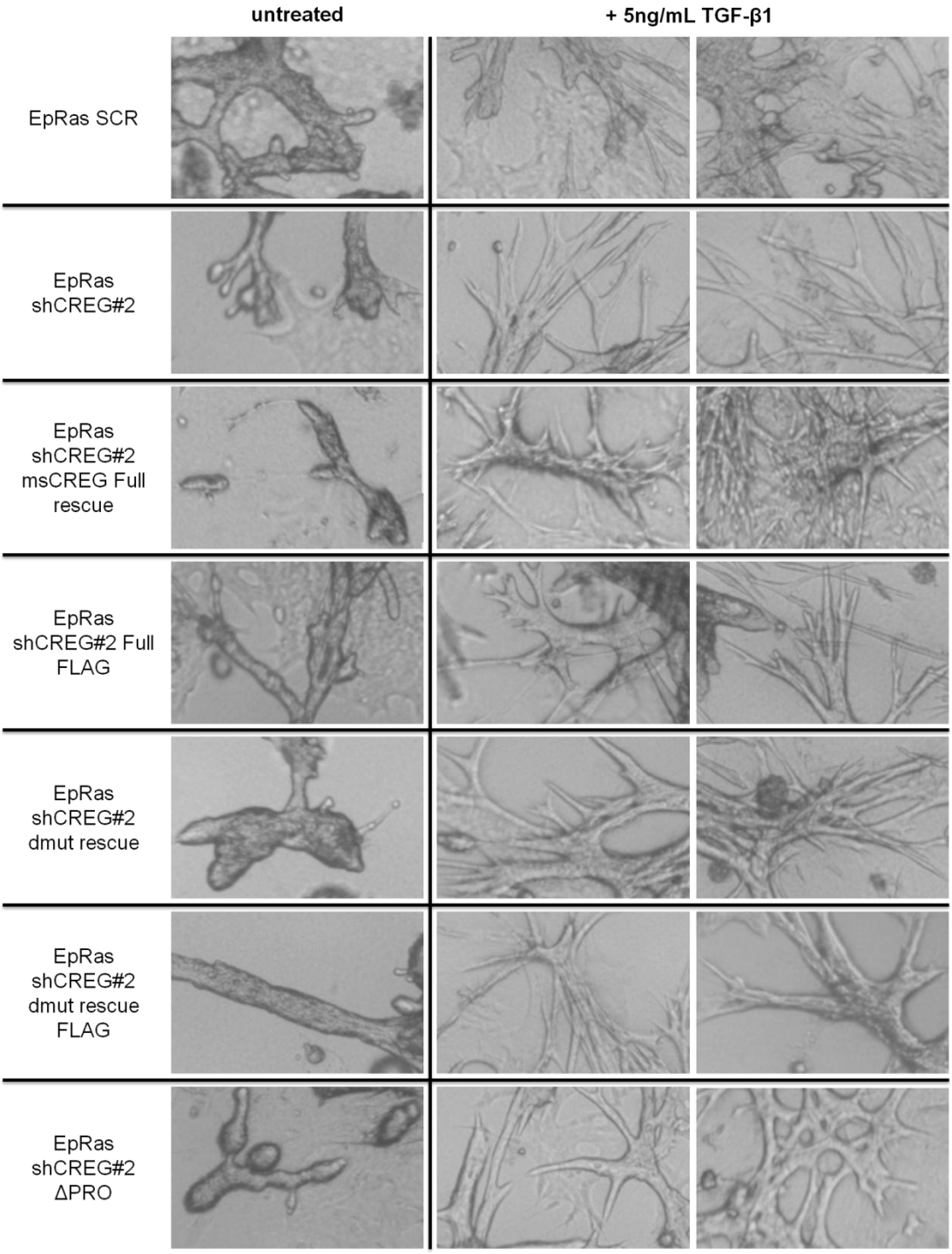


Figure 24: Results of a Collagen Assay of EpRas SCR, EpRas shCREG#2 and reconstituted cell lines after TGF-β1 treatment. The pictures on the left show the untreated cells and the two photos on the right show the behaviour upon TGF-β1 treatment. The pictures were taken 8 days after the start of the experiment and seeding density was 1000 cells/gel.

All displayed cell lines show evidence of EMT – a change towards the typical spiky phenotype and pointy ends – however also the control cell line EpRas shCREG#2, which should display signs of apoptosis, undergoes EMT.

4.2.3.4. Differences in Signal Transduction Pathways in EpRas shCREG#2 cells

SMADs are the only known substrates for type I TGF-β1 receptor kinases that trigger signalling. Eight members of this family are encoded within the human genome and are expressed in all tissues. Three subfamilies are reported – mediator, inhibitory and receptor-activated SMADs. SMAD2 is a member of the latter one, which is phosphorylated and thus activated by type I receptor kinases. It has been recently shown that SMADs convey signals from TGF-β1 through activation of serine/threonine receptor kinases (Moustakas *et al*, 2001).

The cells were treated with TGF- β1 receptor inhibitor, LY294002 (inhibits the PI3K/AKT pathway), PD98059 (inhibits the activation of MEK and therefore the phosphorylation of ERK) and DMSO as a negative control because all inhibitors were diluted in DMSO. The dose dependency of LY and PD was determined and the cells harvested and lysed after 48h when changes in morphology were observed. SDS Page and Western Blot analysis was performed.

