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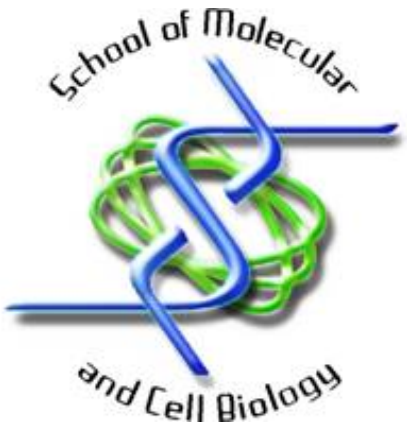

UNIVERSITY OF THE WITWATERSRAND

SCHOOL OF MOLECULAR AND CELL BIOLOGY

M.Sc. Biotechnology Research Report

SHORT HAIRPIN RNA-DIRECTED KNOCK DOWN OF EPIDERMAL GROWTH FACTOR RECEPTOR IN HUMAN OESOPHAGEAL SQUAMOUS CARCINOMA CELL LINES

by

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Submitted to the Faculty of Science, University of the Witwatersrand, in partial fulfillment of the requirements for the degree of Masters of Science

March 2007

Declaration

I hereby declare that this research report is my own, unaided work. It is being submitted in part for the Degree of Masters of Science in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.

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(Signature of candidate)

_____ day of _____ 200 ____

Abstract

Epidermal growth factor receptor (EGFR) is a transmembrane receptor tyrosine kinase which activates, upon EGFR binding, a number of signaling pathways including the mitogenic protein kinase pathway (MAPK) and phosphatidylinositol 3-kinase cascade (PI3K). Over expression of EGFR is a common feature in variety of human cancers including lung, colorectal, breast, pancreatic and oesophageal cancers and results in autonomous cell growth, enhanced metastatic potential, tissue invasion and increased resistance to current cancer therapeutics. Thus EGFR has been identified as a potential target in cancer therapeutics. Using the RNA interference (RNAi) pathway, the aim was to specifically knockdown expression levels of endogeneous EGFR in human oesophageal squamous carcinoma cell (HOSCC) lines. The RNAi pathway was initiated through the transfection of three specifically designed short hairpin RNAs (shRNAs) against human EGFR. The shRNAs were specifically designed using bioinformatics tools and their individual knockdown efficacy determined through the introduction of an exogeneous based target reporter systems, psiCHECK and pcieGFP. Expression levels of EGFR were determined using Western blot analysis followed by densitometry. Knockdown of EGFR was achieved by all three EGFR shRNAs in the three HOSCC cell lines (WHCO1, WHCO5 and WHCO6) despite low transfection levels of ~10%. Greastest knockdown of EGFR (85%) was achieved by EGFR sh2 in WHCO5. EGFR sh2 and sh1 resulted in average knockdown of EGFR of ~ 65% in WHCO1 and WHCO5 respectively. Weakest knockdown of EGFR (~ 20%) was obtained by all three EGFR shRNAs following transfection of WHCO6. RNAi-based approaches therefore show substantial potential for the specific and efficient targeting of EGFR in human cancer cells.

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CHAPTER 1

1.1 Cancer overview

Diseases that are described as being cancerous all share definitive characteristics, including limitless replication or immortalization of cells, their ability to act independently of cellular signals and hormones and lastly their ability to invade the surrounding tissues and metastasize (Rak and Yu, 2004). Cancers are classified according to three broad characteristics, firstly the anatomical site where the cancer occurs, secondly a comparison of appearance to normal cells and, thirdly the extent to which the cancer has spread. Cancers that originate, or stem from, supportive and connective tissues such as bone or muscle are referred to sarcomas. The cancers that stem from epithelial tissues are referred to as carcinomas (Lodish *et al.,* 2000). Epithelial cells cover the outer surface of the body as skin, which are flattened squamous cells known as kertinocytes. Simple squamous epithelial cells are also found to line the lung, vascular system etc. Carcinomas predominate, accounting for the majority of all cancers, with sarcomas predominantly making up the remaining percentage. A very small percentage of cancers belong to the leukemias, lymphomas and myelomas. The squamous cell carcinomas predominate and include oesophageal, colorectal, lung and skin (excluding myelomas) cancers.

1.2 Causative agents in cancer

Cancer develops over time and is a consequence of successive mutations and the growth and replication of these mutants/cancerous cells. Cancer is a disease that is characterized by the loss of cellular growth control, the transformation of normal cells into their cancerous state and the development of tumourgenesis which involves the disruption of normal functioning of the molecular machinery that is responsible for the cell cycle (Kamb, 1995). The mechanisms that regulate the cell cycle are composed of a vast array of elements and interconnecting pathways that function together to precisely regulate the cell cycle and more importantly cell division. The use of multiple pathways in cell cycle control helps to limit the possibility of loss of cell cycle control (Kamb, 1995).

The genetic basis for tumourgenesis has been well documented and includes cellular oncogenes, tumour suppressor genes, DNA mismatch repair genes, genes associated with cell aging and apoptosis which can all be affected by deletions, inversions and translocations. Chromosomal alterations within cells include rearrangements, deletions and amplifications which provide the characteristic cytogenetics of certain cancers (Popescu and Zimonjic, 1997; Fabarius *et al.*, 2003). Most of the structural alterations that occur within the chromosomes, excluding certain leukemias, generate aneuploidy which is described as being an abnormal imbalance of whole chromosomes or segments of chromosomes involving thousands of genes (Fabarius *et al.,* 2003). The general genetic instability of cancerous cells is thought to be a major factor leading to chromosomal aberrations, rearrangements and aneuploidy. However it remains problematic as to whether it is the cause or consequence of the cancer phenotype (Dixon and Kopras, 2004). Due to the complicated nature of cancer as a disease it is difficult to identify specific exogenous agents unless there is specific dose response data available such as sunlight exposure and UV damage (skin cancer), cigarette smoking (lung cancer), excessive alfatoxin exposure (liver cancer), high dosages to radiation and certain bacteria and viruses (Setlow, 2001).

Transformation of a cell to the oncogenic state requires that several key, independent, rare mutations must take place in one particular cell (Nowell, 1976). Six essential alterations within cell physiology have been identified that collectively dictate malignant growth, these include; (i) self-sufficiency in growth signals, (ii) insensitivity to growth-inhibitory signals, (iii) evasion of apoptosis, (iv) limitless replicative potential, (v) sustained angiogenesis and (vi) tissue invasion/ metastasis (Hanahan and Weinberg, 2000). These alterations may be dependent on exogenous factors, including age, race, genetic background, occupation and other physical and life style variables, relating to the cause of cancer in humans (Setlow, 2001). These six key alterations are described below, with particular emphasis placed on self-sufficiency of growth signals, which will be described last, as it pertains to the research project at hand.

1.2.1 Insensitivity to antigrowth signals

Growth inhibitors may be soluble or found immobilized in the surrounding extracellular matrix. As is the case for growth factors, these inhibitors also have receptors located on the surface of these cells which transmit an extracellular signal activating an intercellular pathway. However

this pathway activated by the growth inhibitors blocks proliferation of these cells (Hanahan and Weinberg, 2000). These antigrowth signals may either force proliferate cells into the quiescent stage of the cell cycle (G_0) or permanently inactivate their proliferate capability which causes them to enter into a postmitotic state (Hanahan and Weinberg, 2000). The retinoblastoma protein (pRb), proteins p107 and p130, play the main functional role in these anti-proliferative signals (Hanahan and Weinberg, 2000). pRb has been shown to interact with a number of different transcriptional regulators. pRb may directly interact or bind to tissue specific transcription factors aiding either the activation or repression of their target genes, or pRb inactivates anti-proliferative signals or repressor molecules through direct binding (Korenjak and Brehm, 2005). E2F is responsible for the progression from G_1 to S phase of the cell cycle. Phosphorylation of pRb allows for liberation of E2F transcription factor resulting in cell proliferation (Hanahan and Weinberg, 2000). The transforming growth factor β (TGFβ) governs the anti-proliferative signaling pathway. Insensitivity to anti-proliferative signals arise due to disruption within the TGFβ signaling pathway. The TGFβ receptor may be down-regulated or inactivated resulting in the prevention of the anti-proliferative signal (Hanahan and Weinberg, 2000). TGFβ activates p15 and p21 proteins which blocks phosphorylation of pRb by CDK/cyclin complexes, thereby inactivating pRb (Mittnacht, 1998).

1.2.2 Evasion of apoptosis

The ability of cancerous cells to evade apoptosis is possibly the most important alteration in terms of cancer cell survival and tumour development (Hanahan and Weinberg, 2000). The apoptotic pathway has two distinct signaling pathways, those that stimulate either the apoptotic pathway or the survival pathway. The survival signals are transmitted via the insulin-like growth factors receptors IGF-1/IGF-2. Apoptotic pathways are stimulated through the FAS and tumour necrosis factor (TNF) receptors. Apoptosis may also be activated by internal mechanisms responding to DNA damage, signaling imbalance, hypoxia, oncogene action or loss of cell adherence signals (Hanahan and Weinberg, 2000). These apoptotic signals activate the release of cytochrome C from the mitochondrion. The release of cytochrome C or activation of the FAS receptor results in the downstream activation of cellular proteases known as capases, which are the final stage in apoptosis (Hanahan and Weinberg, 2000). Cancer cells employ the following

strategies to evade apoptosis; the most common of which involve the tumor suppressor protein p53. The p53 protein is a pro-apoptotic protein which amplifies another pro-apoptotic protein Bax, leading to the release of cytochrome C from the mitochondrion. Mutations within the p53 protein have been observed in more than 50% of all human cancers (Harris, 1996). Alternatively up-regulation of survival signaling mechanisms have also been noted in evasion of apoptosis; these include pI3-kinase-AKT/PKB pathways and the oncogene Bcl-2. Bcl-2 opposes the apoptotic effect by controlling the release of cytochrome C from the mitochondrion which is key in preventing the cell from under going apoptosis (Hanahan and Weinberg, 2000).

1.2.3 Limitless replicative potential

Normal cells have a finite replicative potential. Each time the cell passes through the S phase of the cell cycle the 3′ end, comprised of hundreds of 6 basepair (bp) repeats (TTAGGG), is shortened as DNA polymerase is unable to replicate the ends known as telomers (Hanahan and Weinberg, 2000). When deterioration of the telomers reaches a critical stage and no longer serves to protect the 3′ end of the chromosome, genetic instability occurs resulting in the activation of tumor suppressor mechanisms, such as p53 (Rodier *et al.,* 2005). Activation of the p53 protein can result in two possible outcomes, the first being permanent cell growth arrest (senescence) or programmed cell death (apoptosis), both controlled by p53 pathways (Rodier *et al.,* 2005). If disruption of the p53 pathway has already occurred, these cells still need to stabilize their telomers by employing the several strategies. The most common is the reactivation of the telomerase enzyme which adds additional repeats/caps to the ends of the 3′ chromosomal DNA (Kim *et al.,* 1994). An additional strategy that conveys cell immortalization is maintenance of the telomeres just above the critical length, through recombination based inter-chromosomal exchanges of sequence information (Bryan *et al.,* 1995).

1.2.4 Sustained angiogenesis

Normal and cancerous cells both require a sustained supply of nutrients and oxygen from the vascular system. In order for a tissue to increase in size the vascular system must be able to

provide for the expansion, the same is true for a tumour or neoplasmic growth. This process of new blood vessel growth is termed angiogenesis (Hanahan and Weinberg, 2000). Tumourgenic cells are able to activate angiogenesis through up-regulation of angiogenesis and through activating signals such as vascular endothelial growth factor (VEGF) or fibroblast growth factors (FGF), or through down-regulation of inhibitor molecules such as thrombospondin-1 (Hanahan and Weinberg, 2000). The down-regulation of thrombospondin-1 is thought to be as a result in disruption of p53 tumour suppressor pathway, whereas the Ras oncogene is thought to be involved with the heightened expression of VEGF (Hanahan and Weinberg, 2000).

1.2.5 Tissue invasion and metastasis

The ability of cancerous cells to invade surrounding tissues or travel to a new location within the body is a highly complex process. This has been reported to cause 90% of human cancer deaths (Sporn, 1996). Invasion strategies involve alteration to cell adhesion molecules, most commonly E-cadherin which is involved in cell-to-cell adhesion and is responsible for the transmission of antigrowth signals between adjacent cells utilizing the β-catenin activated pathways. E-cadherin function is commonly lost in human epithelial cancers which include carcinomas of the breast, liver, colon, prostate, stomach, skin, kidney, lung and oesophagus (Christofori and Semb, 1999). This may be due to inactivation or truncation of the E-cadherin gene, or proteins involved in the E-cadherin adhesion complex, which include α and β catenin (Christofori and Semb, 1999).

1.2.6 Self sufficiency of growth signals

Normal cells within the body require growth signals that shift the cell cycle from its state of quiescence into a proliferative state. These growth signals have transmembrane receptors that transmit the signal through the membrane activating the internal signaling pathways within the cell (Hanahan and Weinberg, 2000). Many of the oncogenes mimic these growth signals thereby reducing their dependence on exogenously derived signals from their normal microenvironment (Hanahan and Weinberg, 2000). Acquired self-sufficiency of growth signals can utilize three

distinct pathways, firstly alteration of the extracellular growth signals, secondly cellular signal transducers and thirdly activation of intracellular signaling pathways.

Many cancer cells acquire the ability to synthesize their own growth signals, to which they are in turn responsive therefore creating a positive feedback mechanism. Examples of cancers that utilize this strategy are the production of PDGF (platelet derived growth factor) and TGFα (transforming growth factor α) produced by glioblastomas, sarcomas and selected carcinomas (Hanahan and Weinberg, 2000).

Alteration of the growth factor receptors on the surface of the cell is another strategy used to gain independence from exogenous signals. As most of these receptors contain tyrosine kinase activity which activates internal signals within the cell, many of these receptors have been found to be over-expressed in cancer (Arteaga, 2002). Over-expression of these receptors results in a heightened response to fairly low levels of a particular growth factor, inducing proliferation of these cells that would not normally occur.

Other alterations involving these receptors include truncated versions that need not be ligand activated in order to activate the cytoplasmic signaling pathway and hence are constantly active.

Oncogenes that play a role in downstream signaling pathways from these receptors also provide a strategy by which these cells can become independent of growth signals. The most featured signaling pathway in this case is the Ras-Raf-MAP kinase pathway (Bianco *et al.,* 2006). Alteration of the Ras protein has been found to be prevalent in up to 25% of all human cancers (Hanahan and Weinberg, 2000). The Ras-Raf-MAP kinase pathway has been found to be interlinked with a number of other intracellular signaling pathways, thus potentially resulting in multiple cellular changes. Oncogenic viruses that express oncogenes such as c-ras and c-myc, which contribute to their transforming ability have also been identified (Dixon and Kopras, 2004).

One such receptor that is of particular interest with respect to cancer in general and in especially to this study, is the epidermal growth factor receptor (EGFR). Over expression of EGFR occurs in a vast array of tumour types contributing not only to the onset of cancer but also the progression of the disease. The characteristics of EGFR and the downstream signaling mechanism willl be described in greater detail below.

1.3 Epidermal growth factor receptor (EGFR)

Epidermal growth factor receptor (EGFR) is expressed, or over-expressed, in a variety of human tumours, which include non-small lung cancer (NSCLC), breast, head and neck, gastric, colorectal, oesophageal, prostate, bladder, renal, pancreatic and ovarian cancers (Salomon *et al.,* 1995).

1.3.1 Molecular description of EGFR

Epidermal growth factor receptor (EGFR) belongs to the ErbB family of receptor tyrosine kinases. There are four members within this family namely; EGFR (ErbB1/HER1), ErbB2 (neu/HER2), ErbB3 (HER3) and ErbB4 (HER4) (Chan *et al.,* 2006). Epidermal growth factor receptor is a transmembrane glycoprotein, with a molecular mass of between 170 and 180 kDa. The external domain of EGFR is comprised of two cystein rich regions, with a single α-helical transmembrane region and a cytoplasmic region which contains the tyrosine kinase region (Yu *et al.,* 2002). EGFR has number of structurally similar ligands which are able to bind and activate this receptor; these include EGF, transforming growth factor α (TGFα), heparin-binding EGF-like growth factor (HB-EGF), amphiregulin, betacellulin and epiregulin (Marquart *et al.*, 1984; Shoyab *et al.*, 1989; Higashiyama *et al.*, 1991; Shing *et al.*, 1993; Toyoda *et al.*, 1995).

The first step in EGFR activation is binding of EGF to the EGFR, resulting in the homo-or heterodimerization of the ectodomain with other members of the ErbB family (Schlessinger and Ullrich, 1992; Heldin, 1995). This dimerization of the ectodomain activates the receptor resulting in the autophosphorylation of the intracellular C-terminal tyrosine residues resulting in the activation of the receptor. The active receptor allows for the transphosphorylation of various intracellular substrates, as well as the sequestering of particular cytoplasmic proteins which possess Src homology 2 (SH2) and phosphotyrosine binding domains (Khlolodenko *et al.,* 1999). These cytoplasmic proteins are thought to amplify the mitogenic signal (Khlolodenko *et al.,* 1999).

1.3.2 EGFR signalling pathways

Activation of EGFR signalling may result in various biological responses including; proliferation, differentiation, migration, angiogenesis and modulation of apoptosis (Arteaga, 2002; Fischer *et al.,* 2003). These biological responses are mediated through a number of signalling pathways which include the mitogen-activated protein kinase (MAPK), phosphatidylinositol-3 kinase (PI3K) and more recently through G-protein-coupled receptor (GPCR)-mediated mitogenic signalling (Daub *et al.,* 1996; Arteaga, 2002).