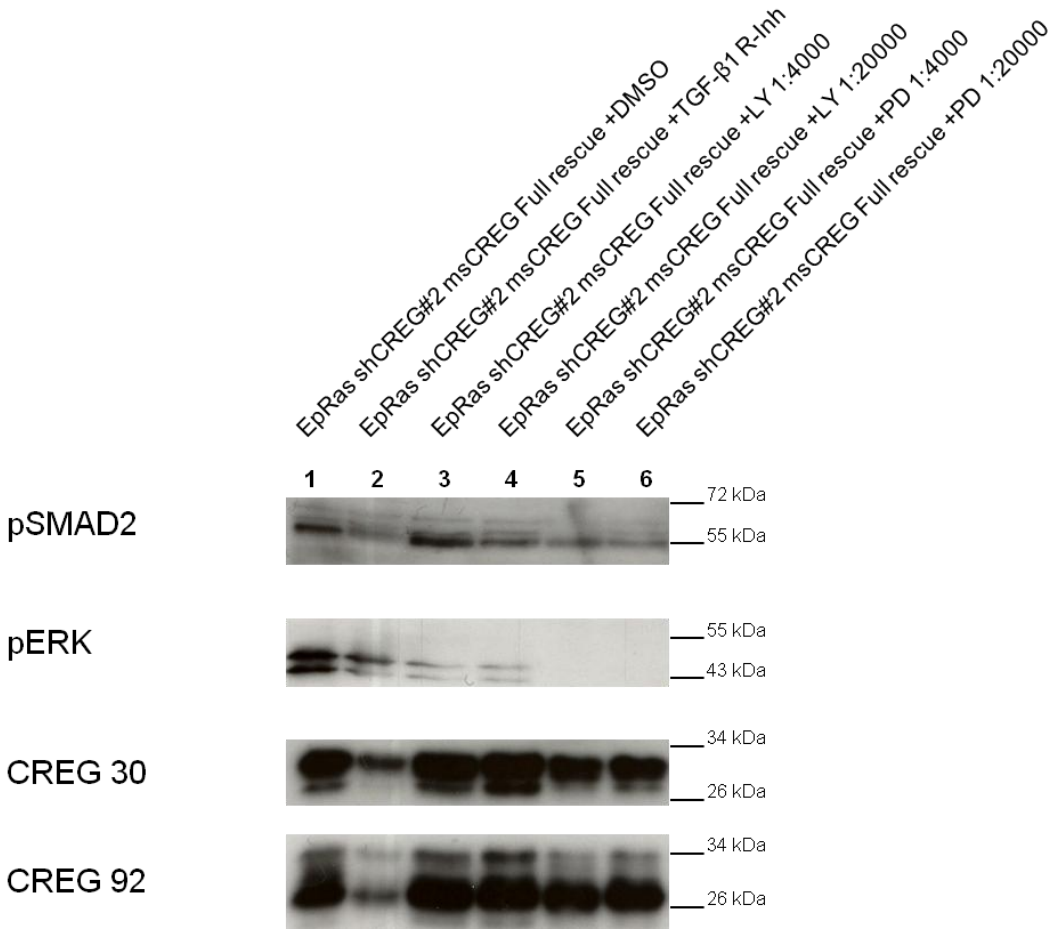


Figure 25: Western Blot analysis of EpRas shCREG#2 msCREG Full cell extracts. Cell extracts (30 µg total protein) were loaded and analysed. Prior to addition of the primary antibodies the membrane was cut into sections to allow simultaneous detection of various antigens. The experiment was performed once.

msCREG Full rescue+ DMSO can be considered as a negative control, therefore all the other samples are compared to its corresponding protein levels. The treatment with TGF- β1 receptor inhibitor results in decreased activation levels of all tested proteins. The addition of LY294002 results in comparatively stable levels of pSMAD2, although the 1:20000 dilution decreases its level. Treatment with PD98059 results in a stronger decrease in activated SMAD2. Contrary to PD, where the CREG expression is unchanged, the PI3 kinase inhibitor increases it. Activated ERK can no longer be detected after treatment with the MEK inhibitor. The effects of PD are dose independent.

4.2.3.5. Invasion Assay of EpRas shCREG#2 cell pools and cell clones

The capability of cells to migrate into the surrounding environment is a hallmark in tumour progression. To test this ability an invasion assay was performed - cells have to migrate through a Matrigel barrier in response to stimulation with chemoattractants.

The cells were seeded on top of the Matrigel and incubated for 24 hours. Afterwards the Matrigel was removed and the invasive cells fixed, stained and counted.

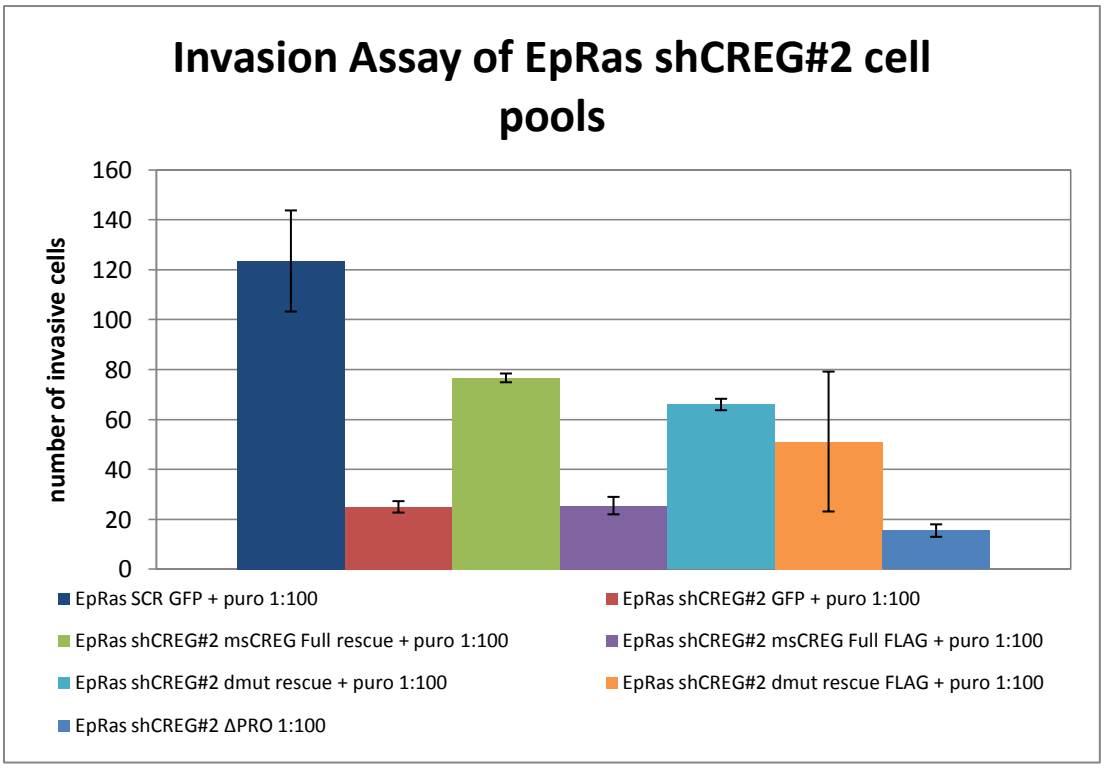


Figure 26: Results of an invasion assay using CREG mutants which were introduced in EpRas shCREG#2 cells. The experiment was performed once using cell pools.

EpRas SCR GFP + puro 1:100 cells were most effective in migrating through the Matrigel. CREG knockdown appears to reduce the invasiveness of EpRas cells. This is not reversed by reconstitution with msCREG Full FLAG and msCREG ΔPRO. msCREG dmut and msCREG Full – which seems to be the more potent one – on the other hand enhance the invasiveness of CREG-knockdown cells. The standard deviation for EpRas shCREG#2 dmut FLAG cells is too big to draw any conclusions.

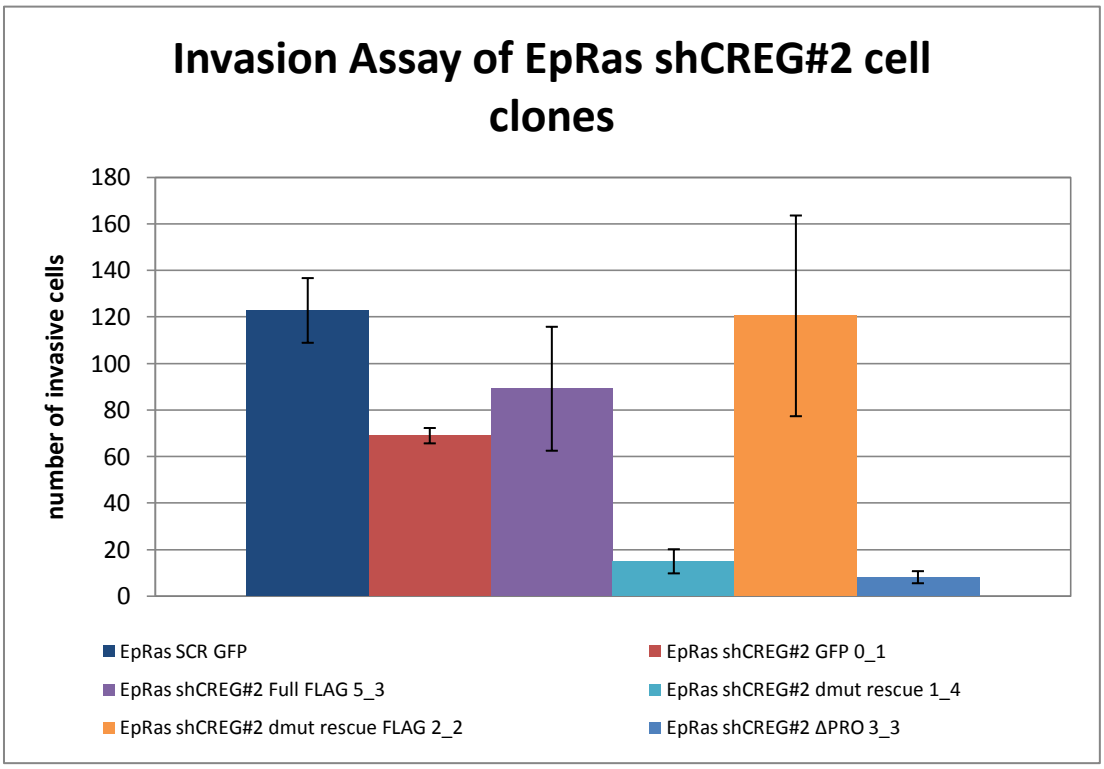


Figure 27: Results of an invasion assay using CREG mutants which were introduced in EpRas shCREG#2 cells. The cells shown in this diagram were obtained from cell-clones which were selected based on phenotype and growth rate. The only exception is the EpRas SCR GFP cell line – these cells were obtained from cell pools. The experiment was performed once.

The amount of EpRas SCR GFP cells that were able to migrate through the barrier is similar to that in the previous experiment. The CREG knockdown in this case is not as efficient in reducing the invasiveness of the cells. The results obtained with cells expressing msCREG Full FLAG and msCREG dmut FLAG cannot be evaluated due to their high standard deviation. In this setting msCREG dmut reduces the invasiveness of the cells, thus showing a result that is the direct opposite to the previously obtained result. The only mutant that behaves as before is msCREG ΔPRO.

Using clones has the advantage of homogeneity, but the risk of obtaining results that are caused by artefacts is higher than it is when using cell pools. Cell pools on the other hand may contain cells that are not reacting in the usual manner but the majority of cells are, thus making cell pools the preferable choice.

4.2.4. CREG knockdown with CREG#3 shRNA

4.2.4.1. CREG levels in EpRas shCREG#3 cells

Cells were grown, harvested and samples prepared as described above (see 4.1.2.1).

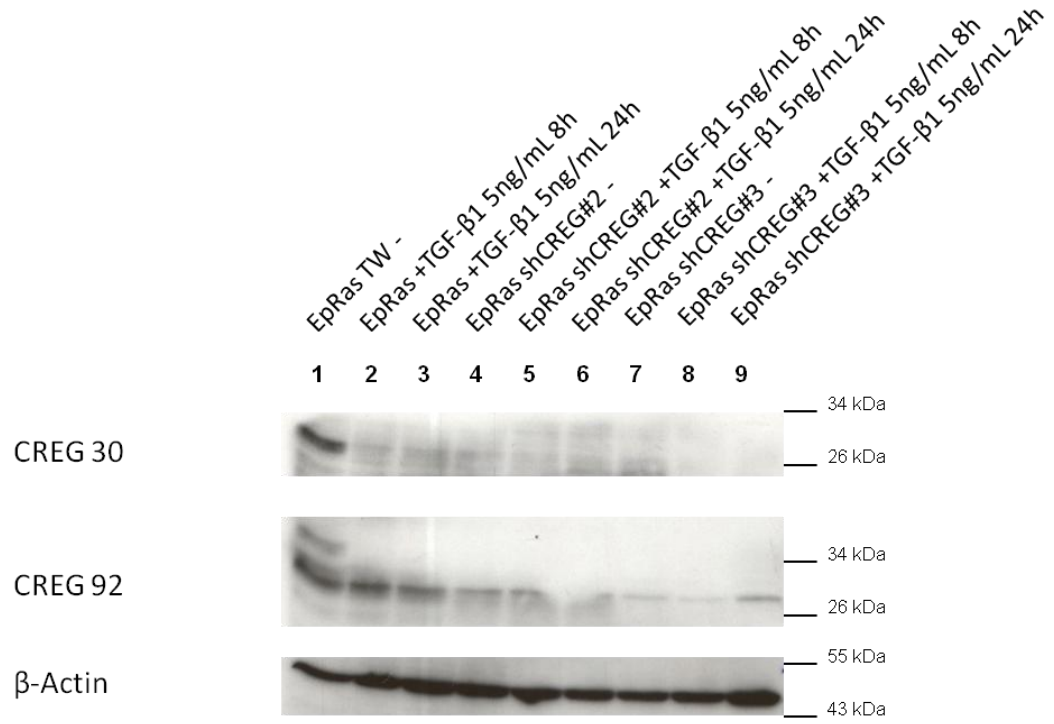


Figure 28: Determination of CREG levels in EpRas shCREG#3 cells using Western Blot analysis. 20 µg total protein of each cell extract were loaded onto the gel. The analysis was performed using rb α CREG 30 and rb α CREG 92 as primary and α rb IgG HRP as secondary antibodies.

EpRas shCREG#3 cells show decreased CREG levels, even lower than those of EpRas shCREG#2 cells. Although it seems that the long term treatment with TGF-β1 increases the CREG level, comparison with β-Actin levels indicates an overall higher protein content of the respective samples.

4.2.4.2. Cloning into pWPI_ML+ GFP

The mutants AtCREG FLAG and CREG hum ΔFMN (data not shown) were created.

Description	Mutation
AtCREG FLAG cDNA encoding full length AtCREG plus a FLAG tag at the C-terminus	+ FLAG (C-terminus)
CREG hum ΔFMN cDNA encoding humCREG lacking the loop that blocks the FMN binding pocket	deletion of amino acids 141 – 144 (DPQS)

Table 18: CREG mutants designed for CREG knockdown #3.

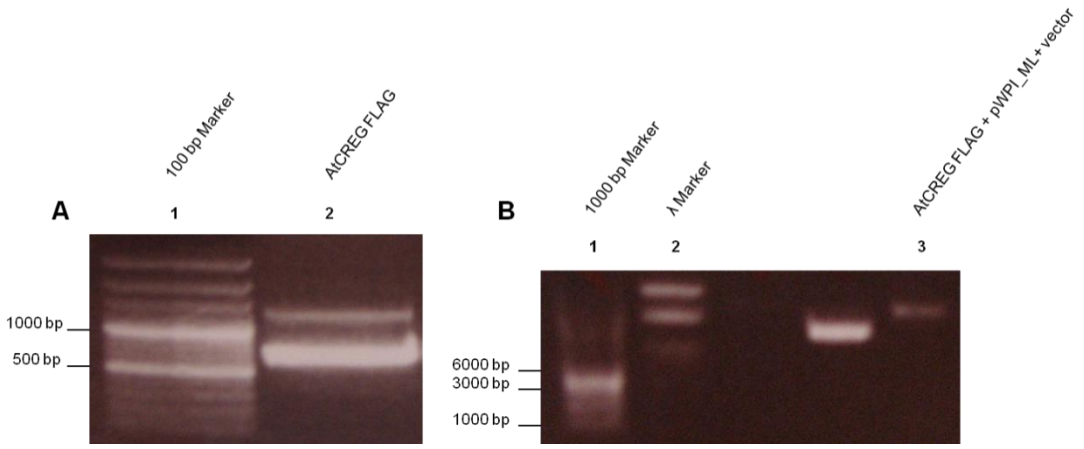


Figure 29: PCR and ligation products of AtCREG FLAG. (A) shows the PCR product of msCREG dmut (~800 bp) (the entire sample was loaded), (B) displays the construct after ligation with pWPI_ML+ vector (1 µL of the sample was loaded). 10 µL GeneRuler™ 100 bp Plus DNA Ladder (Fermentas) and GeneRuler™ 1000 bp DNA Ladder (Fermentas) respectively were applied to determine the size of the bands, additionally 3µL Lambda Eco/Hind III size marker (provided by IMP service department) was applied.