1.3.3 The mitogen-activated protein kinase pathway (MAPK)

The MAPK pathway has been found to be highly conserved in a variety of distantly related organisms, including humans, mice, *Xenopus, Drosophilia* as well as *Caenorhabditis elegans* (Voet and Voet, 1995). Once the receptor has been activated and the cytoplasmic tyrosine residues phosphorylated, the mammalian protein growth factor binding protein 2 (Grb2), binds to these phosphotyrosines via its SH2 domain and simultaneously binds to the proline rich region of the son of sevenless protein (Sos) via its SH3 domain (Voet and Voet, 1995). Activated Sos functions as a guanine nucleotide releasing factor (GRF), which in turn activates Ras, through the exchange of bound GDP with GTP.

Active GTP-Ras then activates a series of serine/threonine (ser/thr) kinases, the first of which being Raf. Raf is activated through direct interaction with GTP-Ras. Activation of Raf results in the phosphorylation of specific ser/thr residues of a protein known as MEK (MAP kinase/ERK-activating kinase) or MAP kinase kinase (MAPKK). MEK in turn phosphorylates a family of proteins referred to as the mitogen-activated protein (MAP) kinases or the extracellular-signal-regulated kinases (ERKs). MEK activate the MAP kinases through phosphorylation of both the threonine (Thr) and tyrosine (Tyr), residues (Voet and Voet, 1995). The MAP kinases are then able to migrate from the cytoplasm into the nucleus where they activate Jun/AP-1, FOS and Myc transcription factors, through phosphorylation (Voet and Voet, 1995).

1.3.4 Phosphatidylinositol 3- kinase cascade

PI3-kinase is a heterodimeric complex, which is comprised of 85 and 110 kDa subunits, referred to as p85 and p110, respectively. The p85 subunit contains a SH3 domain and two SH2 domains. One SH2 domain is involved in the interaction with phosphotyrosines residues of the active receptor and operates as an adaptor. The p110 is the catalytic subunit and is present in three isoforms in mammalian cells, known as α, β and δ, each encoded by separate genes (Vanhaesebroeck and Waterfield, 1999). The PI3 kinases are able to phosphorylate the OH group found in the 3´ position of the inositol ring of phophatidylinositol. The products produced from the PI3 kinases do not act as a substrate for specific cleavage of inositol phospholipids by phospholipase C (PLC) into membrane bound diacylglycerol and inositol triphosphates (Vanhaesebroeck and Waterfield, 1999). Thus there is a disruption in the traditional pathway which results in the release of Ca^{2+} ions from the endoplasmic reticulum into the cytoplasm (Voet and Voet, 1995).

The PI3K signaling pathway activates a protein known as phosphoinositide-dependent kinase-1 (PDK1), activating a protein known as protein kinase B (PKB), through the phosphorylation of Thr 308 and Ser 473 (Vanhaesebroeck and Waterfield, 1999). Activation of PKB, results in specific targets for phosphoryltion that are involved the regulation of cell survival. PKB exerts an anti-apoptotic role through the targeting of key components involved with the cell death machinery. The first example of this, BAD, which inactivates the anti-apoptotic role that the proteins Bcl-2 and Bcl-X play through direct interaction. PKB phosphorylates BAD at Ser 136, preventing BAD from interacting with Bcl-2 and Bcl-X, thus allowing Bcl-2 and Bcl-X, to exert their anti-apoptotic effect and promote cell survival (Vanhaesebroeck and Waterfield, 1999). PKB also targets capase-9, which is critical in the latter stage of apoptosis. Capase-9 is inactivated through phosphorylation by PKB (Vanhaesebroeck and Waterfield, 1999). PKB is also able to inactivate members of the fork head (FH) transcription factor family. It is thought that phosphorylation of the FH transcription factors by PKB, results in their export out of the nucleus. FH transcription factors are involved with the expression of the Fas ligand, which is responsible for cell death upon autocrine or paracrine production. Thus PKB separates the FH transcription factors from their target genes, allowing for cell survival (Vanhaesebroeck and Waterfield, 1999).

1.3.5 Alternative EGFR activation pathways

Ligand binding to the ectodomain of the EGFR causes dimerization of the receptor and results in the autophosphorylation and activation of the cytoplasmic tyrosine kinase domain. This signaling capability can be increased through the formation of heterodimers rather than homodimers of the receptor (Arteaga, 2002). The transformation of HER2 into cancer cells results in the display of aggressive growth in soft agar and in nude mice models and enhances the tumourgenicity and the metastatic potential of these cells. It has also been noted that patients with amplified levels of HER2 (20% of all cancers), have a poor prognosis when compared to those patients that do not (Dowsett *et al.,* 2001).

The EGFR can also be activated through G-protein-coupled receptors (GPCRs). GPCRs constitute the largest group of cell surface receptors and are involved in a variety of biological functions. GPCRs can influence EGFR in a number of ways including activation of matrix metalloproteinases which are able to free membrane-tethered EGFR ligands that stimulate EGFR. EGFR may also be activated indirectly through GPCRs activation of Src which is able to phophorylate tyrosine residues other than those activated through autophosphorylation by receptor dimerization (Arteaga, 2002). Thus the broad diversity of the GPCRs results in the stimulation of the EGFR, and subsequently the signaling pathways that have been mentioned above (Fischer *et al.,* 2003).

1.4 General characteristics of oesophageal cancer in humans

Oesophageal cancer is the eighth most common cancer worldwide after lung, breast, colon and rectum, stomach, prostate, liver and cervical cancers respectively, accounting for 462 000 new cases in 2002 (Parkin *et al.,* 2005). The poor survival rate associated with oesophageal cancer, approximately 16% of patients in the U.S. and 10% in the U.K. survive the first five years, makes it the sixth most common cause of death, accounting for approximately 6% of the total cancer related deaths worldwide in 2002 (386 000 deaths) (Parkin *et al.,* 2005). Oesophageal carcinoma does have an even distribution of incidence throughout the world and has been found to be localized in certain regions based upon various factors including environmental

carcinogens, nutritional deficiencies or a genetic predisposition to oesophageal carcinoma (Parkin *et al.,* 2005).

Oesophageal carcinoma in humans can be divided into two distinct groups, adenocarcinoma and squamous cell carcinoma; this is based upon epithelial cell type from which the cancer or the neoplasmic growth stems.

1.4.1 Adenocarcinoma

The incidence of adenocarcinoma was initially low, accounting for approximately 1-4% of all oesophageal carcinomas, however in recent years, there has been a steady increase of primary associated adenocarcinoma (Gore *et al.,* 1997). The greatest increase in incidence has occurred within white male population with a less dramatic increase within the black male and female populations (Gore *et al.,* 1997). Oesophageal carcinoma may originate from malignant degeneration of metaplastic columnar epithelial cells (Barrett's mucosa) or heterotopic island of columnar epithelial cells or from the oesophageal submucosal glands (Gore *et al.,* 1997). Adenocarcinoma tends to be a disease associated with more developed countries including the U.K and U.S. This sudden increase of adenocarcinoma within these developed countries is thought to be a result of a chronic condition known as Barrett's oesophagus. Barrett's oesophagus refers to chronic gastroesophagus reflux disease, which over time damages these intestinal-type epithelial cells. The prevalence of Barrett's oesophagus has increased over recent years, due to the increased levels of obesity in these developed countries (Parkin *et al.,* 2005).

1.4.2 Human oesophageal squamous cell carcinoma (HOSCC)

Although in recent years there has been an increase in adenocarcinoma of the oesophagus, the majority of oesophageal cancer remains of the squamous cell carcinoma type (Parkin *et al.,* 2005). Human oesophageal squamous cell carcinoma (HOSCC) affects the squamous epithelial cells lining the interior surface of the oesophagus. The majority of HOSCC occurrences affects the middle third of the oesophagus, located approximately 30 cm from the incisors (Gore *et al.,* 1997). Unlike Barrett's adenocarcinoma, HOSCC rarely invades the stomach (Gore *et al.,* 1997).

1.4.3 Epidemiology of HOSCC

The incidence of HOSCC is confined to localized regions distributed throughout the world. These high risk regions include a belt of countries extending from eastern Turkey, through the former Soviet Union countries including Kazakhastan, Turkenistan, Uzbekistan and Tadjikistan, continuing through into Northern China, Hong Kong, Japan and France, Brazil and Bermuda in South America and southeastern Africa (Lam, 2000).

In South Africa, HOSCC is particularly prevalent within certain ethnic populations, particularly in areas of the Eastern Cape (Transkei) (de Groot, 2005). The incidence of HOSCC has been observed in Southern Natal, Western Cape and the highveld areas including Johannesburg, Pretoria, Bloemfontein, Vereening, mainly as a result of migration of people from the Transkei region (McGlanshan, 1988).

The following epidemiological factors have been identified to contribute to HOSCC, alcohol, tobacco, anti-oxidant and vitamin deficient diets, intake of carcinogens (pickled vegetables) and thermal injuries to the oesophageal mucosa. These factors are thought to contribute to the susceptibility of the oesophageal mucosa to various carcinogens that include mycotoxins, nitrosyl compounds and possibly viruses (Lam, 2000). Other factors that contribute to the localized epidemiology of HOSCC include age, sex and race, with HOSCC accounting for approximately 80% of oesophageal malignancy in the black male populations (de Groot, 2005). Pre-existing disorders such as achalasia and genetic predisposition such as tylosis, characterized by the hardening of the palms, soles and papilomas of the oesophagus increases the risk of HOSCC to 90% by the age of 70 years (de Groot, 2005). In the Transkei of South Africa, HOSCC has been linked to improper brewing practices; consumption of *Fusarium sp.* contaminated maize or the comsumption of pipe stems (Pacella-Norman *et al.* 2002; de Groot, 2005; Parkin *et al.,* 2005).

1.4.4 Molecular characteristics of HOSCC

Human oesophageal squamous cell carcinoma is observed to pass through specific histopathological changes which are the result of several genetic alterations. These

histopathological steps include oesophagitis, atrophy, mild to severe dysplasia, carcinoma *in situ* and invasive carcinoma, observed in that order (Mandard *et al.,* 2000). The genetic alterations include the tumour suppressor genes, oncogenes and the gene involved with apoptosis and metastasis.

p53 alteration is common in all human cancers and the incidence in HOSCC is thought to be roughly 50%. These alterations to p53 occur mostly in the conserved domains of the gene, stretching from exon 5-8 (Lam, 2000). Alterations to other tumour suppressor proteins such as p21, p16, p15 and p27 have all been reported in HOSCC (Lam, 2000).

Nuclear factors such as the oncogene *myc*, amplification of cyclin D1 gene and *mdm2* have all been linked to HOSCC. These nuclear factors interfere with the cell cycling process. *myc* encodes for the p62 protein, which dimerizes with the Max protein and is important for activation of G_1 cell cycle phase from G_0. Cyclin D1 is important for cell cycle transitions, thus amplification of this gene results in a growth advantage leading to tumourgenesis. The product of the *mdm2* gene encodes for suppressor of the p53 protein, thus promoting cell survival in cells that have up-regulated levels of the *mdm2* gene (Lam, 2000).

Notably, heightened expression levels of EGFR have also been in observed in HOSCC, and it is thought that between 29-92% of all HOSCC tumours have heightened expression of EGFR. Heightened HER2 expression has also been noted but seems to occur with a maximum incidence of 38% in HOSCC (Lam, 2000).

1.5 Molecular therapeutics in cancer

As with all therapies, whether they are traditional, drug based, or molecular based, identification of a target critical for the pathophysiology of cancer is essential for the success of the chosen therapy (Hermiston and Kirn, 2005). The molecular mechanisms underlying cancer development and progression have not only been useful in classification of some cancers but have ushered in a new era of molecular-based therapies (Mocellin *et al.*, 2006). The following molecular based therapeutics have been identified as possible treatments or are currently undergoing clinical trials for the treatment of cancer.

1.5.1 Antibodies

The ability of an antibody to bind a specific antigen, whether it be a protein, carbohydrate or a synthesized chemical, is of clinical significance. However it was not until the production of monoclonal antibodies through the use of hybridoma technology that the use of therapeutic antibodies became realistic (Kohler and Milstein, 1975). The mouse monoclonal antibodies were unsuccessful as they did not trigger effector functions and would provoke an immune response from the host (Sanz *et al.,* 2004). These problems were overcome by using chimeric antibodies, which contain the human constant region and the murine variable region. This discovery had two important outcomes, firstly it did not provoke an immune response and secondly it extended the half life of these antibodies (Glennie and van der Winkel, 2003). Monoclonal antibodies such as Herceptin® disrupt signaling pathways through binding the target receptor HER-2. Herceptin® is used in the treatment of breast cancer (Glennie and van der Winkel, 2003). Unfortunately solid tumours still remain fairly resistant to antibody based therapeutics, as antibody penetration of tumours remains low (Carter, 2001).

1.5.2 Antisense

Antisense utilizes short, single stranded synthetically produced oligonucleotides, which hybridize to a complementary coding RNA sequence inside the cell. Translation is disrupted through a number of mechanisms; firstly disruption of ribosomal activity, secondly, specific cleavage of the targeted RNA strand by RNase H and thirdly, competition for the ribosomal binding of the 5′ end (Rojanasakul, 1996). Antisense oligonucleotides have an advantage over traditional drugs that are usually directed against proteins, as proteins have complex three-dimensional structures and may possess more than one functional domain. By using antisense oligonucleotides that target mRNA, design is governed by sequence (Rojanasakul, 1996). Various chemical modifications have been made to these antisense oligonucleotides either through the modification of the phosphodiester backbone, or the ribose sugar of the nucleotide portion. The chemical modifications made to these antisense oligonucleotides improve the stability of these RNA molecules against nuclease degradation, improves target affinity and pharmacokinetics (Kurreck, 2003). As antisense oligonucleotides reduce the RNA levels of the mRNA target sequence, their use as cancer therapeutics has focused on targeting particular

oncogenes. For example, apoptosis inhibition includes targeting of the Bcl-2 family of proteins and indirectly through targeting of *mdm*2 via p53 pathway. Signal transduction disruption in cancer incorporates the targeting of Ras and Raf in solid tumours. Other antisense oligonucleotides targets include cytoprotective chaperones (HSP27) and DNA enzymes (methyltransferase) (Wacheck and Zangemeister-Wittke, 2006). These antisense targets are still in clinical or pre-clinical trials, and many of the problems and hurdles that are encounted in antisense oligonucleotide therapy are common to siRNAs and will be discussed later.

1.5.3 Tyrosine kinase inhibitors

Tyrosine kinase inhibitors are synthetic, mainly quinazoline-derived, low molecular weight molecules (Harari, 2004). Tyrosine kinase inhibitors interact with the intracellular tyrosine kinase domain of this family of receptors, of which EGFR is a member. The mechanism by which tyrosine kinase inhibitors operate is through inhibition of the ligand-induced, autophosphorylation of the internal tyrosine kinase of the receptor through competing for intracellular Mg-ATP-binding site and thus blocking the signal transduction pathway which has been associated with tumour proliferation and survival (Harari, 2004).

1.6 Molecular therapies targeting EGFR

The epidermal growth factor receptor has been identified as an ideal target for anti-cancer based therapies. The rationale behind targeting EGFR has been reviewed in many articles dealing precisely with this topic (Ciardiello and Tortora 2003; Baselga 2006; Fish-Steagall *et al.,* 2006 and Normanno *et al.,* 2006). This receptor has been found to be highly expressed in numerous human solid tumours including breast, head and neck, non-small cell lung, prostate, colorectal, gastric, bladder renal, pancreatic, ovarian and oesophageal tumours (Salmon *et al.,* 1995). High expression of EGFR has been assosciated with advanced tumourgenesis as well as poor prognosis and resistance to traditional therapeutics including hormone and radiation therapies (Ciardello and Tortora, 2003). Epidermal growth factor receptor and the downstream signaling pathway may result in a wide variety of cellular outcomes including; cell proliferation, migration, maturation, differentiation, metastasis, angiogenesis and apoptosis inhibition

(Woodburn, 1999). Different tactics have been explored in the therapeutic targeting of EGFR, including those therapies which target the extracellular domain of the receptor and those targeting the intracellular tyrosine kinase domain. A description of currently available, molecular based therapeutics designed specifically to target high expression of EGFR follows:

Cetuximab, a chimeric mAb specific for EGFR. Cetuximab was approved for usage in humans by the FDA in 2004 and directly competes for the natural ligand-binding sites resulting in receptor internalization and downregulation. It has been shown to inhibit growth of various numbers of tumour cells lines including colon, head and neck, non-small cell lung cancer and pancreas (Harari, 2004; Baselga, 2006).

ZD1839 (Iressa®, AstraZeneca) is a small-molecule EGFR-tyrosine kinase inhibitor that can be administered orally. It has been clinically approved for the treatment of NSCLC, but has been shown to have a broad anti-tumour effect in various solid tumours including colorectal, breast, head and neck and oesophageal (Teraishi *et al.*, 2005). In addition a cooperative effect has been seen both *in vitro* and *in vivo*, when ZD1839 is used in conjunction with traditional chemotherapeutic agents or radiology treatments (Teraishi *et al.*, 2005).

1.7 RNA interference

RNA interference (RNAi) is sequence specific, post-transcriptional gene silencing which is mediated through the formation of double-stranded RNA (dsRNA). Although RNAi had been observed in plants it was the not until work done on *Caenorhabditis elegans* in the 1990's revealed its mechanism (Fire *et al*., 1998). The process of RNAi is as follows:

Double stranded DNA is important for the initiation of the process and is derived from integrated transposons, replicating viruses or an endogenous mechanism of gene regulation known as micro RNAs (miRNAs) (Leung and Whittaker, 2005). This dsRNA is cleaved by Dicer which belongs to the RNase III family of enzymes into 21-25 RNA nucleotide fragments which are termed small-interfering RNAs (siRNAs). The Dicer protein is an ATP dependent protein, which has an N-terminal helicase domain, a PAZ domain (domain conserved through evolution that is found in Piwi/Argonaute/Zwille proteins in Drosophilia and Arabidopsis associated with developmental control), dual RNAse III domain and double stranded RNA-binding domain (Bernstein et al., 2001). These siRNAs generated through the action of the Dicer are incorporated into a multi-

component protein complex known as RISC (RNA-induced silencing complex). The RISC complex is composed of eIF2C1, eIF2C2, RNA helicase Gemin3 and Gemin4. The RISC complex then unwinds the siRNA duplex utilizing its helicase activity, the antisense siRNA is then used to target other specific mRNAs for endonucleolytic cleavage. Further cleavage of mRNAs is achieved through cellular exonuclease activity (Elbashir *et al*., 2001). The result of RNAi is very similar to that seen when using antisense oligonucleotides, however the process by which the target mRNA is silenced differs substantially.

miRNAs are comprised of approximately 22 bp in length nucleotide, non-coding sequence found in eukaryotes. The miRNAs, initially produced as long miRNA precursors by RNA polymerase II, are subsequently processed into smaller fragments via various nucleases. The miRNAs are mediated through the action of nuclear RNase III enzyme Drosha and RNase III enzyme Dicer, which generate the 22 bp duplex. The final short RNA duplex shows a high degree, but not perfect sequence complementary to more than one mRNA target transcript (Gartel and Kandel, 2006). These miRNAs are responsible for the regulation of endogenous genes in mammalian cells. The function of these miRNAs remains uncertain, however evidence suggests that these molecules are localized in cancer-associated genomic regions, and questions their involvement as tumour suppressor or oncogenes (Gartel and Kandel, 2006).

1.8 Exploiting the RNAi pathway.

RNA interference promises to be an invaluable tool, not only providing a greater understanding gene function but also the possible future use in the therapeutic field (Heale *et al*., 2005). Initiation of the RNAi pathway can be done in number of ways, however, unlike *C. elegans* and *D. melanogaster* models where long dsRNAs (> 500 bp) induce specific silencing, mammalian models prove to be more difficult. Double stranded RNA more than 30 bp in length induce the γ-interferon (IFN) which is part of the innate immune response, generally triggered by viruses (Leung and Whittaker, 2005). For this reason siRNAs have been employed to imitate the RNAi pathway. These siRNAs maybe chemically synthesized or produced as a result of enzymatic cleavage from expression systems (shRNAs). Due to their relatively small size 21-23 bp these siRNAs do not trigger the interferon response and mimic the dicer products inducing the RNAi pathway (Leung and Whittaker, 2005). The majority of siRNAs are generated from vector

systems containing RNA polymerase III promoters. The siRNAs can be generated either by transcribing the sense and anti-sense sequences separately off two separate promoters (tandem type) or by transcribing the sense and anti-sense sequence connected via loop sequence from a single promoter sequence (shRNA type). The transcribed products are then process by Dicer to generate siRNAs (Leung and Whittaker, 2005). These vector based systems are advantageous in that they may be used to create stably transfected cell lines by means of incorporating a drug resistance gene into the plasmid. A disadvantage of these vector based systems is the low transfection efficiency which has lead to the development of viral vectors (Rubinson *et al*., 2003).

Since the discovery of the RNAi pathway (Fire *et al.,* 1998), exploitation of the RNAi based technologies has not only played a pivitol role in current research methodologies, but also in the development of future therapeutics against human diseases such as viral infection, cancer or any other gene related disease e.g. Alzheimers, diabetes or heart disease (Robinson, 2004). The fact that the RNAi pathway has a high specificity and efficient down-regulation of the target gene, makes RNAi based therapeutics ideal, and prevents unwanted off-target effects and side effects. Currently there are no RNAi based therapies in clinical trials nor RNAi based treatments. Numerous experiments have shown promising results in the treatment of various cancers, targeting a number of different oncogenes. Targeted oncogenes can be divided into groups based on their functional role in the cell including oncogenic pathways, apoptosis, cell cycle regulators, cell senescence, chemotherapy resistance and tumour-host interaction which includes angiogenesis, metastasis, invasion and immune evasion (Masiero *et al.*, 2007). Specifically targeted oncogenes proposed for cancer treatment have been reviewed several times (Gartel and Kendel, 2006; Masiero *et al.*, 2007). Following is the description of three examples of oncogenes that have been targeted using RNAi based technology:

1. Bcl-2

   The Bcl-2 protein plays a regulatory role in the mitochondria-mediated apoptotic pathway and other cell death stimuli which includes gamma radiation and chemotherapy. Therefore, high expression levels of Bcl-2 has not only been observed in many cancers but also proves to be an obstacle in the treatment of these tomours with traditional chemotherapeutic approaches, due to the enhanced survivability attributed to high expression levels of Bcl-2. Recent work utilizing shRNAs to target and knockdown over-

expressed protein levels of Bcl-2 in the A549 lung cancer cell-line increased the susceptibility of the A549 cells to gamma radiation chemotherapy (He *et al.,* 2007).

2. CXCR4

   The chemokine receptor CXCR4 has been identified as a critical factor in the metastatic spread of breast cancer to the regional lymph nodes, bone marrow, lungs and liver in an organ-selective process. The breast cancer metastatic process occurs through the interaction of the chemokine recptor CXCR4 and its interaction with ligand, stromal cell-derived factor-1 (SDF-1) (Muller *et al.,* 2001). Recently it has been shown that by targeting CXCR4 with two specific siRNAs results in the inhibition of breast cancer metastasis in an animal model (Liang *et al.,* 2005).

3. Telomerase

   Telomerase is a ribonucleoprotein complex that is responsible for maintaining the heterochromatic regions (telomers) that protect the ends of the chromosomes. Human telomerase is comprised of two sub-units, the human telomerase RNA (hTR) which holds an 11 nucleotide in length sequence and acts as the template for the synthesis of additional telomeric repeats and the catalytic sub-unit known as the human telomerase reverse transcriptase (hTERT). Telomerase is usually repressed in normal tissue within the body, the exception being the germ, stem and those cells found in the basal layer of the epidermis. However, the telomerase enzyme has been found to be reactivated in many human tumours resulting in an almost limitless replicative potential as the telomere erosion that is part of natural cell division and aging is counteracted by the telomerase activity. Recently the catalytic sub-unit of the telomerase ribonucleoprotein complex (hTERT) was targeted using siRNAs. Not only were protein expression levels of hTERT and telomerase activity decreased but the two prostate cancer cell lines showed a significant decrease in cellular proliferation following transfection (Gandellini *et al.,* 2007).

The scope for RNAi approaches to modern cancer therapeutics is vast as there are numerous potential targets for treatment. The three examples discussed above show that RNAi is at the forefront of many modern anti-cancer therapeutics whether it is by decreasing cancer cell

proliferation, resistance to anti-apoptotic signals, chemotherapy resistance or reducing the metastatic ability of certain tumours. Despite the efficiency and specificity of RNAi based therapeutics inroads still need to be made into the fields of target cell delivery of the siRNAs and their *in vivo* stability (Masiero *et al.,* 2007).

1.9 Objectives

Human oesophageal squamous cell carcinoma is prevalent in South Africa and the HOSCC cell lines used in our lab have been shown to over express the low-affinity EGFR (Veale and Thornley, 1989). EGFR over expression has been shown to play a significant role in cancer development and resistance to traditional treatments (Zhang *et al.*, 2005). RNAi has been shown to be an effective method in knocking down EGFR expression in lung cancer cell models (Zhang *et al.*, 2004; Zhang *et al.*, 2005; Bai *et al.*, 2006). Using RNAi, and in particular shRNAs the aim is to specifically knock-down EGFR expression in the WHCO series of HOSCC cell lines.

1.10 Aims

The specific aims of the project are as follows:

- Design and construct effective shRNAs that specifically target EGFR.
- Test the efficiency of the shRNAs against synthetically produced target sequence.
- Show efficient and specific knockdown of endogenous EGFR expression in HOSCC lines.

Chapter 2

2.1 Design and selection of RNA's to be used for knockdown

2.2 Small interfering and short hairpin RNAs (siRNA and shRNA)

Since the discovery of the RNAi pathway the use of siRNAs has not only become a powerful research tool but is also forming the basis a new class of molecular therapeutics (Kurreck, 2006). siRNAs may be synthesized chemically or produced as a result of enzymatic cleavage, transcribed from shRNA expression systems, to mimic the Dicer products resulting in specific post-transcriptional gene silencing (Leung and Whittaker, 2005). Although great success has been observed through many RNAi based approaches, the ability to design highly effective siRNA effector molecules against specific gene targets still remains a major hurdle in this field. In an effort to overcome this hurdle much of the recent research has been directed towards a greater understanding of the RNAi pathway, as well as the creation of many databases for analyzing siRNA sequence information regarding experimentally verified siRNAs (Jagla *et al.* 2005; Chalk *et al.* 2005). Although design guidelines for siRNAs have improved the efficacy, the designed siRNAs must still be experimentally tested for potency (Kumar *et al.,* 2003). It should also be noted that use of chemically synthesized siRNAs, or siRNAs generated as result of cleaving plasmid born shRNAs, results in gene knockdown and not knockout, which is transient in mammalian cells. This transient expression is due to mammalian cells lacking the RNA-dependent RNA polymerase which amplifies the siRNAs, therefore over time the siRNAs numbers become diluted as a result of cell division (Leung and Whittaker, 2005). For this reason vector based systems have been developed that are able to induce stable siRNAs in the targeted cells. These vector systems are controlled by RNA polymerase III promoters that express either sense and antisense strands from separate promoters, or expression of shRNAs which are subsequently cleaved by Dicer to produce siRNAs (Leung and Whittaker, 2005).

Another advantage of using a vector-based system, apart from the associated stability, is the control of over-expression, since siRNAs synthesis can be induced. This has been achieved by using the tetracycline operon which is cloned in the position between the promoter and in this case, the specific siRNA. Using cells that express the tetracycline repressor will prevent gene

transcription through binding of the repressor to the operon. Doxycycline is able to bind to the repressor, preventing the repressor from binding to the operon and thus allowing RNA Pol III to bind to the operon and allowing transcription of genes downstream (van der Wetering *et al.* 2003).

2.3 siRNA/ shRNA design

In designing effective siRNA sequences against human EGFR the following design criteria were considered:

i. Sequence uniqueness: This is important in preventing non-specific degradation of other mRNA transcripts within the transfected cell that share sequence similarity with the target mRNA transcript. The sequence uniqueness is obtained by subjecting the proposed siRNAs to a BLAST search and only accepting those siRNAs that show no sequence similarity to any other human genes except for the target.

ii. Bias selection of the anti-sense sequence of the siRNA duplex: Bias selection of the anti-sense is done through the software programs developed to calculate the thermodynamic instability between the 5′ end of the sense strand versus the 5′ end of the anti-sense strand. It has been shown that a lower thermodynamic stability of the 5′ end of the anti-sense strand versus the 5′ end of the sense strand determines which strand is incorporated into the RISC complex and thus ultimately determines the targeting specificity of the siRNA (Khvorva *et al.* 2003). Further destabilization of the 5′ anti-sense end of the siRNA duplex can be achieved through the addition of G:U wobble mutations. The anti-sense sequence remains unaltered while corresponding uracil or guanine bases in the sense strand near the 3′ end can be mismatched in this manner (Holen *et al.,* 2005).

iii. RNA polymerase III promoter sequence requirements: In this study the human U6 promoter sequence is used to generate the shRNAs *in vivo*. Important design criteria include the sense sequence of the siRNA duplex starting with a guanine residue since transcription is initiated at this position. Within the shRNA sense-loop-anti-sense sequence there should be no more than three consecutive thymine residues as this may result in a premature termination signal leading to the truncation of the shRNA.

iv. General siRNA design: Optimized production of these siRNAs has revealed necessary structures to improve the efficacy, which includes the incorporation of a two basepair overhang on the 3´ ends of the duplex (Elbashir *et al.*, 2001).

For this investigation, a total number of three shRNAs directed against human EGFR were finally designed and synthesized. The design criteria mentioned above, were used in the phase leading up to the production of the shRNA sequences. The method used to synthesize these shRNA expression cassettes was done according to Castanotto *et al.* 2002.

2.4 Materials and methods

2.4.1 siRNA selection

The full length cDNA sequence of the human epidermal growth factor receptor (erythroblastic leukemia viral (v-erb-b) oncogene homologue) was obtained from the Nation Centre for Biotechnology Information (NCBI) website: www.ncbi.nlm.nih.gov/ (accession number NM 005228.)

The coding sequence of EGFR (247–3879) was inserted into an online software program. A recent attempt to access the site has revealed that it has closed down, however the algorithm on which this program is based was published by Heale *et al.* 2005. For this investigation 19 bp oligomer sequences with a two nucleotide overhang on either side were selected. The two nucleotide overhang is not completely necessary, as shall later be shown, as this may be substituted with a different sequence of nucleotides to create the loop or termination sequence. The program divides the coding sequence of human EGFR into 19 bp oligomer sequences. For each 19 bp oligomer, the software tool calculates the difference in thermodynamic potential between the 5´ ends of the sense and anti-sense strands.

The RNA Consortium (TRC) is located on the Broad Institute website http//www.broad.mit.edu. The TRC is an RNAi library, which designs siRNAs based upon an algorithm, developed separately for selected sequences obtained from the NCBI website. The selected siRNA

sequences, designed by the TRC, for the EGFR can be found at the following website: http://www.broad.mit.edu/genome_bio/trc/publicGetTransFromGeneName.php?geneName=egfr
Small interfering RNA sequence selection was based on the following criteria:

i. a positive thermodynamic value
ii. no sequence homology to any other human genes expect EGFR
iii. no more than three consecutive thymine/uracil residues.

Those siRNA sequences that did not meet the above criteria were discarded. The narrowed down selection of the siRNAs is shown in the results section.

2.4.2 shRNA design

Once the siRNA sequences had been selected, primers need to be designed for each of the sequences, in order to generate the shRNAs expression cassette. The shRNAs are produced by modification of the siRNAs, through the addition a 9 nucleotide (nt) loop sequence between the sense and anti-sense strands of siRNAs, and a termination signal comprising six consecutive thymine residues following the anti-sense sequence. The modification of siRNAs to shRNAs is shown in Figure 2.1. The modification of the three siRNAs into shRNAs directed aginst human EGFR were named appropriately EGFR sh1, EGFR sh2 and EGFR sh3. The technique used for generating these shRNA followed that of Castanotto *et al.,* 2002. The 9 nt loop sequence used in the three shRNAs differed slightly. The sequences used were: UUCAAGAGA for EGFR sh 1 and sh3 and AUCAAGAGU for EGFR sh2. Once the final hairpin sequence had been designed the reverse compliment of the sequence is needed. This must include the reverse primer sequence for the human U6 promoter (sense strand 5′ TTGTGGAAAGGACGAAACACC 3′) in order to generate the shRNA expression cassette (Figure 2.2). The G:U wobble mutations were introduced into the sense sequence (Figure 2.3).

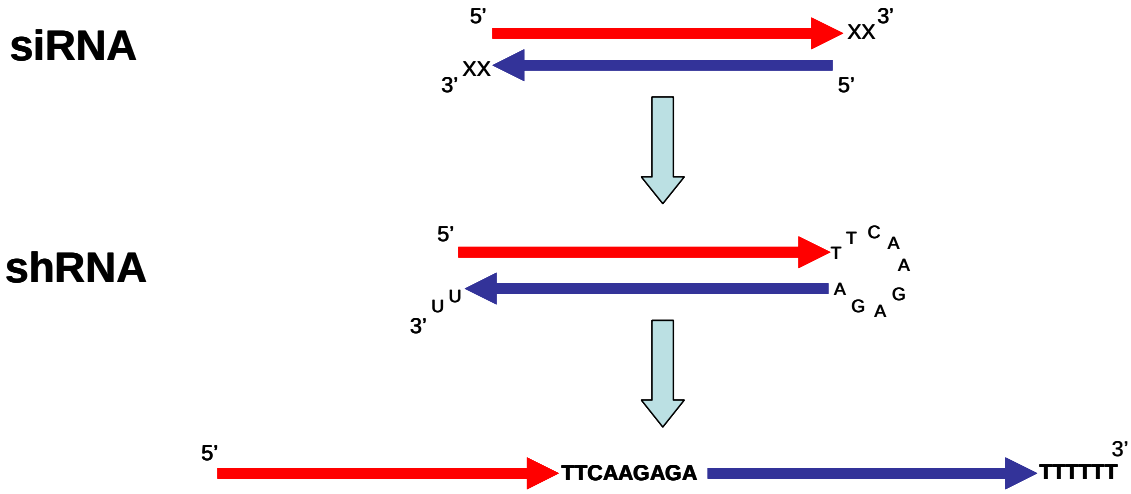


Figure 2.1: Schematic diagram indicating the modification strategy used in converting siRNA into shRNAs. The 'xx' indicates the two nucleotide overhang on the 3' of the sense (red) and anti-sense (blue) strand. The siRNA is modified through the addition of a 9 nt loop sequence (TTCAAGAGA) and a termination signal (TTTTTT) following the anti-sense strand.

2.4.3 shRNA synthesis:

The three chosen EGFR shRNAs were synthesized by Inqaba Biotech, South Africa, using the following 74 nucleotide, reverse compliment sequences (Inqaba Biotech, South Africa), to generate the three shRNAs (Figure 2.3). The technique used to generate the three EGFR shRNAs followed that of Castanotto *et al.* 2002 (Figure 2.2).

EGFR shRNA 1:

5′ - AAAAAAGCTGGATGATAGACGCAGATCTCTTGAATCTACGCCTATCATCCAGCGGTGTTTCGTCCTTTCCACAA - 3′

EGFR shRNA 2:

5′ - AAAAAAGCCACAAAGCAGTGAATTTACTCTTGATAAACTCACTGCTTTGTGGCGGTGTTTCGTCCTTTCCACAA - 3′

EGFR shRNA 3:

5′ - AAAAAAGTCGCTATCAAGGAATTAATCTCTTGAATCAACTCCTTGATAGCGACGGTGTTTCGTCCTTTCCACAA - 3′

The universal forward primer used for generating all three EGFR shRNAs was as follows:

5′ - GATCGGGCCCGTCGACAAGGTCGGGCAGGAAGAGGGCCT – 3′.