Other constructs that were used during the experiments were already available.
As described in 4.1.3.2 the PCR product was checked for its size, purified, digested (*Mlu1* and *BamH1*), ligated in the vector and then introduced into DH5α. Afterwards the cells were plated onto ampicillin agar plates, colonies were picked and plasmids harvested. When the sequence was confirmed the plasmids were used to transduce EpRas cells.

4.2.4.3. Collagen Assay of EpRas SCR and reconstituted EpRas shCREG#3 cell lines

The experiment was performed as described above (see 4.1.2.2.).

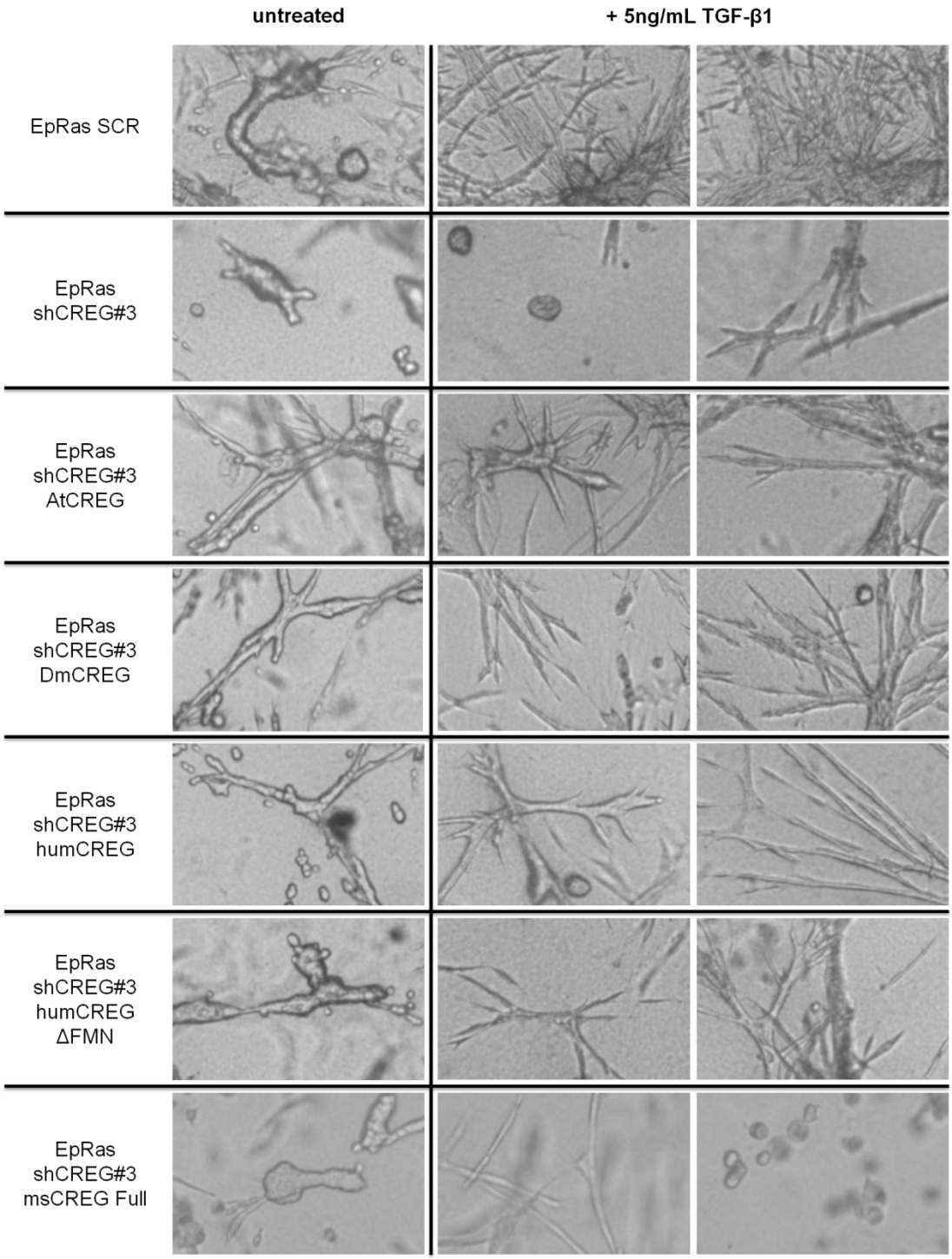


Figure 30: Results of a Collagen Assay of EpRas SCR, EpRas shCREG#3 and reconstituted cell lines after TGF-β1 treatment. Photos on the left show untreated cell lines and the two on the right show structures after TGF-β1 treatment. The pictures were taken 7 days after the start of the experiment (seeding density was 1000 cells/gel).

Figure 30 shows the result of this experiment. Changes upon TGF-β1 treatment can be observed for every reconstituted cell line, although differences regarding the change are evident.
EpRas SCR cells show typical signs of full EMT – spiky, almost spindle-like structures and detached cells on the ends of the branches. The knockdown cell line shows a more slender phenotype, and a lot of the cells are dying (indicated by the round structures). The phenotype of EpRas shCREG#3 msCREG Full cells resembles the knockdown cell line. However, structures of the remaining reconstituted cell lines – AtCREG, DmCREG, humCREG and humCREG ΔFMN – show changes in their structures similar to the positive control, the EpRas SCR cell line.

4.2.4.4. Differences in Signal Transduction Pathways in EpRas shCREG#3 cells

The cell lines were grown with and without addition of 5 ng/mL TGF-β1. After 24 hours, when changes in the phenotype of the treated cells occurred, the cells were harvested and lysed. Samples were prepared and SDS Page followed by Western Blot analysis was performed.
The goal of this experiment was to determine whether TGF-β1 treatment alters the activity of several signal transduction pathways.

It has been recently shown in MCF10CA1h – a human breast cancer cell line - that the p38 and Rho/ROCK pathways both affect the phosphorylation of SMAD2/3 and are thus indispensable for TGF-β1 mediated growth inhibition (Kamaraju *et al*, 2005). There is also evidence that p38 exhibits anti-mitogenic characteristics because of its ability to (1) activate Hsp70 (heat shock protein 70) that inhibits cell proliferation (2) decrease the expression of cyclin D1 – a protein that regulates cell proliferation. Furthermore p38 is mostly activated by cytokines, indicating a role in proliferation control within the immune system (Chen *et al*, 2000).