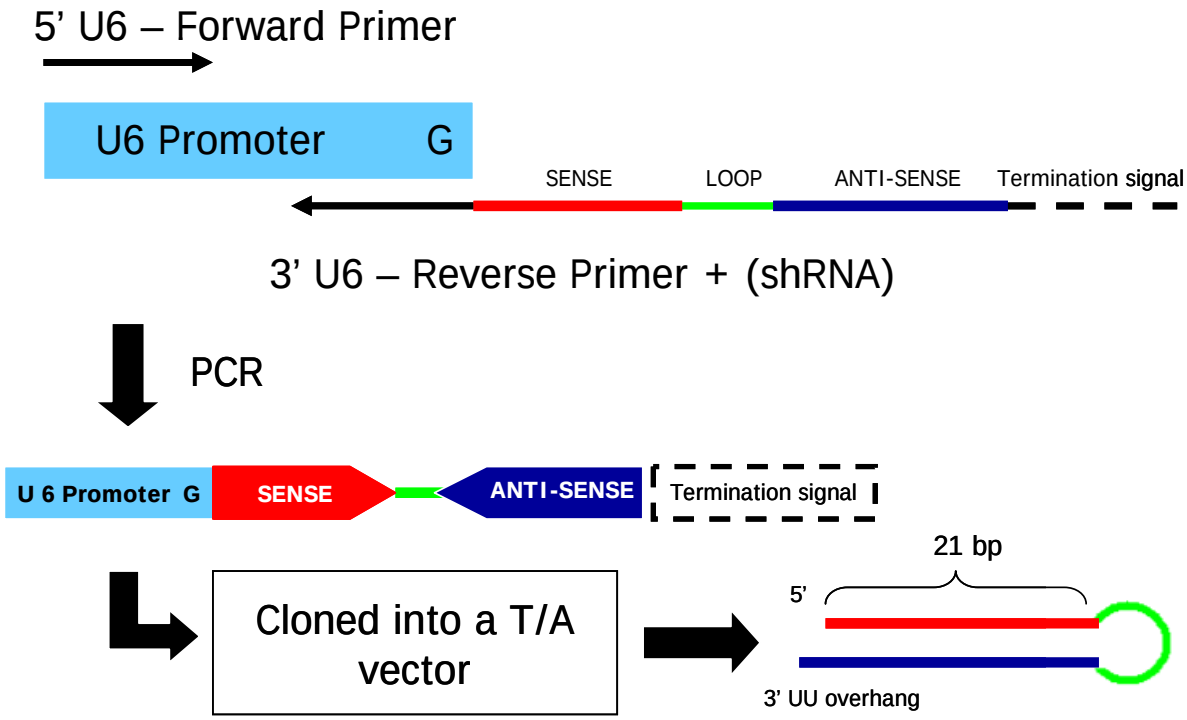


Figure 2.2: Schematic diagram of the proposed PCR strategy that will be used to construct the EGFR targeted shRNA. The 5′ forward primer is complementary to the RNA Pol III U6 promoter sequence. The 3′ reverse primer is complementary to the 3' end of the U6 promoter region, conjugated to a sense, loop, anti-sense and RNA Pol III termination signal. The PCR product will contain the RNA pol III promoter sequence U6 followed by the sense, loop, anti-sense and RNA Pol III termination signal and referred to as an shRNA expression cassette. Once cloned and transfected into a mammalian cell, the shRNA sequence is transcribed to produce the shRNA.

2.4.4 Verification of complete shRNA expression cassette

The shRNAs were cloned, ligated and inserted by Inqaba, Biotech South Africa into the T/A vector pTZ57R/T (Fermentas). To ensure that each of the shRNA expression cassettes contained the reverse human U6 promoter, sense, loop and anti-sense sequence, the three EGFR shRNA plasmids were sequenced from the T7 promoter site. The sequences obtained were aligned according to a hypothetical reverse complement sequence for each EGFR shRNA designed. Alignments were done using Vector NTI Distributive version 10, freely available from the Invitrogen website: www.invitrogen.com

2.5 Results

Small interfering RNA design requires the use of bioinformatics tools available online. The full length gene sequence encoding the Human Epidermal Growth Factor Receptor was obtained from the NCBI website (www.ncbi.nlm.nih.gov/) accession number NM 005228. This preliminary siRNA selection resulted in more than 50 sequences that were subsequently narrowed down based on the following criteria; the sequences must have a positive thermodynamic potential which is greater than an assigned value of two, and no more than three consecutive thymine bases. The siRNAs obtained from The RNA Consortium, via the Broad Institutes website, were also subjected to the same selection criteria even though they were selected using a different algorithm.

The selection was further narrowed down by blasting the sequence using the BLASTn tool. Small interfering RNAs that showed no sequence homology with any other human gene from the online database except for human epidermal growth factor receptor were chosen, and are shown in Table 1. A guanine residue at the start position of the sense strand is also favourable, as the human U6 promoter initiates transcription at this residue. However if the siRNA sequence lacked the guanine initiation residue, it was possible to alter the sequence so as to incorporate the relevent guanine residue.

Table 1: siRNAs against mRNA of human epidermal growth factor receptor (EGFR)

Description of siRNA	Sense/ Anti-sense sequence	Thermodynamic differential (Free enegry)
The RNA consortium (The Broad Institue)	5' - CGCAAGUGUAAGAAGUGCGaa - 3' 3' - agGCGUUCACAUUCUUCACGC - 5'	-1.1
	5' - GCUGGAUGAUAGACGCAGAua - 3' ***** 3' - caCGACCUACUAUCUGCGUCU - 5'	2.5
	5' - GCCACAAAGCAGUGAAUUUau - 3' ***** 3' - cgCGGUGUUUCGUCACUUAAA - 5'	8.7
Personal Selection	5' - GUCGCUAUCAAGGAAUUAAga - 3' ***** 3' - ggCAGCGAUAGUUCCUUAAUU - 5'	7.6
	5' - UCCAGAGGAUGUUCAAUAAcu - 3' 3' - ggAGGUCUCCUACAAGUUAUU - 5'	6
	5' - GAUGGAGAUGUGAUAAUUUca - 3' 3' - caCUACCUCUACACUAUUAAA - 5'	7.2
	5' - UCCACAGGAACUGGAUAUUcu - 3' 3' - cuAGGUGUCCUUGACCUAUAA - 5'	6.1
	5' - GUGACUUUCUCAGCAACAUgu - 3' 3' - guCACUGAAAGAGUCGUUGUA - 5'	3.6
	5' - GUGAGUUGAUCAUCGAAUUcu - 3' 3' - ggCACUCAACUAGUAGCUUAA - 5'	5.6

***** indicates the finally selected siRNAs.
Lower case sequence front indicates the two base pair overhang.

2.5.1 Modification of siRNAs to shRNAs

The three selected siRNAs (see * in Table 1) were converted to shRNAs through the modifications shown in Figure 2.3. The two nucleotide overhang following the sense sequence was discarded and replaced with a 9 nt loop sequence linking the sense and anti-sense sequences. The loop sequence used to generate EGFR sh2 differed slightly in sequence to that used in EGFR sh1 and sh3. This adjustment in sequence was made to avoid the occurrence of three consecutive thymine residues. A termination signal of six consecutive thymine residues was added to the 3′ end following the anti-sense sequence.

The U6 promoter sequence is incorporated into the shRNA expression cassette through the PCR strategy used by Castanotto *et al.* 2002. Addition of a 21 nt sequence preceding the sense strand of the shRNA is incorporated and acts as the reverse primer in the PCR reaction. This is possible as this sequence is complimentary to the 3′ end of the human U6 promoter. Amplification of this sequence ultimately generates the shRNA expression cassette (Figure 2.2).

Figure 2.3 shows the introduction of mismatch basepairs, in which deliberate G:U wobble mismatches were introduced into the sense strand, while the anti-sense sequence is unaltered.

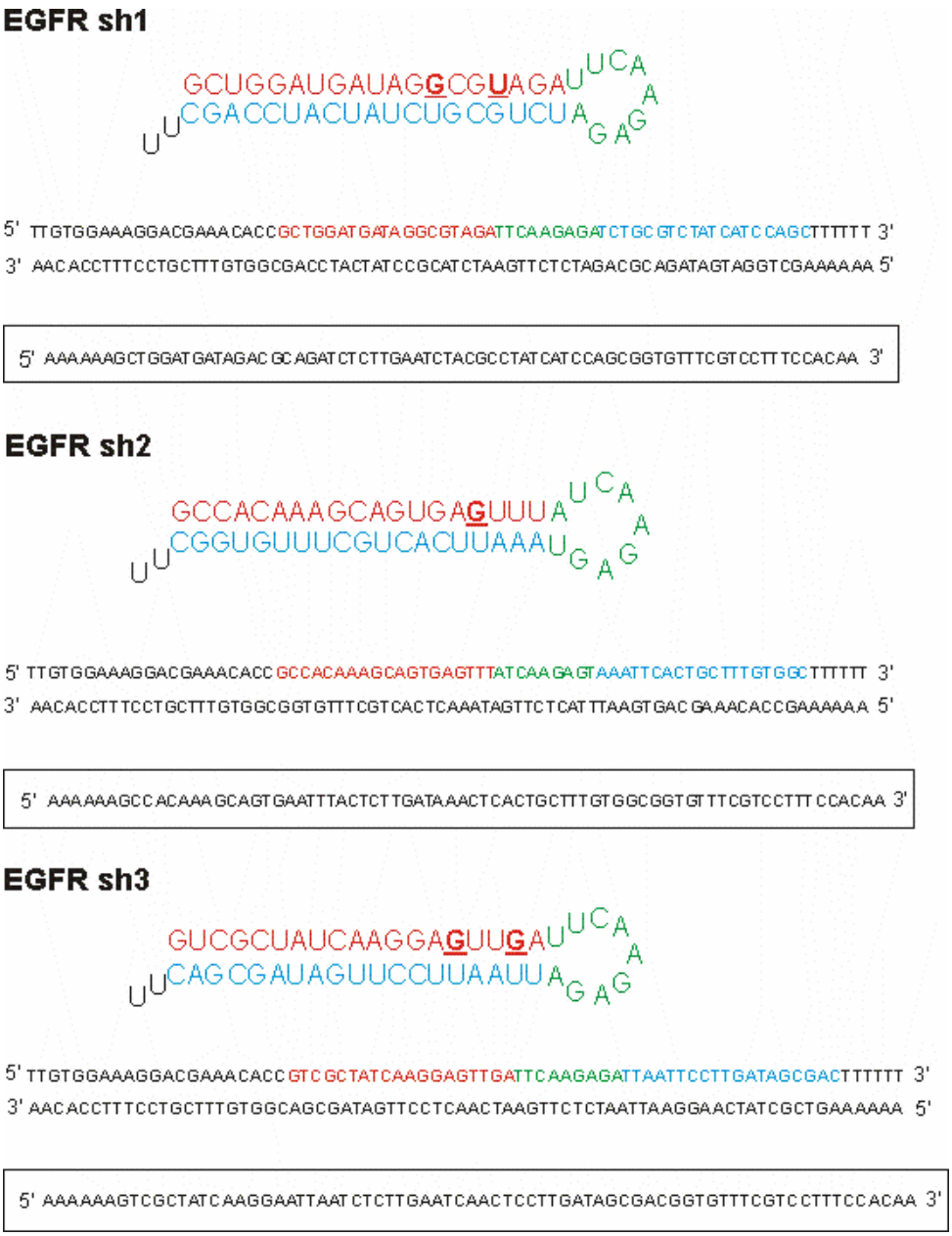


Figure 2.3: Modification of chosen EGFR siRNAs into shRNAs.

The sense strand is shown in red, 9 bp loop sequence in green and the anti-sense strand in blue. The bold, underlined basepairs found in the sense strand indicate introduced mismatch basepairs. The reverse complement 74 nt oligonucleotide sequence for each of the shRNAs, EGFR sh1, EGFR sh2 and EGFR sh3 that were ordered are indicated on the diagram.

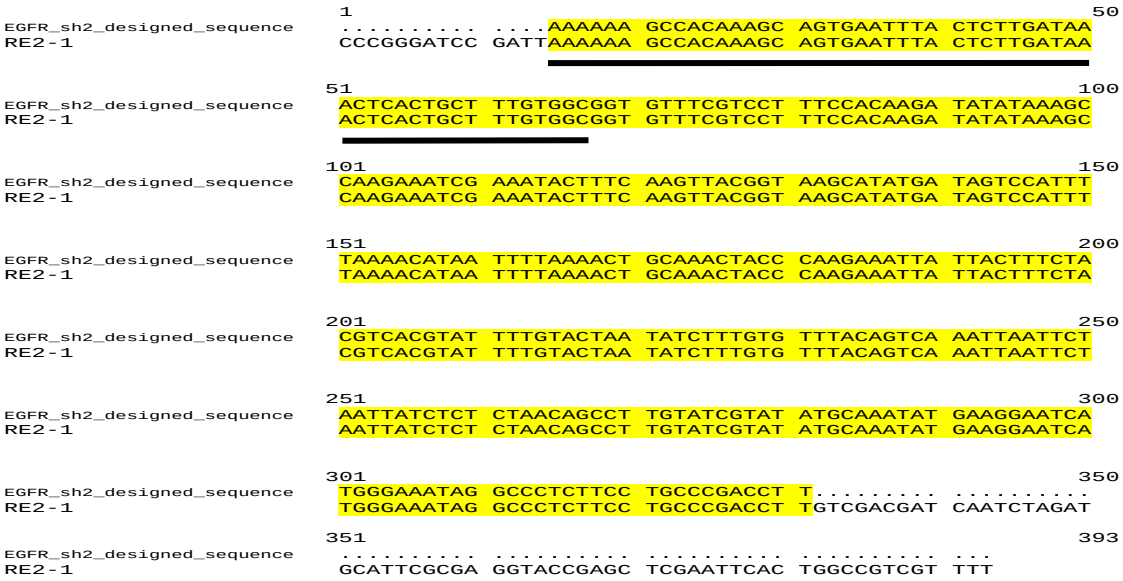
2.5.2 Verification of the shRNAs cassette sequences

Confirmation of the complete shRNA expression cassette sequence containing the human U6 promoter, sense, loop, anti-sense sequence and termination signal (TTTTTT) cloned correctly into the plasmid pTZ57R/T, was determined by sequencing from the T7 promoter site of pTZ57R. Alignments were performed using Vector NTI distributive version 10, against the designed hypothetical sequence of each shRNA. The sequence alignments are shown below for each of the EGFR shRNAs (Figure 2.4)

A.

```
                          1                                                  50
EGFR_sh1_designed_sequence\  .......... .AAAAAAGCT GGATGATAGA CGCAGATCTC TTGAATCTAC
RE1-2                     GGGATCCGAT TAAAAAAGCT GGATGATAGA CGCAGATCTC TTGAATCTAC

                         51                                                 100
EGFR_sh1_designed_sequence\  GCCTATCATC CAGCGGTGTT TCGTCCTTTC CACAAGATAT ATAAAGCCAA
RE1-2                        GCCTATCATC CAGCGGTGTT TCGTCCTTTC CACAAGATAT ATAAAGCCAA

                        101                                                 150
EGFR_sh1_designed_sequence\  GAAATCGAAA TACTTTCAAG TTACGGTAAG CATATGATAG TCCATTTTAA
RE1-2                        GAAATCGAAA TACTTTCAAG TTACGGTAAG CATATGATAG TCCATTTTAA

                        151                                                 200
EGFR_sh1_designed_sequence\  AACATAATTT TAAAACTGCA AACTACCCAA GAAATTATTA CTTTCTACGT
RE1-2                        AACATAATTT TAAAACTGCA AACTACCCAA GAAATTATTA CTTTCTACGT

                        201                                                 250
EGFR_sh1_designed_sequence\  CACGTATTTT GTACTAATAT CTTTGTGTTT ACAGTCAAAT TAATTCTAAT
RE1-2                        CACGTATTTT GTACTAATAT CTTTGTGTTT ACAGTCAAAT TAATTCTAAT

                        251                                                 300
EGFR_sh1_designed_sequence\  TATCTCTCTA ACAGCCTTGT ATCGTATATG CAAATATGAA GGAATCATGG
RE1-2                        TATCTCTCTA ACAGCCTTGT ATCGTATATG CAAATATGAA GGAATCATGG

                        301                                                 350
EGFR_sh1_designed_sequence\  GAAATAGGCC CTCTTCCTGC CCGACCTT.. .......... ..........
RE1-2                        GAAATAGGCC .TCTTCCTGC CCGACCTTGT CGACGATCAA TCTAGATGCA

                        351                                                 398
EGFR_sh1_designed_sequence\  .......... .......... .......... .......... ........
RE1-2                        TTCGCGAGGT ACCGAGCTCG AATTCACTGG CCGTCGTTTT ACAACGTC
```

B.



C.



Figure 2.4: Sequence alignment of reverse complement of the three EGFR shRNAs against the reverse compliment shRNA designed sequence.

Clones were sequenced from the T7 promoter of the plasmid pTZ57R. A. EGFR sh1 reverse complement designed sequence aligned against clone sequence RE 1-2, B. EGFR sh2 reverse complement designed sequence aligned against clone sequence RE 2-1 and C. EGFR sh3 reverse complement designed sequence against clone sequence RE 3-3. Consensus sequence of the alignment is shown in yellow. Short hairpin RNA sequence is underlined.

Sequence alignments verified the correct orientation and sequence of the three EGFR shRNAs. The only aberration detected was the deletion of a cytosine residue in the EGFR sh1 reverse sequence at position 311 (Figure 2.4), which occurred in the Human U6 promoter region. However, this was not cause for concern and was carried forward into the next phase as it was thought, and later shown, that it was likely to have little if any effect on the transcription and performance of EGFR sh1.

2.6 Discussion

The ability to design highly efficient siRNA/shRNA molecules against desired targets has become central to the use of the RNAi pathway in research and possible clinical application (Kurreck *et al.,* 2006). Recent research efforts have focused on determining the necessary intrinsic siRNA factors as well as a better understanding of the RNAi pathway with specific focus on the silencing mechanism. This research has resulted in the setup of many siRNA databases with associated stastical analyses. Intrinsic design factors that are important in siRNA design include:

i. target sequence uniqueness
ii. anti-sense strand bias selection
iii. RNA polymerase III requirements
iv. two nt overhang on the 3′ end of the sense and anti-sense strands

The initial step used in the design of shRNAs is to locate those siRNAs which show a lower thermodynamic instability between the 5′ end and the 3′ of the anti-sense strand, known as duplex end stability (Heale *et al*., 2005). This differential between the two ends of the anti-sense strand was calculated using the software which is based on the algorithm published by Heale *et al.* 2005. This asymmetric thermodynamic relationship between the two strands making up the siRNA duplex has been hypothesized to be the basis upon which strand is incorporated into the RISC complex. This process is thought to be determined by a RNA helicase that dissociates the siRNA duplex at the 5′ end with the lower thermodynamic stability (Schwarz *et al.,* 2003). The selection of the anti-sense strand of the siRNA/shRNA and its incorporation in the RISC

complex is essential in determining the specificity of mRNA degradation through the mRNA pathway (Shabalina *et al.,* 2006). It is the anti-sense sequence of the duplex that provide the RISC complex with its specificity in degrading mRNA transcripts that are complementary to anti-sense sequence (Heale *et al*., 2005). Preliminary selection of the EGFR siRNA sequences was done by selecting only those siRNAs that showed a favorable thermodynamic duplex end stability of the anti-sense strand, above a free energy value of two (Table 1).

The siRNA sequences were further narrowed down by discarding those sequences that showed sequence similarity with other human genes or if they contained more than three consecutive thymine residues. Sequence uniqueness is essential so that there is no occurrence of non-specific degradation of intrinsic cellular mRNA other than the desired target mRNA sequence which in this case is human EGFR. Sequence uniqueness was determined by performing a BLAST search of each siRNA duplex strand. The elimination of siRNA sequences that had three or more consecutive thymine residues was done to prevent premature transcription termination by RNA polymerase III.

The siRNA sequences shown in Table 1 all show promise in knocking down human EGFR. However the first Broad Institute siRNA sequence was unfavorable due to the negative thermodynamic free energy calculated. Thus the two remaining siRNA sequences from the Broad Institute and an additional one from the personal selection were selected to be modified into shRNAs (Table 1). The Broad Institute sequences were selected as Sigma-Aldrich Corporation utilizes the TRC to generate their Mission™ shRNA sequences and thus provide a possible positive control.

The three siRNA sequences selected all start with guanine residues which is important as transcription is initiated at the first guanine residue following the U6 promoter sequence.

The modification of the selected siRNAs into shRNA is shown in Figure 2.3, these shRNAs were renamed accordingly EGFR sh1, EGFR sh2 and EGFR sh3. The 9 nt loop sequence in the modification of EGFR sh2 differed slightly from the 9 nt loop sequence used for EGFR sh1 and sh3. This was done to prevent the incorporation of four consecutive thymine residues which may result in a premature termination signal, as mentioned previously.

Basepair mutations were also introduced into the shRNA design (Figure 2.3). The mismatch basepair mutations were all G:U wobble mutations designed to be asymmetrically situated towards the 3′ end of the sense strand (Figure 2.3). These mutations were introduced into the sense strand of duplex. Rationale for this is that the anti-sense sequence of this asymmetric

siRNAs/shRNAs duplex is efficiently incorporated into the RISC complex (Tang, 2005). The anti-sense strand determines the specific degradation of the RISC complex, through sequence complementarity between the anti-sense and mRNA target sequence (Schwarz *et al.*, 2003.). Thus altering the anti-sense sequence would alter the degradation specificity induced by the siRNA duplex.

As indicated in Figure 2.3, EGFR sh1 and sh3 show two G:U wobble mutations while EGFR sh2 only has one G:U mismatch. This was due to the fact that the last five nt of the sense sequence is A/U rich (Figure 2.3) and thus did not require the addition of more than one G:U wobble mismatch, as the 5′ end of the anti-sense demonstrated desirable asymmetry/thermodynamic instability.

The three EGFR shRNAs expression cassette sequences were verified through sequencing (Figure 2.4). A deletion of cytosine residue at position 311 (Figure 2.4) was observed for EGFR sh1. This was not a concern as the deletion of the cytosine basepair occurred towards the beginning of the U6 promoter sequence. Based upon the function of promoter sequences this provides sequence recognition for RNA polymerase III transcription and single basepair deletion should not affect this.

This chapter discussed the construction of three specific shRNAs targeting human EGFR. The shRNAs were designed according to several criteria that aim to ensure effective transcription of the shRNAs by RNA Polymerase III but also effective initiation of the RNAi pathway *in vivo*.

CHAPTER 3

3.1 Construction and validation of reporter systems

3.2 siRNA/shRNA target efficacy reporter system

Despite the significant advances that have been made in effective siRNA design, using a number of bioinformatical databases and online software tools, the efficiency of designed siRNA and shRNAs still need to be tested experimentally against the appropriate target sequence in order to validate the knockdown effects (Kumar *et al.,* 2003).

The design of siRNA/shRNA efficiency target reporter systems used for screening potential siRNAs/shRNAs requires the consideration of the following criteria as discussed by Malik *et al.* 2006:

i. Analysis of the predicted siRNA/shRNA duplexes using the reporter system should be able to be performed within a reasonable time frame.

ii. The reporter gene used should produce a protein product that is either directly visible, such as the use of fluorescent proteins, or produces a detectable signal that can be quantified, such as an enzyme based assay.

iii. The cloning of the target sequence behind the reporter gene should not result in translation fusion as this may disrupt the reporter protein.

iv. The contrast between the expression of the reporter gene when in the presence or absence of the target specific siRNA/shRNA, should be adequate to distinguish between the efficiency of the tested siRNAs/shRNAs in terms of knockdown ability.

v. Plasmid born siRNA/shRNAs target reporter efficiency system should allow for the accommodation of large target sequences enabling the possible formation of secondary structure in mRNA transcript which may influence knockdown.

vi. Off-target effects should be avoided, such as using a cell line that lacks an interferon response.

3.3 Reporter system description

In this study two reporter systems were used, the first being a dual luciferase plasmid psiCHECK 2.2 available from Promega. This psiCKECK 2.2 plasmid contains two luciferase genes which are under control of separate promoters. The *Renilla* (*Renilla reniformis*) luciferase is under control of the SV 40 enhancer promoter, whereas Firefly (*Photinus pyralis*) luciferase is under the control of the Herpes simplex virus (HSV) promoter. Essentially these two luciferase genes act independently from one another allowing for relative expression of the two independent genes to be measured (Promega, 2005).

The second reporter system utilizes the mammalian expression vector pCI-neo plasmid backbone (Brondyk, 1995), into which the sequence of eGFP (enhanced green fluorescent protein) from the jellyfish *Aequorea victoria*, has been cloned between the cytomegalovirus promoter sequence (CMV) and SV 40 poly-adenylation signal.

The actual mechanism of how these siRNA/shRNA target efficiency reporter systems work will be described in greater detail in the next chapter where they are used to determine the efficiency of the three design EGFR shRNAs. This chapter focuses on the construction of these reporter systems.

3.4 Materials and methods

3.4.1 Design of the shRNA efficacy reporter system

A dsDNA oligonucleotide was designed containing the three EGFR shRNAs sense (target) sequences flanked by *Xho*I and *Not*I overhangs on the 5′ and 3′ ends respectively and an *Eco*RV restriction site following the *Xho*I overhang. This target sequence was ordered from Inqaba Biotech, South Africa as a forward and reverse sequence:

3xEGFR target forward sequence (Figure 3.1A.):

5′ TCGAGATATCGCTGGATGATAGACGCAGATGCCACAAAGCAGTGAATTTAGTCGCTATCAAGGAATTAAGC 3′

3xEGFR target reverse sequence:

5′ GGCCGCTTAATTCCTTGATAGCGACTAAATTCACTGCTTTGTGGCATCTGCGTCTATCATCCAGCGATATC 3′

3.4.2 Construction of the shRNA efficacy reporter system

The 3xEGFR target DNA oligonucleotides were prepared and annealed for cloning as follows: the forward and reverse 3xEGFR target sequences were re-suspended in SABAX dH_2O to yield a final concentration of 100 µM. The forward and reverse oligonucleotides were phosphorylated separately in a final volume of 20 µl, using 2 µl of 100 µM DNA oligonucleotide stock, 2 µl 10x phosphonucleotide kinase (PNK) buffer, 2 µl 10mM ATP, 0.5 µl PNK (New England Biolabs) and 13.5 µl SABAX dH_2O. Ten microlitres of the phosphorylated forward and reverse 3xEGFR target oligonucleotides were mixed together and incubated at 75°C for 10 minutes to inactivate the PNK enzyme. The complementary reverse and forward 3xEGFR target oligonucleotides were annealed to one another by gradually cooling from 75°C to room temperature.

3.4.3 Plasmid preparation for shRNA efficacy reporter system

Once the dsDNA oligonucleotides containing all three of the shRNA target sequences (3xEGFR target) had been designed, synthesized and annealed together, the 3xEGFR target was separately cloned into two different reporter plasmid systems, namely psiCHECK 2.2 (Promega) and pCIeGFP (pCI-neo plasmid back bone obtained from Promega, the eGFP was cloned in by Dr. Marco Weinberg, (HBV Laboratory, WITS Medical School, South Africa). psiCHECK2.2 was prepared as follows: a final concentration of 2 µg of plasmid was linearized using a double digestion with 1 µl *Not*I (Fermentas) and 1.5 µl of *Xho*I (Fermentas) in 3 µl of the 10x Orange Buffer (Fermentas), and prepared in a final volume of 30 µl and incubated for 1hr 30mins at 37°C. pCIeGFP plasmid was linearized using 2 µg of vector backbone and a double digestion performed using *Sal*I (Fermentas) and *Not*I (Fermentas). The linearized psiCHECK and pCIeGFP plasmid samples were then separated on a 0.8% agarose gel. 0.8% Agarose gel was prepared by dissolving 0.24 g of agarose in 30 ml of Tris acetic acid EDTA buffer (80 mM Tris, 30 mM sodium acetate, 20 EDTA and pH adjusted to 7.2 using acetic acid. The psiCHECK and pCIeGFP plasmid bands were excised from the agarose gel and purified using the Qiagen MiniElute Gel Extraction Kit. The Qiagen MiniElute Gel Extraction Kit functions through a number of different solutions; initially Buffer QG solubilizes the agarose gel creating the

appropriate high chaotrophic salt concentration and pH (≤ 7.5) suitable for the adsorption of DNA to the silica-gel membrane. This is followed by a wash step using Buffer PE, which contains ethanol to remove excess salts. The silica-gel bound DNA is then eluted using Buffer EB (10 mM Tris-Cl, pH 8.5), as elution is most efficient under basic conditions and low salt concentrations. The linearized psiCHECK and pCIeGFP plasmids were eluted separately in 10 µl from the silica-gel membrane. The linearized psiCHECK and pCIeGFP plasmids were then dephosphorylated by adding 2 µl antartic phosphatase 10 x buffer, and 1 µl antartic phosphatase to the 10 µl eluted from the gel extraction and made up to a final volume of 20 µl with best quality H_2O. This was incubated at 37°C for 1 hour. After incubation the reaction was driven to completion by adding 1 µl of antartic phosphatase 10x buffer, 1 µl antartic phosphatase and made up to a final volume of 30 µl with best quality H_2O and incubated for a further 1 hour period at 37°C. The dephosphorylation reaction was then inactivating by heat the reaction to 65°C for 15 minutes.

3.4.4 Ligation of 3xEGFR target into the psiCHECK and pCIeGFP plasmid

The ligation reactions for the insert of the 3xEGFR target phosphorylated dsDNA oligonucleotide into the psiCHECK and pCIeGFP dephosphorylated vectors was done as follows; 2 µl of eluted, dephopshorylated, digested psiCHECK or pCIeGFP vector (50 ng), 5 µl of the 200 nM phosphorylated 3xEGFR target dsDNA oligonucleotides, 2 µl of the 10x ligation buffer, 1 µl T_4 ligase (Fermentas) and 10 µl dH_2O. Ligation controls containing no phosphorylated dsDNA 3xEGFR target oligonucleotides, were set up as follows; 2 µl of prepared psiCHECK and pCIeGFP vectors (50 ng), 2 µl of the 10x ligation buffer, 1 µl of T_4 ligase and 15 µl of dH_2O. Ligation reactions were incubated for 4 hours at 16°C, after which the T_4 ligase was heat inactivated at 65°C.

3.4.5 Transformation of Escherichia coli DH5α

Escherichia coli DH5α cells were cultured overnight in 5 ml Luria Bertani (LB) broth at 37°C. Secondary culture was prepared by adding the 5 ml of primary culture to 45 ml of fresh LB broth and incubated further at 37°C until an absorbance reading of 0.5 was obtained at a wavelength of 600 nm. The cells were harvested through centrifugation at 3 000 g for 15 minutes at 4°C. The

supernatant was discarded and the bacterial pellet re-suspended in 5 ml transformation buffer (10 mM PIPES-HCl, pH 7.0, 100 mM $CaCl_2$ and 15 % glycerol) that had been chilled on ice for 20 minutes before use. The bacteria were harvested again through centrifugation at 1 000 g for 10 minutes and at 4°C. The supernatant was discarded and the bacterial pellet re-suspended in 2 ml transformation buffer. One hundred microlitres of *E.coli* DH5α competent cells were added to the ligation reactions, mixed gently and incubated on ice for 30 minutes. The cells were heat shocked at 42°C for 90 seconds and placed on ice for a further 5 minutes. Bacteria were plated on LB plates containing 1 mg/ml final concentration of ampicillin, and incubated overnight at 37°C.

3.4.6 Confirmation of insert

All possible transformants for the psiCHECK and pCIeGFP were cultured in 5 ml of LB broth containing 1 mg/l final concentration of ampicillin, overnight at 37°C. A sample of each of the possible transformants was plated on LB plates containing 1mg/l final concentration of ampicillin. Cells were harvested through centrifugation at 10 000 g for 10 minutes. DNA plasmid isolation was performed on each of the possible clones using the high pure plasmid isolation kit (Roche). This DNA plasmid isolation kit is based on the alkaline lysis method of plasmid isolation from bacterial cultures. In the presence of chaotrophic salt, DNA will selectively bind the glass fiber fleece. The DNA is then washed several times to remove impurities, followed by elution in a low-salt solution.

The possible psiCHECK 3xEGFR target transformants were each digested in a final volume of 25 µl: 10 µl of mini preparation psiCHECK, 11.5 µl dH_2O, 2.5 µl 10x Red buffer (Fermentas) and 1 µl *Eco*RV (Fermentas). pCIeGFP 3xEGFR targets were double digested in a final volume of 25 µl as follows: 10 µl of isolated DNA plasmid preparation pCIeGFP, 10.5 µl dH_2O, 10x Red Buffer (Fermentas), 1 µl *Eco*RV (Fermentas) and 1 µl *Xho*I (Fermentas). Digestion reactions were incubated at 37°C for 1 hour and the samples were separated on a 2% agarose gel.

3.5 Results

Two efficiency reporter systems were constructed to test the knockdown efficiency of the three shRNAs. The psiCHECK 2.2 system is a dual luciferase system, while pCIeGFP has the gene encoding for enhanced Green Fluorescent Protein (eGFP) which fluoresces green when excited at a wavelength of 488 nm. The reporter system contains the three target sequences corresponding to the three constructed shRNA against human EGFR. The 3xEGFR target sequence was designed based on three hairpins from chapter 2 with flanking XhoI and NotI restriction overhangs, with an EcoRV restriction site following the XhoI overhang (Figure 3.1A.).