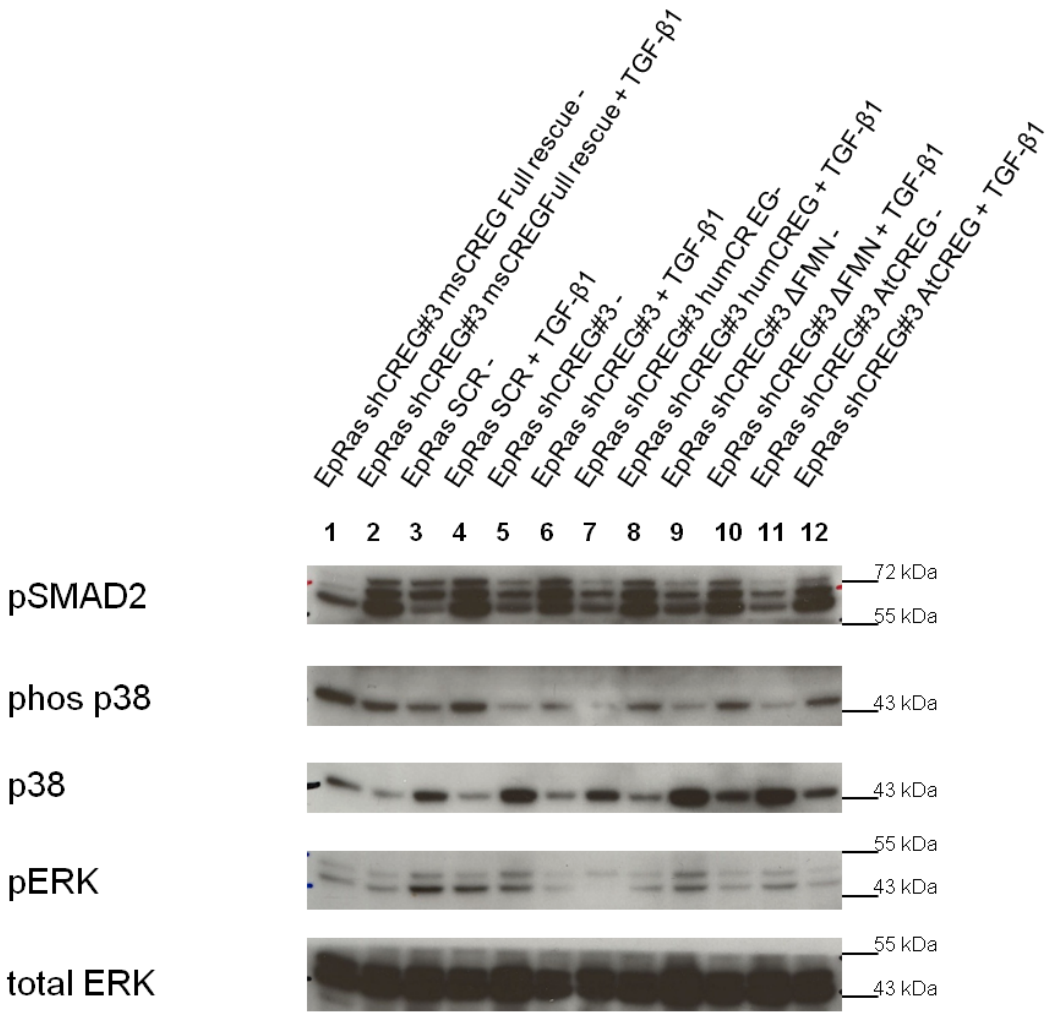


Figure 31: Western Blot analysis of EpRas shCREG#3 cell extracts. Cell extracts (20 µg total protein) were loaded and analysed. Prior to addition of the primary antibodies the membrane was cut into segments to allow simultaneous detection of different antigens.

Comparing the pSMAD2 levels of TGF-β1 treated and untreated EpRas shCREG#3 cells expressing different CREG constructs, no significant differences can be observed. Phosphorylated and thus active p38 levels increase upon TGF-β1 treatment in all cell lines and show an opposite pattern compared to total p38. Abundance of pERK and total ERK is higher in untreated cells but no significant differences can be observed regarding the different cell lines.
To verify the uniformity of sample loading Ponceau staining was performed after blotting onto the PVDF membrane.