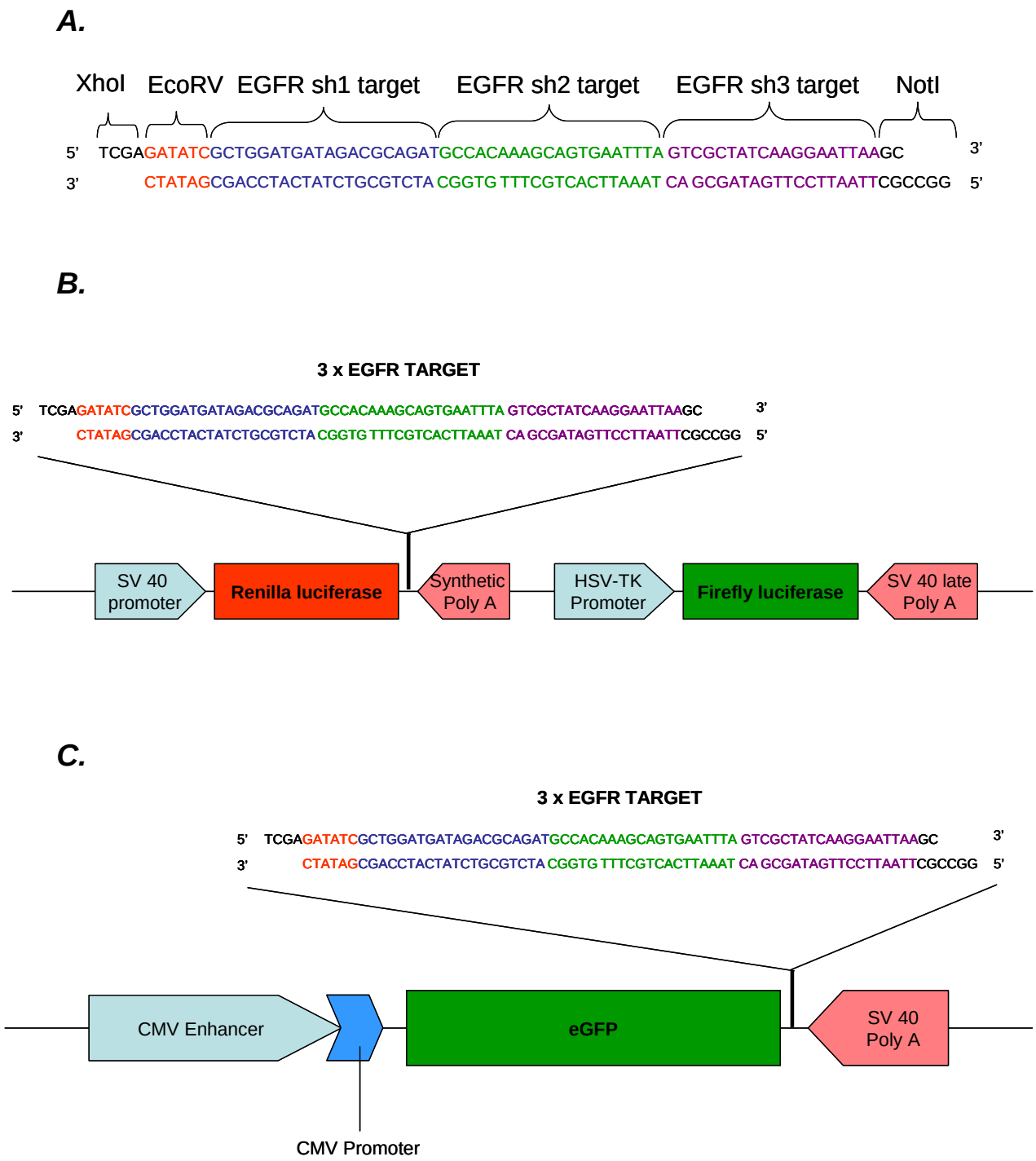


Figure 3.1: Design and cloning strategy used in constructing the EGFR shRNA efficiency reporter system.

A. Diagrammatic representation of the 3xEGFR target dsDNA oligonucleotide sequence, showing the XhoI, NotI overhangs and EcoRV restriction site. The three target sequences for each of the EGFR shRNAs are also indicated. B. Linear representation of psiCHECK 2.2 plasmid into which the 3xEGFR target sequence was cloned. C. Linear representation of pCIeGFP plasmid showing the position that the 3xEGFR target dsDNA oligonucleotide was cloned.

Following ligation of the 3xEGFR target insert into the psiCHECK and pCIeGFP efficiency reporter systems, seven possible transformants were obtained for the psiCHECK and two possible transformants for pCIeGFP shRNAs reporter systems, respectively.

The 3xEGFR target dsDNA oligonucleotide was annealed, phosphorylated and then directionally cloned into the psiCHECK 2.2 and pCIeGFP vectors which had been linearized using a double digestion XhoI, NotI and SalI and NotI respectively. Clones were screened for inserts by plasmid preparation followed by restriction endonuclease analysis. psiCHECK 3xEGFR target possible clones were digested with EcoRV only while pCIeGFP 3xEGFR targets possible clones were digested by EcoRV and XhoI. Insert was confirmed by separating these digestions on a 2% agarose gel shown in Figure 3.2:

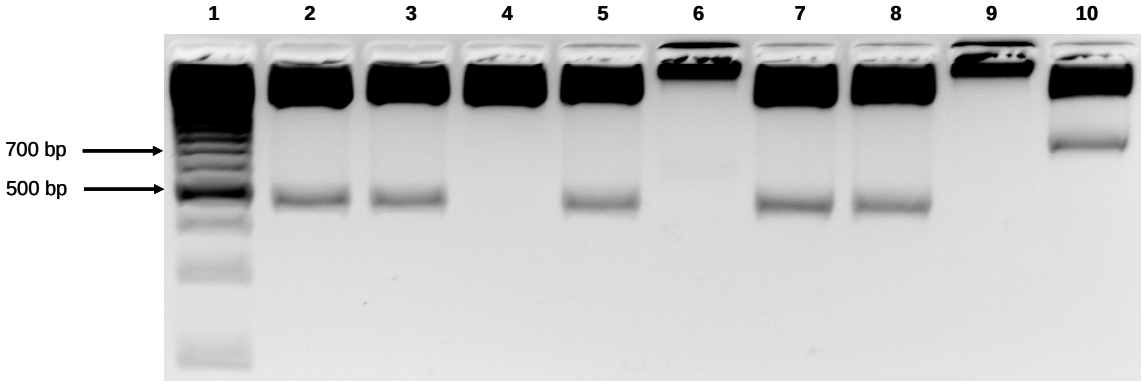


Figure 3.2: Screening of possible clones for 3xEGFR target sequence insert.

Lane 1, Marker, Lane 2-8 psiCHECK 2.2 clones, Lanes 9 and 10 pCIeGFP clones. Possible psiCHECK2.2 transformants were screened by digesting with EcoRV only, while possible pCIeGFP transformants were digested by EcoRV and XhoI. Positive clones for psiCHECK 3xEGFR target can be seen in lanes 2, 3, 5, 7 and 8 while lane 10 shows the only positive pCIeGFP 3xEGFR target clone.

Restriction analysis was performed on possible clones to ascertain the presence of an insert. Restriction analysis of positive psiCHECK clones containing the 3xEGFR target sequence by EcoRV yielded a fragment of approximately 500 bp (Figure 3.2).

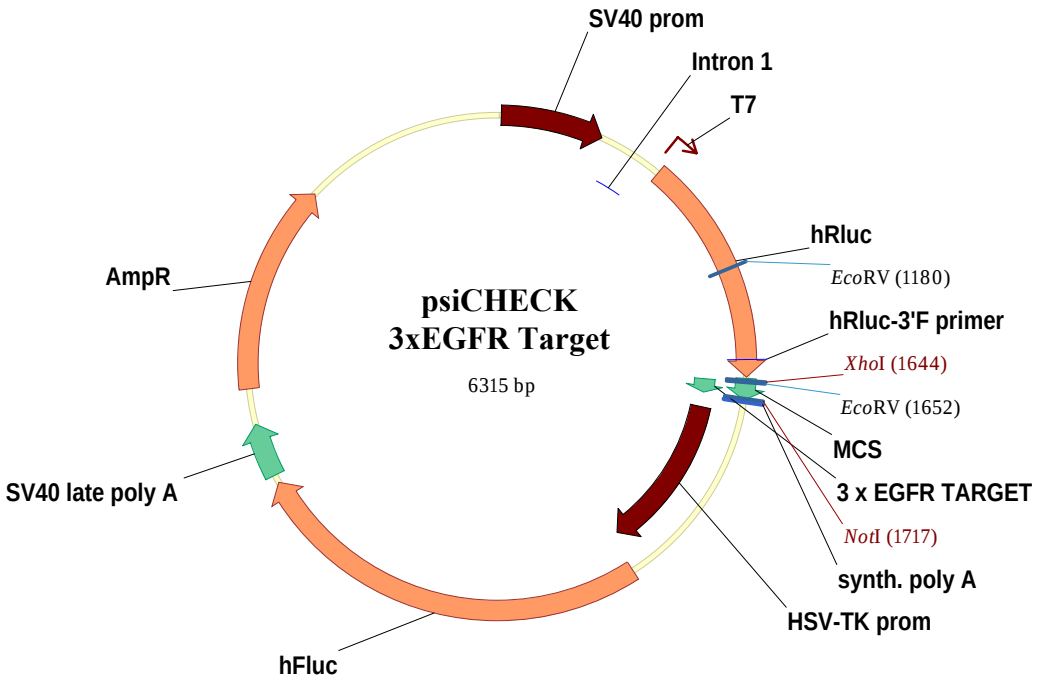
Double restriction analysis was performed on possible pCIeGFP clones containing the 3xEGFR target sequence using XhoI and EcoRV. Digestion of positive pCIeGFP clones containing the 3xEGFR target insert yielded a fragment roughly 700 bp in size (Figure 3.2).

3.6 Discussion

The use of siRNA/shRNAs target efficiency reporter systems have been developed to provide a rapid and accurate, preliminary assessment of knockdown by selected siRNA/shRNA duplexes against a synthetic target sequence. In this study two separate reporter systems were constructed to determine the knockdown efficiency against a target sequence produced specifically for this purpose.

The 3xEGFR target sequence was designed so that EGFR sh1, EGFR sh2 and EGFR sh3 could target this sequence (Figure3.1A). The 3xEGFR target sequence was directionally cloned into the reporter systems psiCHECK 2.2 and pCIeGFP (Figures 3.1B. and 3.1C.). The position into which the 3xEGFR target sequence was cloned in such that the target sequence is sequentially inserted following the respective gene, Renilla luciferase in psiCHECK and eGFP in pCIeGFP. The target sequence and reporter gene was linked sequentially by cloning the target sequence between the stop codon of the reporter gene and the poly-adenylation signal. This prevents translational fusion and possible functional disruption of reporter protein.

A.



B.

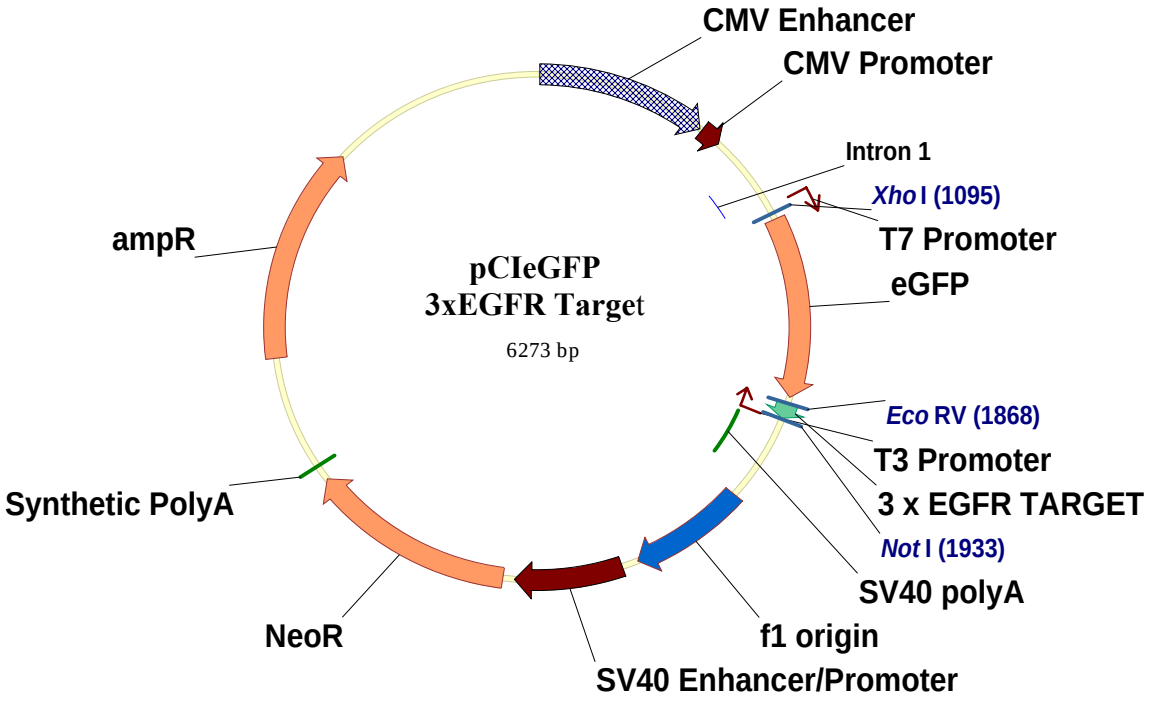


Figure 3.3: Plasmid maps of the two shRNA efficiency reporter systems psiCHECK and pCIeGFP containing the 3xEGFR target insert for testing efficiency of the EGFR shRNAs.

A. psiCHECK 3xEGFR target report system showing the features simian virus (SV) 40 promoter, Renilla Luciferase (hRluc), multiple cloning site (MCS), the 3xEGFR target sequence, Herpes Simplex virus promoter (HSV-TK) Firefly luciferase (hFluc) and ampicillin resistance (AmpR). B. pCIeGFP 3xEGFR target reporter system showing the features; cytomegalovirus (CMV) promoter and enhancer, enhanced green fluorescent protein (eGFP), the 3xEGFR target sequence, simian virus (SV) 40 enchancer promoter, neomycin resistance (NeoR) and ampicillin resistance (ampR). In both images useful restriction sites pertaining to this research are indicated, position shown in brackets.

The 3xEGFR target sequence was designed with an EcoRV restriction site following the XhoI overhang (Figure 3.1A.). This EcoRV restriction site was used to confirm insertion of 3xEGFR target in these transformants.

Positive psiCHECK 3xEGFR target clones will contain two EcoRV restriction sites, as there is an EcoRV site intrinsic to the psiCHECK 2.2 backbone. Thus digestion of positive psiCHECK containing the 3xEGFR target is predicted using Vector NTI, distributive version 10, as yielding

a fragment 471 bp in size (Figure 3.3A.) which is confirmed by the 500 bp marker band in Figure 3.2.

pCIeGFP plasmid does not contain EcoRV restriction site therefore verification of the insert in the positive clones needed to be performed via a double digestion. The EcoRV restriction site that has been incorporated into the 3xEGFR target sequence allows for double digestion using EcoRV and XhoI to analyze positive pCIeGFP clones. Based on the prediction by Vector NTI, double digestion of a positive pCIeGFP containing 3xEGFR target sequence clone should yield a fragment 773 bp in size(Figure 3.3B.), which corresponds to the fragment band size observed in Figure 3.2, Lane 10.

Due to the fact that the 3xEGFR target sequence was directionally cloned into the reporter systems using the XhoI and NotI restriction overhangs it is not necessary to check for orientation.

This chapter describes the design and cloning procedure of a synthetically produced target sequence which was cloned into two separate shRNA efficiency repoter systems. The report system provides us with information on the likelihood of efficiency of knockdown by each of the designed EGFR shRNAs.

CHAPTER 4

4.1 EGFR shRNAs knockdown efficiency assay

This chapter examines the knockdown efficiency of the three shRNAs; EGFR sh1, EGFR sh2 and EGFR sh3, against a synthetically designed target sequence which was cloned into two different plasmid born reporter systems (Chapter 3). This necessitates a discussion of the mechanism by which the reporter system psiCHECK and pCIeGFP plasmid operates.

4.1.1 psiCHECK 2.2 dual luciferase reporter system

psiCHECK 2.2 dual luciferase reporter system contains two individual luciferase genes that are under control of separate promoter systems, located on the same plasmid. The mechanisms by which the psiCHECK 2.2 reporter system operates is that a target sequence, to which siRNA/shRNAs have been designed to knockdown, is cloned into the multiple cloning site located down stream of *Renilla* luciferase gene and preceding the synthetic poly-adenylation signal. This effectively fuses the *Renilla* luciferase gene with the target sequence. Following cloning the psiCHECK vector containing the desired target sequence is co-transfected with the siRNAs or shRNAs of interest. If the siRNA or shRNA duplex is effective in initiating the RNAi pathway the fused *Renilla* luciferase-target sequence mRNA will be cleaved and degraded thus decreasing the *Renilla* luciferase signal. The Firefly luciferase gene acts as background control against which the *Renilla* luciferase signal can be normalized. Due to the distinct evolutionary origins of the *Renilla* and Firefly luciferase enzymes they have dissimilar structures and substrate requirements needed to produce their respective bioluminescent reactions. The Dual-Luciferase Reporter Assay System (Promega) operates by the addition of a substrate necessary for the Firefly bioluminescent reaction to occur. Once this signal is quantified, the Firefly reaction is stopped and the *Renilla* luciferase bioluminescent reaction is activated and the corresponding signal quantified (Promega, 2006).

4.1.2 pCIeGFP reporter system

The pCIeGFP reporter system has a similar mechanism however this reporter system encodes for a fluorescent protein (eGFP) and isn't based on an enzymatic reaction. Again the target sequence is cloned into the pCIeGFP plasmid after the eGFP stop codon preceding the poly-adenylation signal. Thus, the siRNA/shRNA target sequence is fused to the eGFP gene. The pCIeGFP plasmid is then co-transfected with a siRNA/shRNA of interest into the appropriate cell line. If the siRNAs/shRNAs initiate the RNAi pathway, there will be degradation of the eGFP fused target sequence mRNA, results in a decrease in eGFP fluorescence when compared to the control.