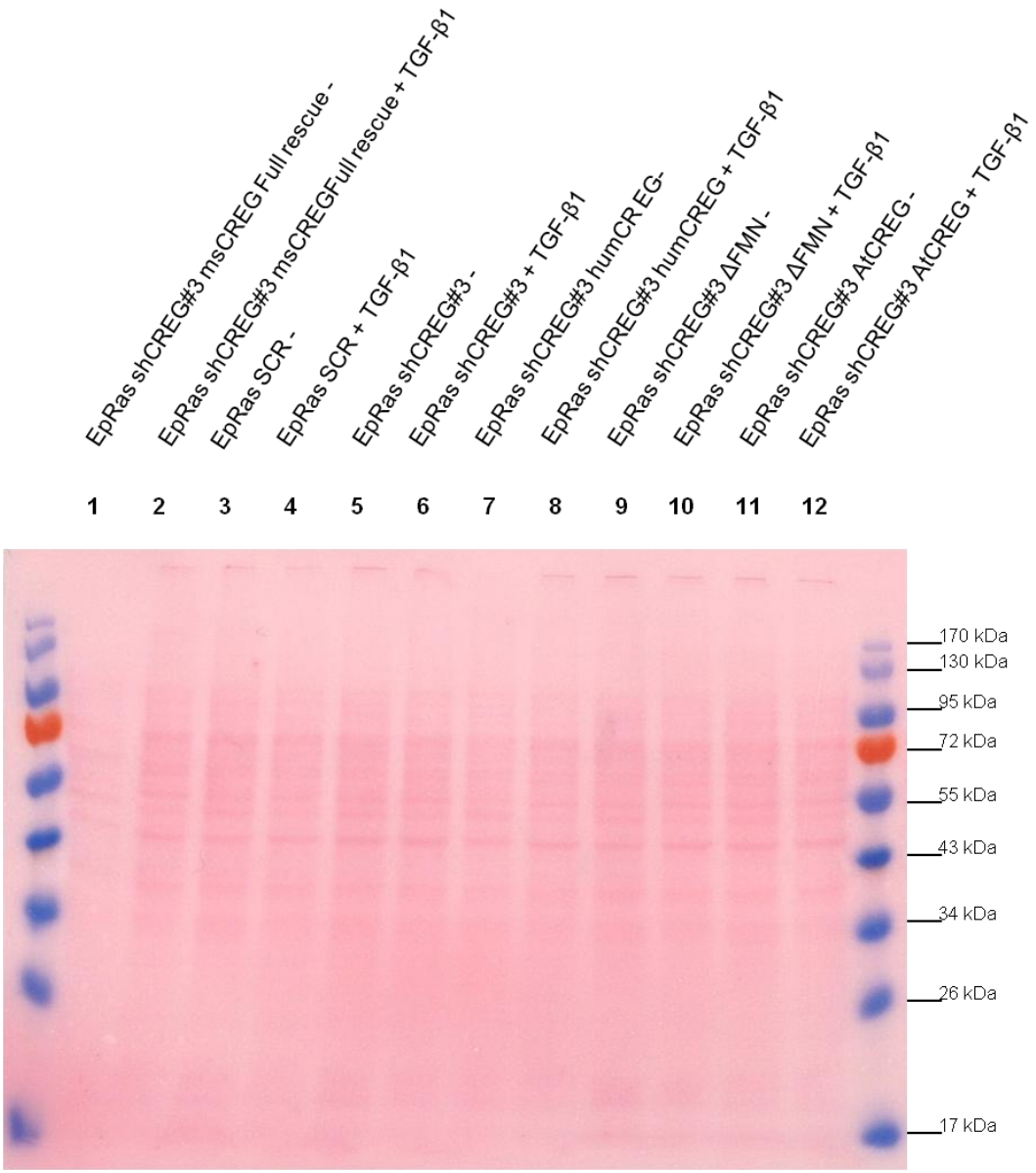


Figure 32: Loading control of EpRas shCREG#3 cell extracts. Ponceau staining of the PVDF membrane was used as loading control. The staining was performed directly after blotting before blocking of the membrane. Uniform loading (except lane 1 EpRas shCREG#3 msCREG Full -) was achieved.

4.2.4.5. Invasion Assay of reconstituted EpRas shCREG#3 cell lines

The experiment was performed as described in 4.1.3.4 and performed once.

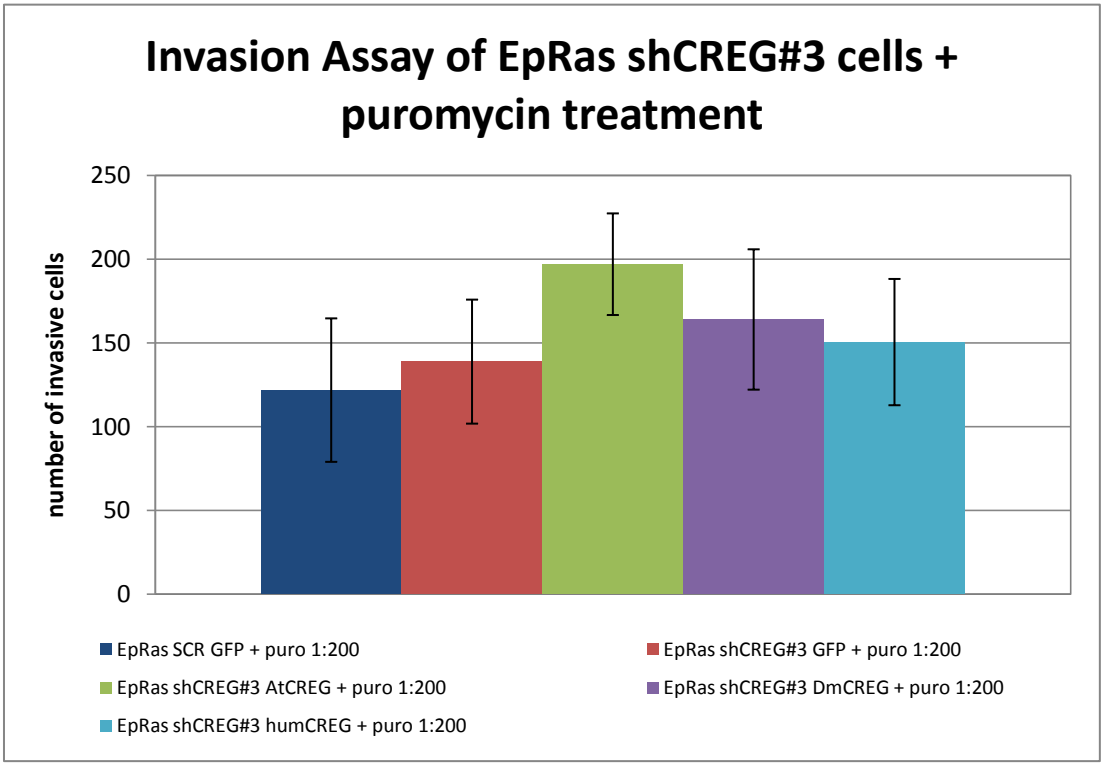


Figure 33: Results of an invasion assay using CREG mutants which were introduced in EpRas shCREG#3 cells. The cells were treated with puromycin. Although it is an antibiotic that is used for selective growth of transduced cells it also induces stress. The cells used for this experiment were obtained from cell pools. The experiment was performed once.

CREG knockdown does not reduce the number of invasive cells as observed in the previous experiment using cell lines created with shRNA CREG#2. Reconstitution with the constructs AtCREG, DmCREG and humCREG results in an increased invasiveness of the resulting cell lines.

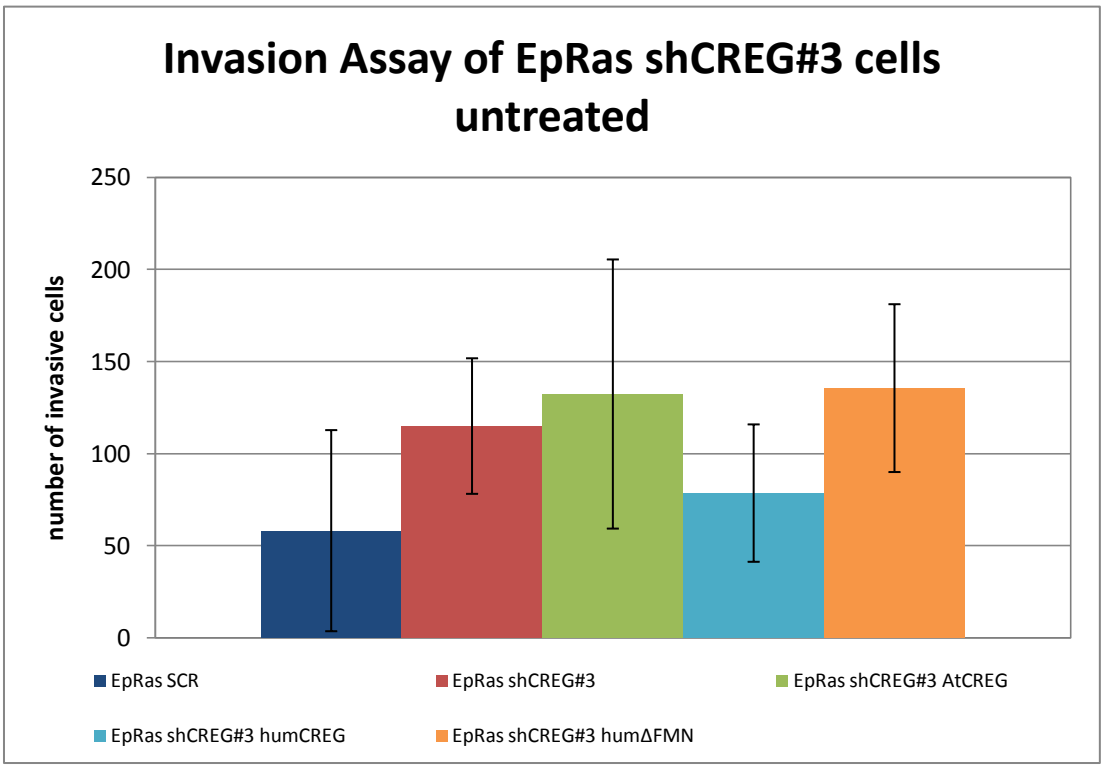


Figure 34: Results of an invasion assay using CREG mutants which were introduced in EpRas shCREG#3 cells. The cells used for this experiment were obtained from cell pools. The experiment was performed once.