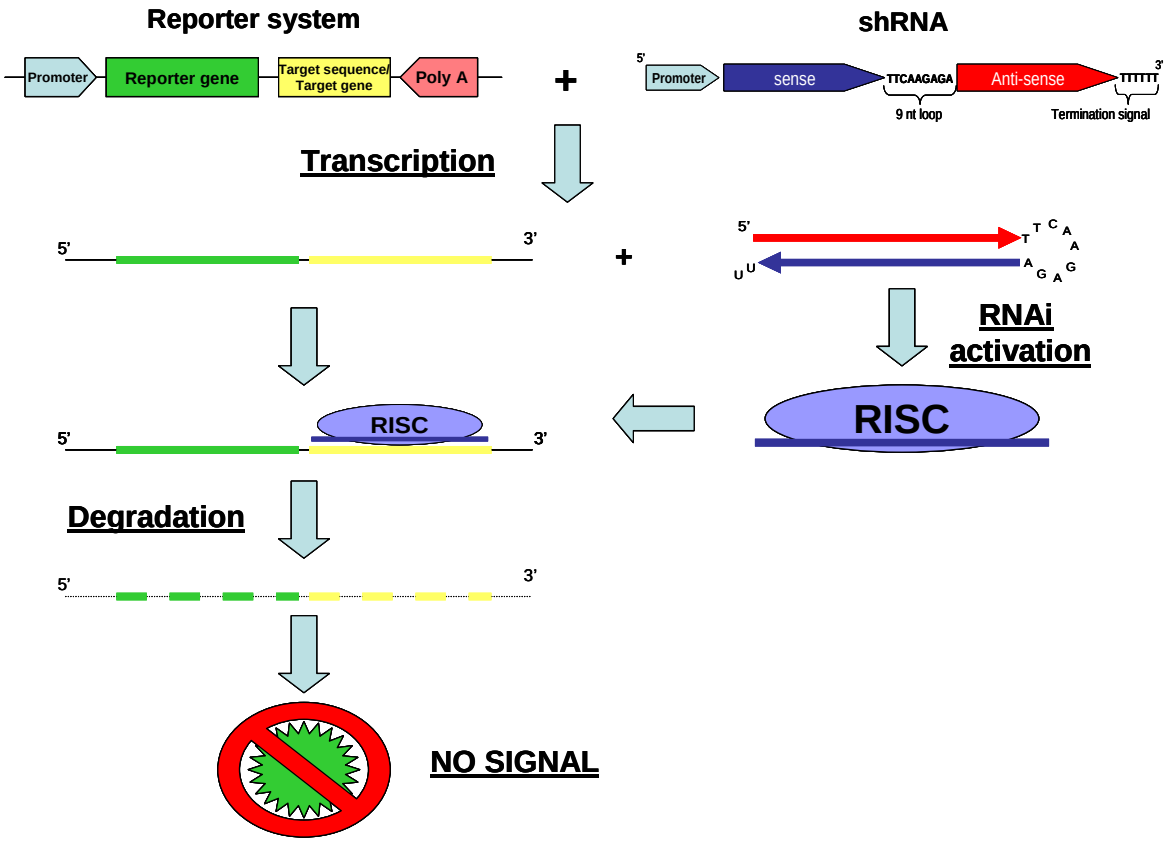


Figure 4.1: General schematic representation of a knockdown reporter system.

Cell line of choice is transfected with the knockdown efficiency reporter system containing the target sequence (reporter system) and the shRNA expression cassette (shRNA) designed against the target sequence. Following transcription, if the shRNA initiates the RNAi pathway there will be specific degradation of the target sequence. Since the reporter gene is sequentially linked to the target sequence, the reporter gene will also be degraded and, thus no detectable signal.

In this study the knockdown reporter assay was done in the Human embryonic kidney (HEK) 293 cell line. The reasons for this is that the HEK 293 cells is a particularly easy line to work with as they have relatively simpler growth requirements, but more importantly the HEK 293 cell line shows good transfection efficiency. The HEK 293 cell lines lack the interferon response (Malik *et al.,* 2006), which prevents cellular shut down and off target effects and makes the line a good candidate in testing the knockdown of reporter systems.

4.2 Materials and methods

4.2.1 Mammalian transfection plasmid description

The design and synthesis of the three EGFR shRNA expression cassettes were described in chapter 2 and the construction of the shRNA knockdown efficiency report systems psiCHECK 3xEGFR target and pCIeGFP 3xEGFR target was described in chapter 3. Sh5, pCIeGFP, pDsRED and pTZ U6+1 were control plasmids used in this experiment, all four plasmids were kindly provided by Dr. M. Weinberg, HBV laboratory, WITS, South Africa. Sh5 was used as the non-specific hairpin with the following sequence (Carmona *et al.,* 2006):

sense sequence: 5′ GUCGUGUGCGCCUUUGCUUCGCCUCUG 3′

anti-sense sequence: 5′ CAGAGGUGAAGCGAAGUGCACACGG 3′.

The pTZ U6+1 comprises the pTZ57R plasmid which has had the human U6 promoter sequence only cloned in. pCIeGFP is the unaltered reporter, having no-target sequence cloned into it and pDsRED plasmid expresses Red fluorescent protein.

4.2.2 Suitable DNA plasmid isolation for mammalian cell transfection

DNA plasmid isolation of the following plasmids were prepared for mammalian cell transfections EGFR sh1, EGFR sh2, EGFR sh3, pCIeGFP 3xEGFR target, psiCHECK 3xEGFR target, pTZ U6+1, sh5, pCIeGFP and pDsRed, and were performed using the Qiagen Maxi Plasmid Purification kit. The plasmid purification principle is based upon alkaline lysis of the

bacterial cells and precipitation of the genomic DNA followed by the binding of the DNA to the Qiagen Anion-exchange resin under low-salt and the appropriate pH conditions. RNA, protein and other low weight molecular molecules are removed through a series of medium salt washes. The plasmid DNA is eluted from the resin using a high salt concentrated buffer, followed by isopropanol precipitation and ethanol wash steps.

4.2.3 DNA Quantification

Plasmid preparations purified from the Qiagen Maxi prep kit were re-suspended in a final volume of 100 µl of SABAX dH_2O. The DNA concentration of each sample was determined using the NanoDrop (ND-100) UV-VIS spectrophotometer, and working stock of each was prepared at concentration of 0.5 µg/µl.

4.2.4 Mammalian cell culture and transfection

HEK293 cells were cultured in Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum and cultured at 37°C in a 5% CO_2 rich atmosphere. Twenty four hours prior to transfection cells were subcultured and counted using a haemocytometer and seeded 120 000 cells per well in a 24-well dish, in 500 µl of complete medium per well.

4.2.4.1 psiCHECK 3xEGFR target efficiency report system transfection

Transfection of the HEK293 cells was carried out in 24-well dish using a 5:1 ratio of EGFR shRNAs to psiCHECK 3xEGFR target and pCIeGFP as a background control. Thus total of 1 µg of total DNA was used in the transfection in each well of which 100 ng was background transfection control. Transfection protocol differed slightly from that recommended by the manufacturer. A ratio of 1:1 DNA final concentration to Lipofectamine 2000 volume was used mixed with 1/5th the volume of culture volume used in OptiMEM (Gibco).

4.2.4.2 The pCIeGFP 3xEGFR target reporter system transfection

Transfection of the HEK293 cells was done according to the same ratios as those used in the psiCHECK 3xEGFR target transfection, however pDsRed was used as background control instead of pCIeGFP. Triplicates of each transfection were prepared and the details are shown in Table 2.

One hundred microlitres of the 1 µg DNA/1 µl Lipofectamine 2000 in OptiMEM was added to each well of the 24-well dish, and incubated for 24 hours, after which the media was discarded and replaced with fresh complete media.

Table 2: Details of the DNA concentration used in the HEK293 cell transfection in determining the EGFR shRNA efficiency reporter system.

psiCHECK transfection description	DNA amount (ng)	pCIeGFP transfection description	DNA amount (ng)
psiCHECK 3xEGFR target	150	pCIeGFP 3xEGFR target	150
pTZ U6+1	750	pTZ U6+1	750
pCIeGFP	100	pDsRed	100
psiCHECK 3xEGFR target	150	pCIeGFP 3xEGFR target	150
EGFR sh1	750	EGFR sh1	750
pCIeGFP	100	pDsRed	100
psiCHECK 3xEGFR target	150	pCIeGFP 3xEGFR target	150
EGFR sh2	750	EGFR sh2	750
pCIeGFP	100	pDsRed	100
psiCHECK 3xEGFR target	150	pCIeGFP 3xEGFR target	150
EGFR sh3	750	EGFR sh3	750
pCIeGFP	100	pDsRed	100
psiCHECK 3xEGFR target	150	pCIeGFP 3xEGFR target	150
sh5	750	sh5	750
pCIeGFP	100	pDsRed	100

4.2.5 Dual luciferase assay

Twenty four hours post-transfection of the HEK293 cells, the knockdown efficiency of the three EGFR shRNAs and control plasmid pTZ U6+1 and sh5 were determined using the psiCHECK 3xEGFR target plasmid. Luciferase activities were determined using the Dual-Luciferase®

Reporter Assay System (Promega) according to the manufacturer's specifications and the luciferase signal was quantified using the Veritas Microplate Luminometer, Turner BioSystems. Cells were first lysed using the passive lysis buffer, which releases the *Renilla* and Firefly luciferase reporter enzymes into the cell lysate. The Firefly luciferase signal is activated by the addition of Luciferase Assay Reagent II (LARII) which is then quantified by the luminometer. The Firefly luciferase signal is then quenched and the *Renilla* luciferase signal activated through the addition of Stop & Glo to the same reaction which is subsequently quantified by the luminometer. The results were obtained using three independent lysates generated from three independent mammalian cell transfections and represented as a normalized Firefly to *Renilla* luciferase values. The results are a normalized representation of Firefly to *Renilla* luciferase values.

4.2.6 pCIeGFP EGFR shRNA efficiency reporter system

Twenty four hours following the HEK293 cell transfection, the knockdown efficiency was determined visually by using the Zeiss LSM confocal microscope. GFP fluoresces when excited at a wavelength of 488 nm. The Red Fluorescent protein (transcribed from the pDsRed plasmid) was excited at a wavelength of 558 nm.

4.3 Results

Two shRNAs efficiency reporter systems were constructed to test the efficacy of the design EGFR shRNAs versus a target sequence that had been inserted downstream of a reporter gene. The target sequence is a synthetic dsDNA oligonucleotide, containing the three target sequences corresponding to the three EGFR shRNAs designed and synthesized in chapter 2 and 3.

4.3.1 psiCHECK dual luciferase reporter system

The 3xEGFR target sequences were cloned into the MCS which is located between the *Renilla* luciferase gene stop codon and the synthetic poly A tail. Co-transfection psiCHECK 3xEGFR target plasmid and one of the EGFR shRNAs should result in knockdown of the *Renillia*

luciferase gene in relation to the Firefly luciferase which acts as a background control for the experiment. Luminometer data show an average normalized ratio of Firefly luciferase to *Renilla* luciferase (Figure 4.2).

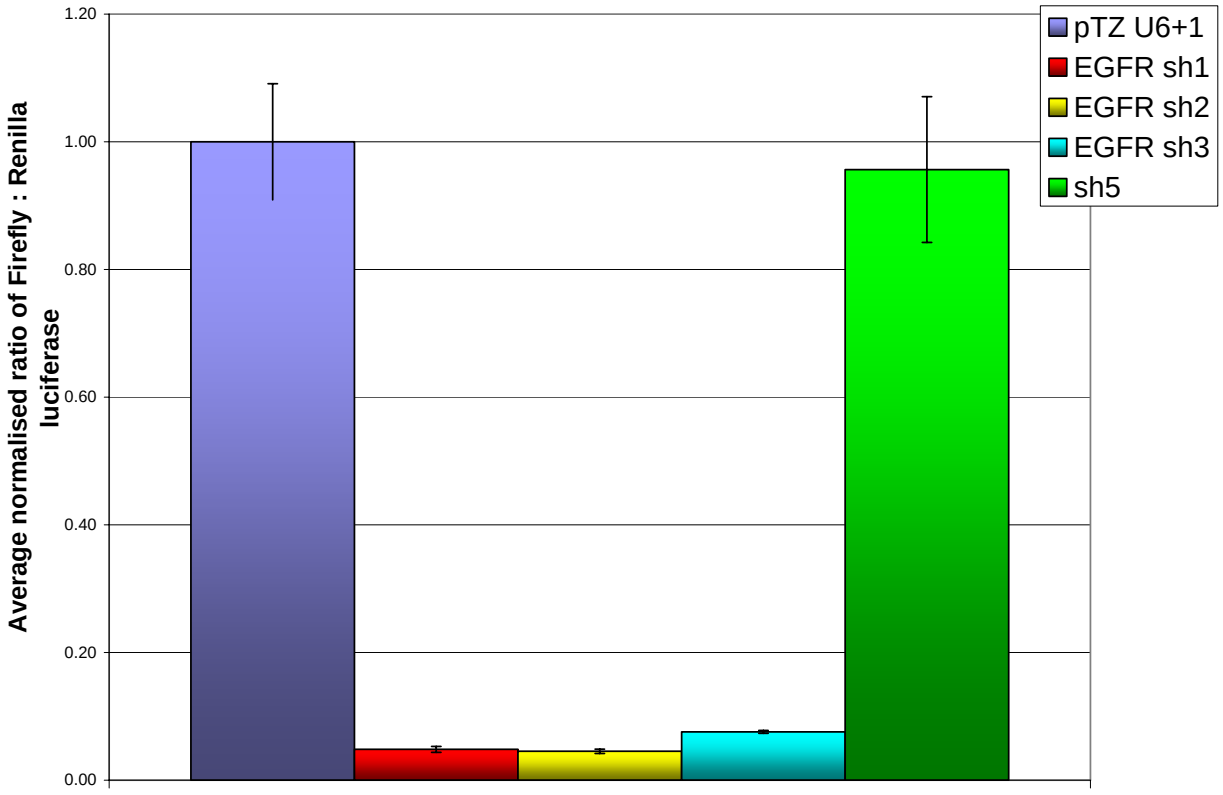


Figure 4.2: Knockdown efficiency of the three EGFR shRNAs against the psiCHECK 3xEGFR target plasmid dual luciferase reporter system.

Renilla luciferase values were normalized against Firefly luciferase values and standardize against pTZ U6+1 co-transfection. EGFR sh1 and EGFR sh2 resulted in 95% knockdown and EGFR sh3 showed 92% knockdown of the psiCHECK 3xEGFR target reporter system. Error bars produced by stastical comparison of means.

Knockdown of the psiCHECK 3xEGFR target plasmid system was extremely efficient by all three EGFR shRNA designed, although a slight variation was observed between the three shRNAs (Figure 4.2). EGFR sh1 and EGFR sh2 resulted in a 95% knockdown of *Renilla* luciferase gene compared to the Firefly luciferase, while EGFR sh3 showed a slightly less knockdown ratio of 92%. The normalized values were all standardized according to the pTZ U6+1 values. The non-specific hairpin sh5 showed negligible decrease in *Renilla* luciferase compared to the three EGFR specific shRNAs.

4.3.2 pCIeGFP 3 x EGFR target reporter system

The 3xEGFR target sequences were cloned into the pCIeGFP plasmid between the eGFP stop codon and the poly adenylation signal. Co-transfection of this pCIeGFP 3 x EGFR target plasmid and one of the shRNA effector molecules and the control hairpin and promoter sequence yielded the following results (Figure 4.3.).

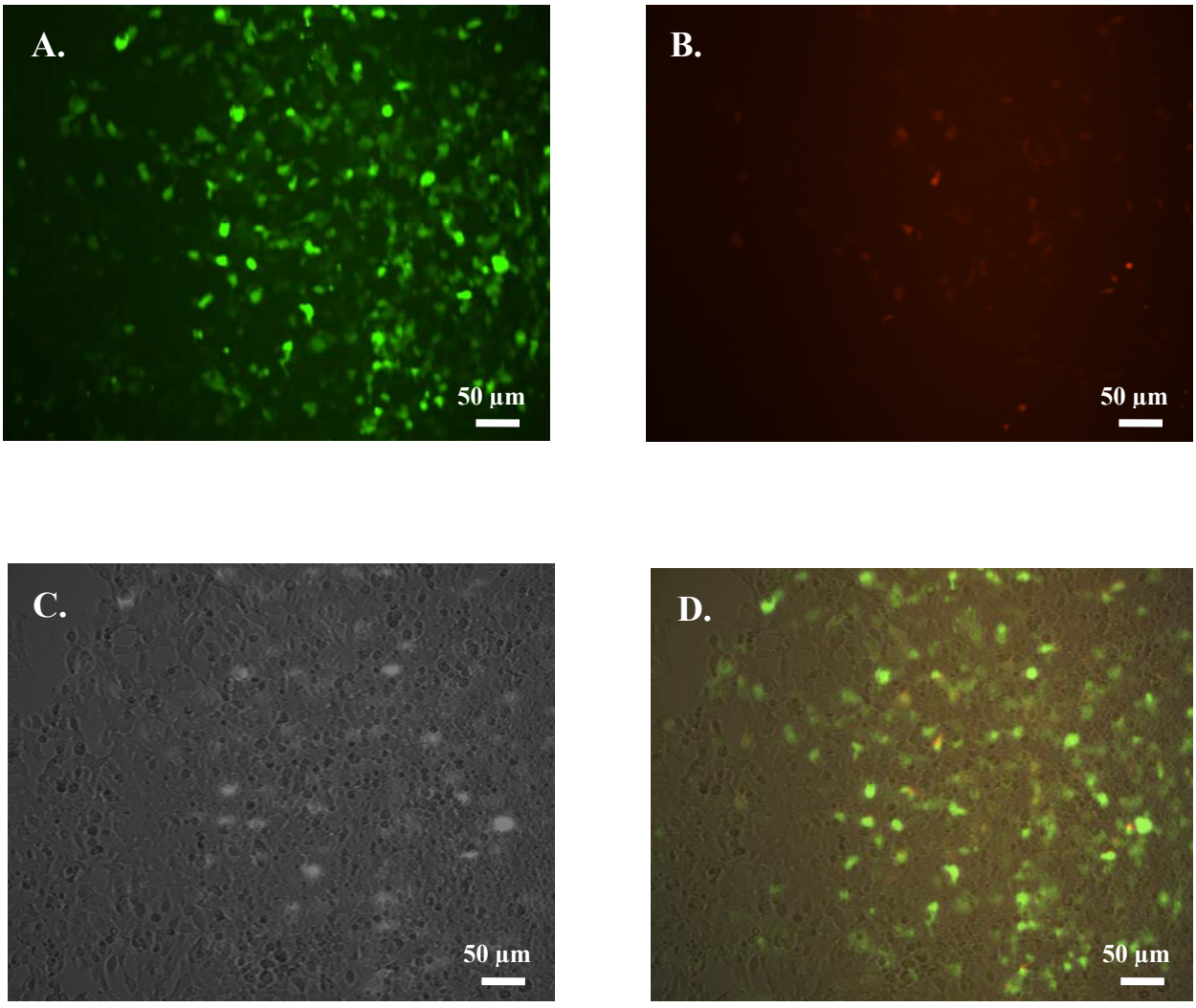


Figure 4.3.1 Fluorescent images showing the knockdown efficiency of the pCIeGFP 3xEGFR target, co-transfected with pTZ U6+1 and pDsRed as background control.