A similar trend can be observed when using unselected cells. AtCREG and humCREG ΔFMN enhance the ability of EpRas shCREG#3 cells to migrate through the Matrigel barrier – however, the number of invasive EpRas shCREG#3 cells is decreased upon expression of humCREG.

4.2.4.6. Detection of EMT Markers in reconstituted EpRas shCREG#3 cell lines

Preparations of cells were performed as described above (see 4.1.2.4.)

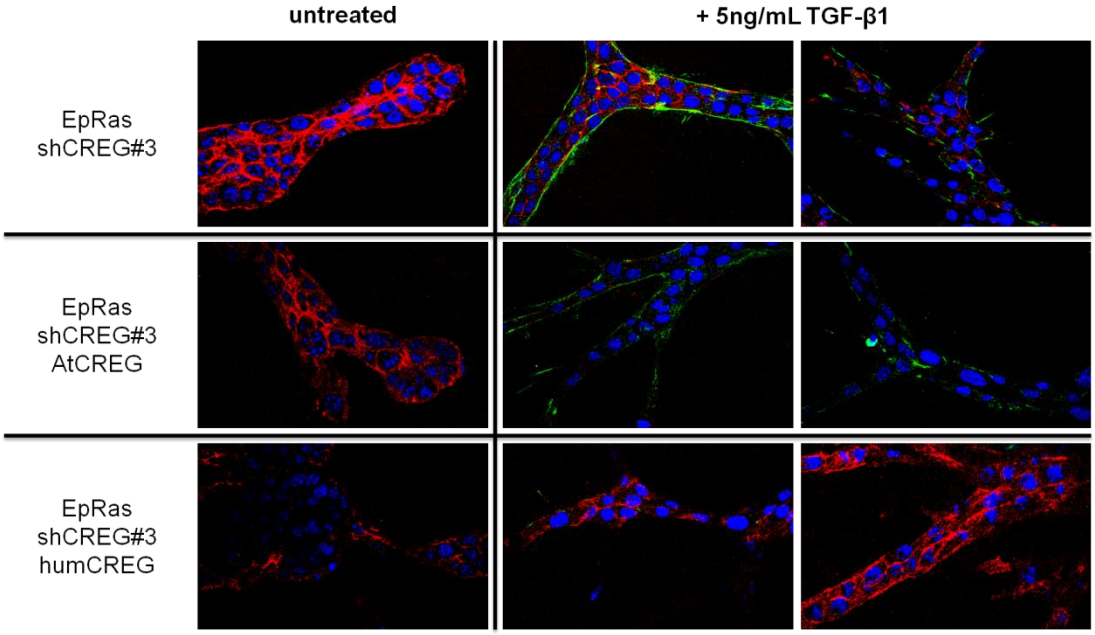


Figure 35: Results of immunofluorescent staining of EpRas shCREG#3 cell lines. The pictures on the left show untreated cell lines, the photos on the right the reactions induced by TGF-β1 treatment. E-Cadherin is shown red and Fibronectin green. The nucleus was stained using DAPI. Overlapping green and red signals are shown yellow. The cells were grown on cover slips. The experiment was performed once.

The negative control, EpRas shCREG#3 cells, is able to survive TGF-β1 treatment and to express Fibronectin, whereas the reconstituted cell lines EpRas shCREG#3 AtCREG and EpRas shCREG#3 humCREG display lower and no detectable Fibronectin levels respectively. Nearly no E-Cadherin can be detected in AtCREG cells upon TGF-β1 treatment.

5. Discussion

The claim that CREG expression is increased upon undergoing EMT (Jechlinger et al, 2002) could be verified using qRT-PCR and EpRas and EpRas XT cell lines. Expression in XT cells, which were retrieved from lung metastases, was increased when compared to normal EpRas cells, thus indicating that CREG plays an important role in tumour progression.

CREG knockdown can result in reduced survival of EpRas cells upon TGF-β1 treatment

It has been recently shown that msCREG overexpression in NMuMG cells – normal murine mammary gland cells – induces resistance to TGF-β1 induced apoptosis. This was not observed for an unglycosylated msCREG mutant, indicating a direct link between glycosylation status, hence localisation, and biological activity (Schönbacher, 2010).

TGF-β1 treatment causes EpRas SCR cells to undergo EMT, whereas it results in reduced survival of EpRas knockdown cell lines EpRas shCREG#0 and EpRas shCREG#3. Reconstitution with CREG variants protects the resulting cell lines from TGF-β1 induced apoptosis.
The structures formed in Collagen gels seem to have undergone EMT but this must be verified using immunofluorescent staining. E-Cadherin and Fibronectin levels have to be determined, the former an epithelial marker that is present in cell-cell adhesion structures and the latter a mesenchymal marker (Acloque *et al*, 2008, Lee *et al*, 2006, Guarino *et al*, 2007).

EpRas shCREG#0 cells show no signs of apoptosis upon TGF-β1 treatment. A similar effect was observed in the cell lines reconstituted with msCREG Full, msCREG dmut, msCREG ΔFMN and msCREG ΔPRO, however the phenotype is altered towards a spikier shape.
Although shCREG#3 shows E-Cadherin in cell-cell adhesion structures, Fibronectin is detectable as well. Changes in the shape of the structures are visible, but the nuclei keep their shape. Almost no E-Cadherin but Fibronectin expression is visible

when reconstituting with AtCREG. Reestablishment with humCREG showed only traces of Fibronectin, and E-Cadherin was not internalized.

The data obtained from Collagen gels suggest that CREG expression may be of importance for protection from TGF-β1 induced apoptosis.

CREG does not influence the activation status of various signal transduction pathways in EpRas cells

The reconstitution of EpRas shCREG#0 cells with the constructs msCREG Full, msCREG Full FLAG, msCREG dmut and msCREG ΔFMN alters the activation of AKT and ERK. Full FLAG, dmut and ΔFMN CREG expressing cell lines show increased levels of phosphorylated ERK, whereas dmut and ΔFMN CREG enhance and Full FLAG CREG reduces the activation of AKT.
Treatment of EpRas shCREG#2 msCREG Full rescue cells with the TGF-β1 receptor inhibitor decreases pSMAD2, pERK and CREG levels. The latter one is increasing when cells were treated with LY294002, though activated ERK is decreased. The effects of LY seem to be dose dependent, the 1:20000 dilution results in increased CREG and decreased pSMAD2 levels. PD98059 treatment reduces SMAD2 as well as ERK activation, which is undetectable after treatment with this inhibitor.
Levels of activated SMAD2 were higher upon treatment of EpRas shCREG#3 cell lines (msCREG Full rescue, AtCREG, humCREG and hum ΔFMN) with TGF-β1 as compared to EpRas SCR. Treatment results in increased phosphorylation of SMAD2 and p38, whereas untreated cells show higher levels of pERK.

When considering the results of the signal transduction pathway analysis, modification of CREG expression did not alter the activation patterns in a significant way, although differences regarding the reconstitution of shCREG#0 cells can be observed. Nevertheless signal transduction studies may be of importance once active forms of CREG mutants are identified.

Downregulation of CREG can reduce the invasiveness of EpRas cells

One of the hallmarks in tumour progression is the ability of cells to invade the surrounding microenvironment. Because CREG is known to play an important role

during EMT and tumour progression, several mutants were created and tested for their ability to migrate through a Matrigel barrier.

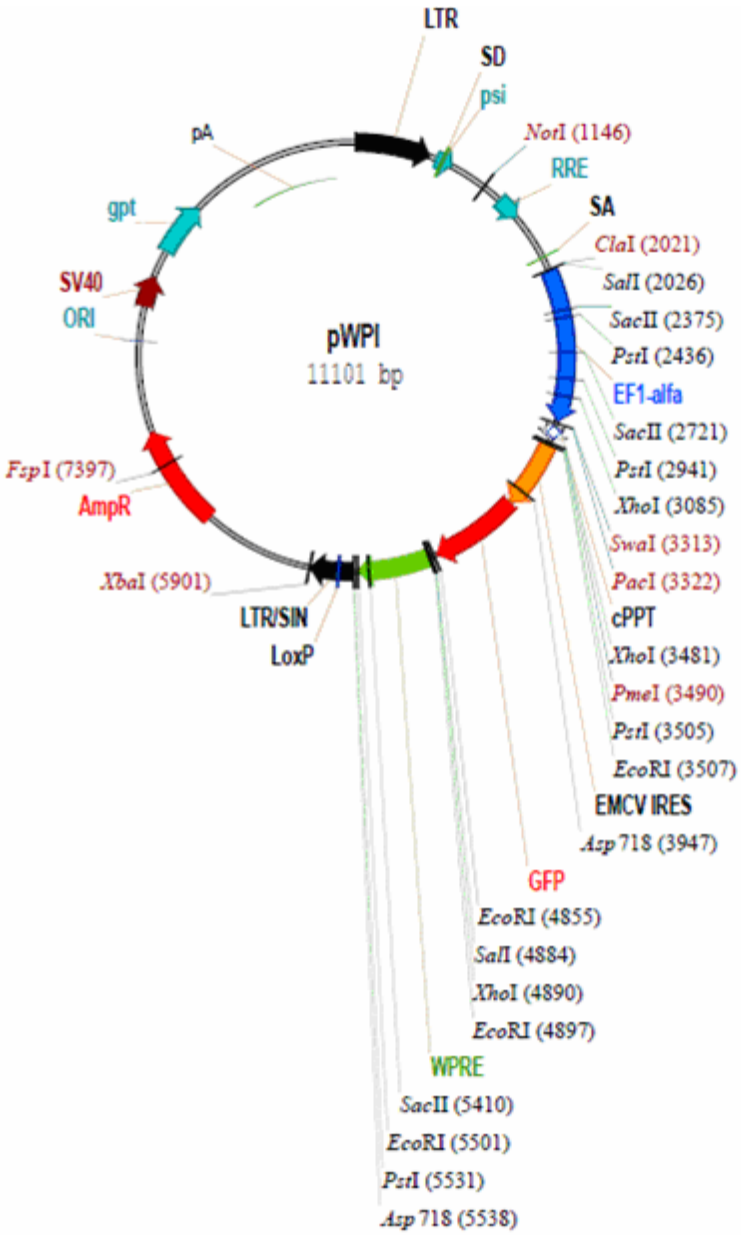
Expression of shCREG#2 in EpRas cells decreases their invasiveness. This could be reversed by reconstitution with msCREG Full rescue and msCREG dmut rescue. Numbers of migrating Full FLAG and ΔPRO CREG-expressing cells are unchanged compared to shCREG#2 cells.
Contrary to shCREG#2, shCREG#3 expression increases the number of invading cells.

In the future it may be of interest to use Cre-Lox recombination for introducing the reconstitution construct into the cells before inducing CREG knockdown. Once the knockdown occurs, Cre cleaves the loxP sites and removes the repressor that inhibits the expression of the reconstitution construct. The Cre gene, under the control of an inducible promoter, ensures an expression with utmost precision upon knockdown. The concept is to prevent the selection of knockdown cells whose survival and changes are independent of CREG expression, and thus to reduce the likelihood of artefacts.

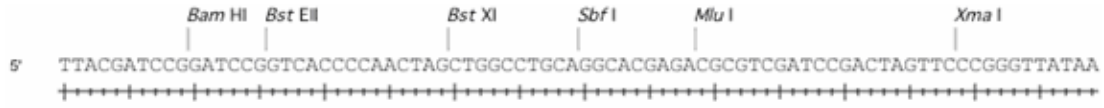
6. Appendix

6.1. Vector Map

pWPI_ML+



The multiple cloning site (MCS) region is exchanged by the following sequence:



6.2. Primer Sequences

6.2.1. Cloning of Constructs

AtCREG FLAG forward primer (*BamH*I)

5´ – GGCC GGA TCC ACC ATG GAA CTT CAA GTC CTT – 3´

AtCREG FLAG reverse primer (*Mlu*I)

5´ – GGCC ACG CGT TTA CTT GTC GTC ATC GTC CTT GTA GTC TAA AAA GGA AGC GAG TTT GAT CGA CTT – 3´

msCREG dmut forward primer (*BamH*I)

5´ – GGCC GGA TCC ACC ATG GCT GCC CGT GCT CCT – 3´

msCREG dmut rescue reverse primer (*Mlu*I)

5´ – GGCC ACG CGT TCA CTG CAG CGC GAC – 3´

msCREG dmut rescue FLAG reverse primer (*Mlu*I)

5´ – GGC CAC GCG TTC ACT TGT CGT CAT CGT CCT TGT AGT CCT GCA GCG CGA CGT TAA AAT ATT CTT – 3´

humCREG ΔFMN forward primer 1 (*BamH*I)

5´ – GGC CGG ATC CAC CAT GGC CGG GCT ATC CCG – 3´

humCREG ΔFMN reverse primer 2 (*Mlu*I)

5´ – TAT GTG AAC ACA AAG GGG AAA TCC ATG TTT – 3´

humCREG ΔFMN forward primer 3 (*BamH*I)

5´ – AAA CAT GGA TTT CCC CTT TGT GTT CAC ATA – 3´

humCREG ΔFMN forward primer 4 (*Mlu*I)

5´ – GGCC ACG CGT TCA CTG AAC TGT GAC ATT ATA ATA TTC – 3´

6.2.2. qRT-PCR Primers

msCREG forward primer
5´ – GCG GAC ATC ATC TCA ATC AGT – 3´

msCREG reverse primer
5´ – TC AGC GTA GCC TCT GGA TTT – 3´

β-Actin forward primer
5´ – GTG GGC CGC TCT AGG CAC CAA – 3´

β-Actin reverse primer
5´ – TCGT TGC CAA TAG TGA TGA CTT GGC – 3´

GAPDH forward primer
5´ – TGCA CCA CCA ACT GCT TAG – 3´

GAPDH reverse primer
5´ – GAT GCA GGG ATG ATG TTC – 3´

HPRT forward primer
5´ – CT GGT GAA AAG GAC CTC TCG – 3´

HPRT reverse primer
5´ – CA CAG GAC TAG AAC ACC TGC – 3´

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