HEK293 cells were co-transfected with pCIeGFP 3xEGFR target, pTZ U6+1 and pDsRed as a background control. A. fluorescing eGFP at 488 nm, B.DsRed fluorescence at 558 nm, C. transmission image and D. overlay of all three channels.

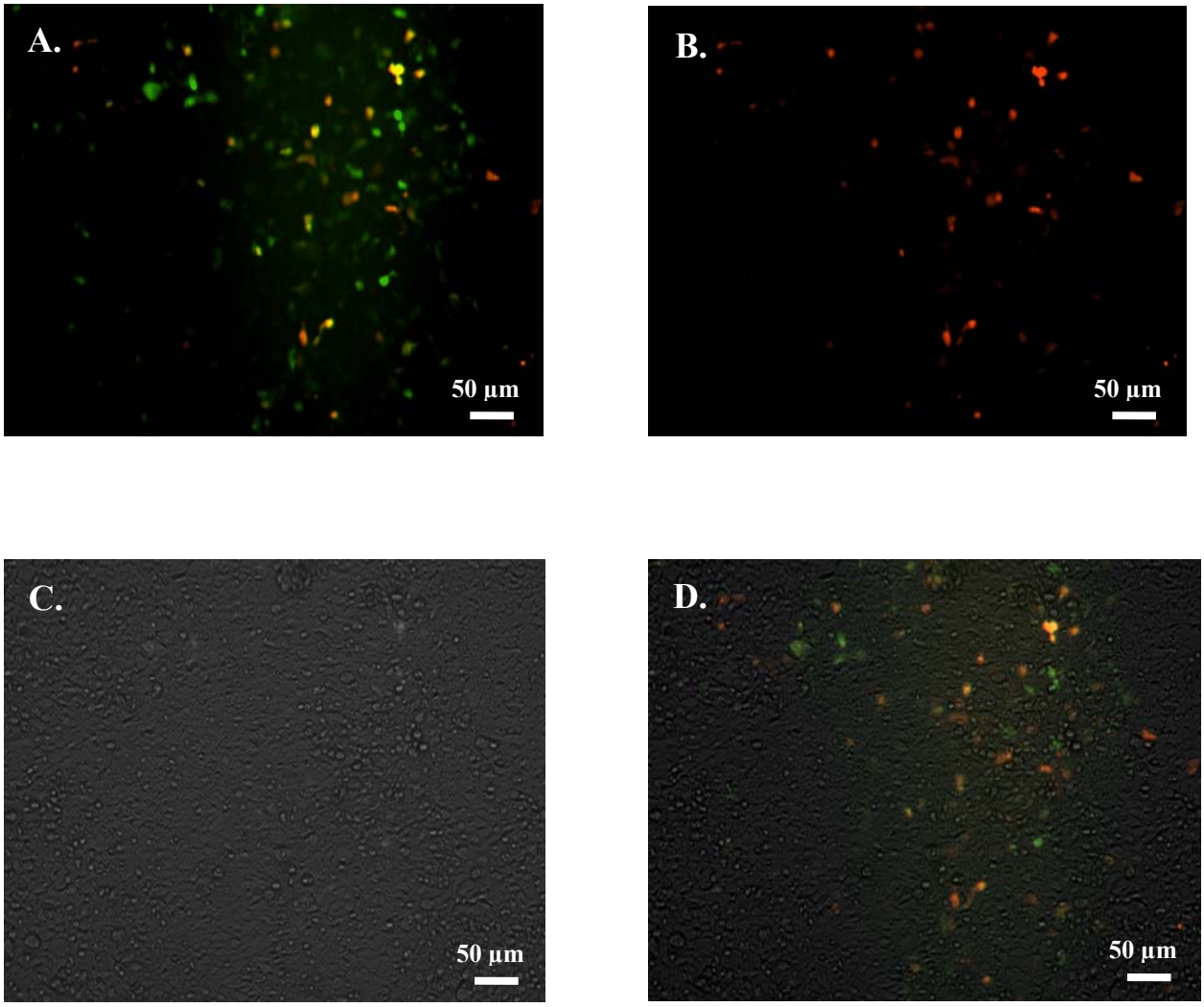


Figure 4.3.2: Fluoresecent images showing the knockdown efficiency of the pCIeGFP 3xEGFR target, co-transfected with EGFR sh1 and pDsRed as background control.

HEK293 cells were co-transfected with pCIeGFP 3xEGFR target, EGFR sh1 and pDsRed as a background control. A. eGFP fluorescence at and DsRed fluorescence overlay, B.DsRed fluorescence, at 558 nm C. transmission image and D. overlay of all three channels.

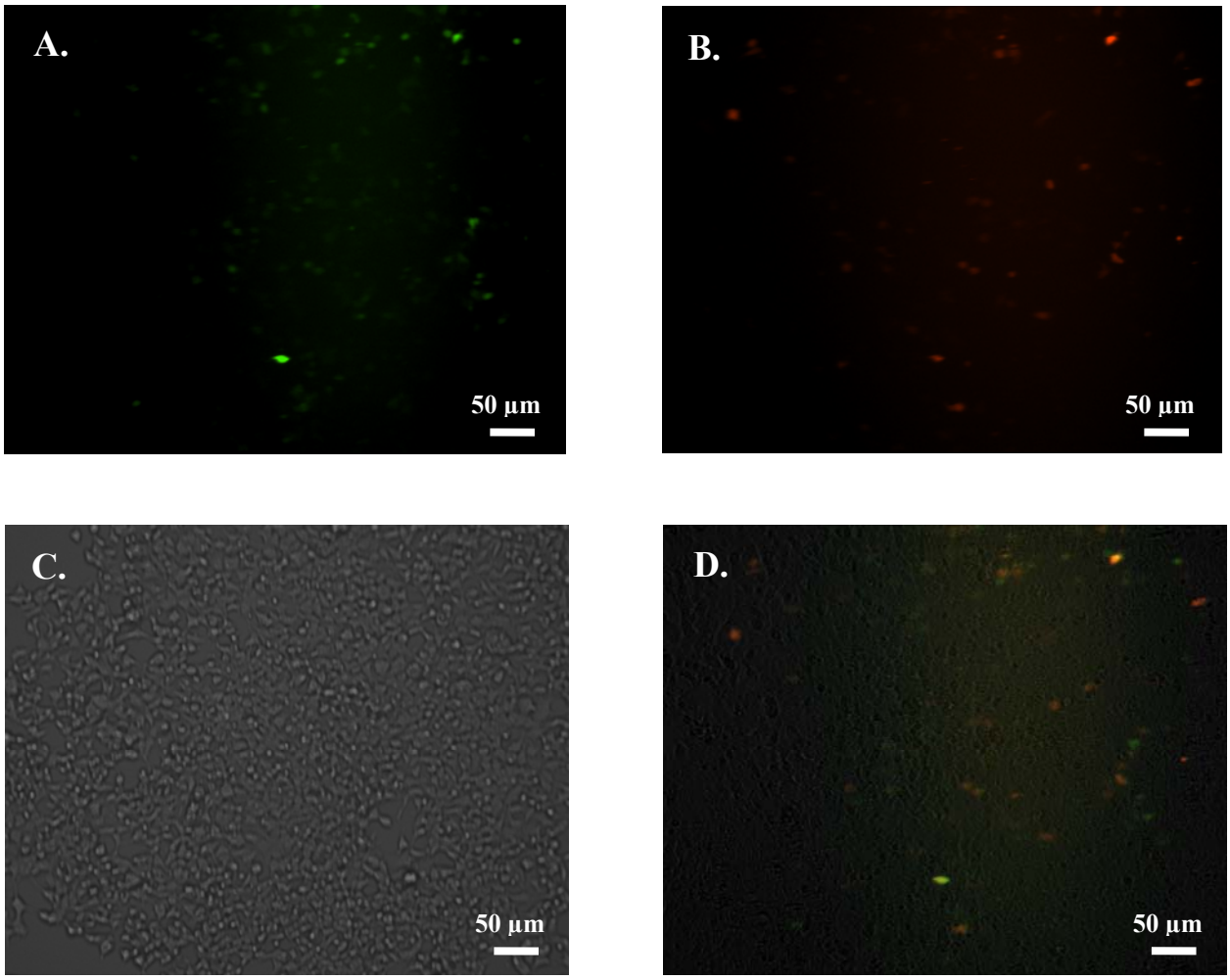


Figure 4.3.3: Fluoresecent images showing the knockdown efficiency of the pCIeGFP 3xEGFR target, co-transfected with EGFR sh2 and pDsRed as background control.

HEK293 cells were co-transfected with pCIeGFP 3xEGFR target, EGFR sh2 and pDsRed as a background control. A. eGFP fluorescence at 488 nm, B.DsRed fluorescence at 558 nm, C. transmission image and D. overlay of all three channels.

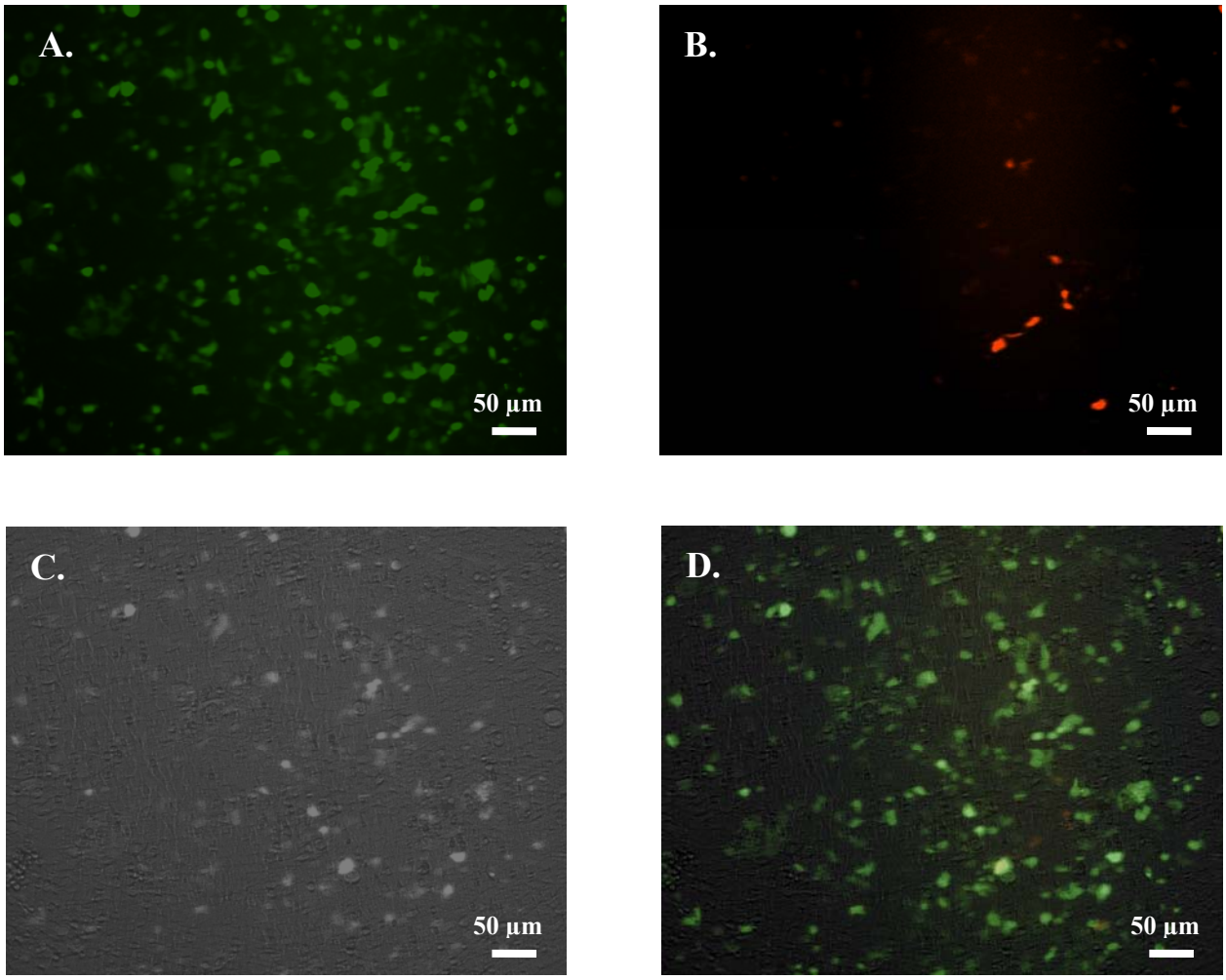


Figure 4.3.4: Fluorescent images showing the knockdown efficiency of the pCIeGFP 3xEGFR target, co-transfected with EGFR sh3 and pDsRed as background control.

HEK293 cells were co-transfected with pCIeGFP 3xEGFR target, EGFR sh3 and pDsRed as a background control.A. eGFP fluorescence at 488 nm, B.DsRed fluorescence at 558 nm , C. transmission image and D. overlay of all three channels.

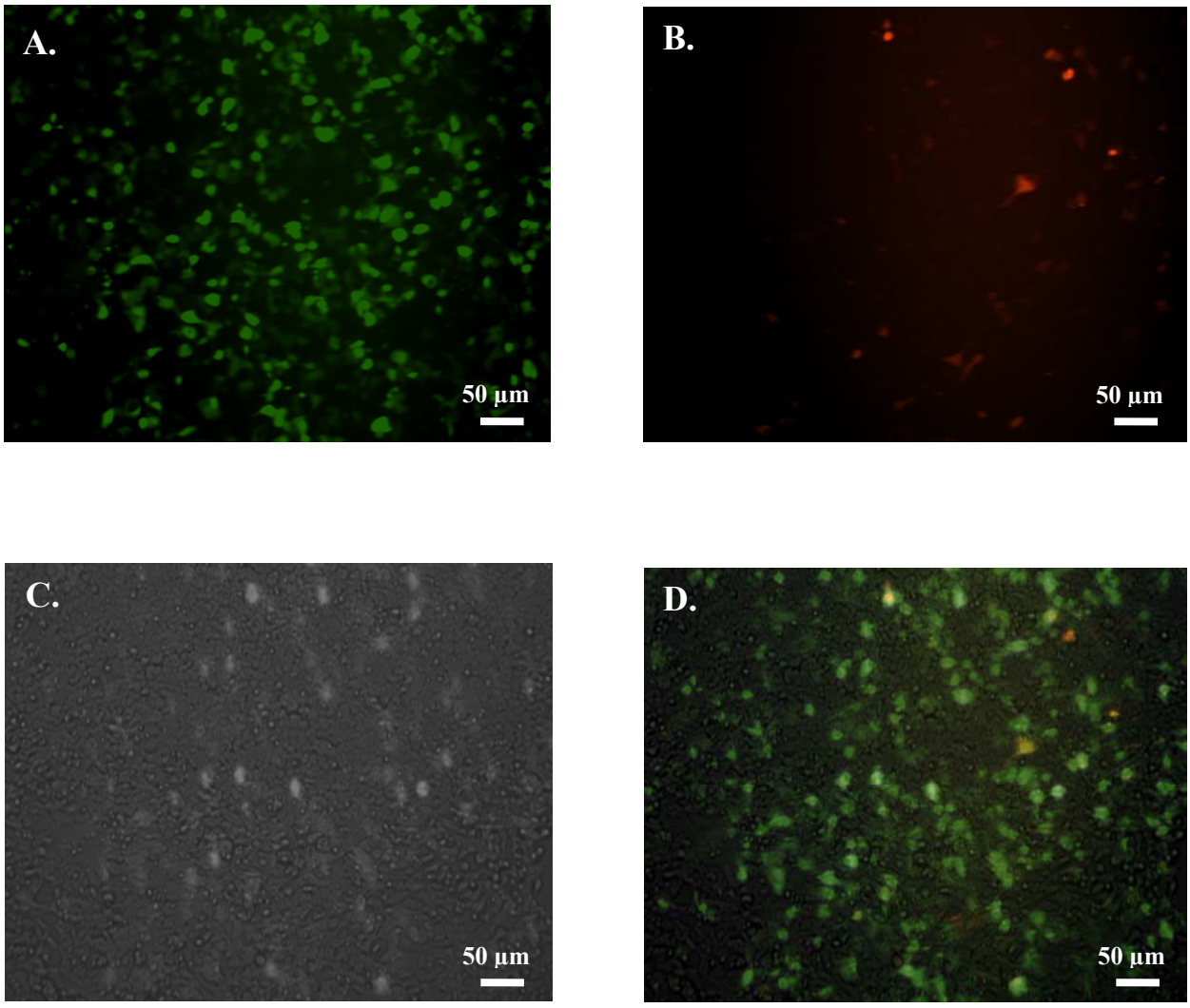


Figure 4.3.5: Flourescent images showing the knockdown efficiency of the pCIeGFP 3xEGFR target, co-transfected with sh5 and pDsRed as background control.

HEK293 cells were co-transfected with pCIeGFP 3xEGFR target, sh5 and pDsRED as a background control. A. eGFP fluorescence at 488 nm, B.DsRED fluorescence at 558 nm , C. transmission image and D. overlay of all three channels.

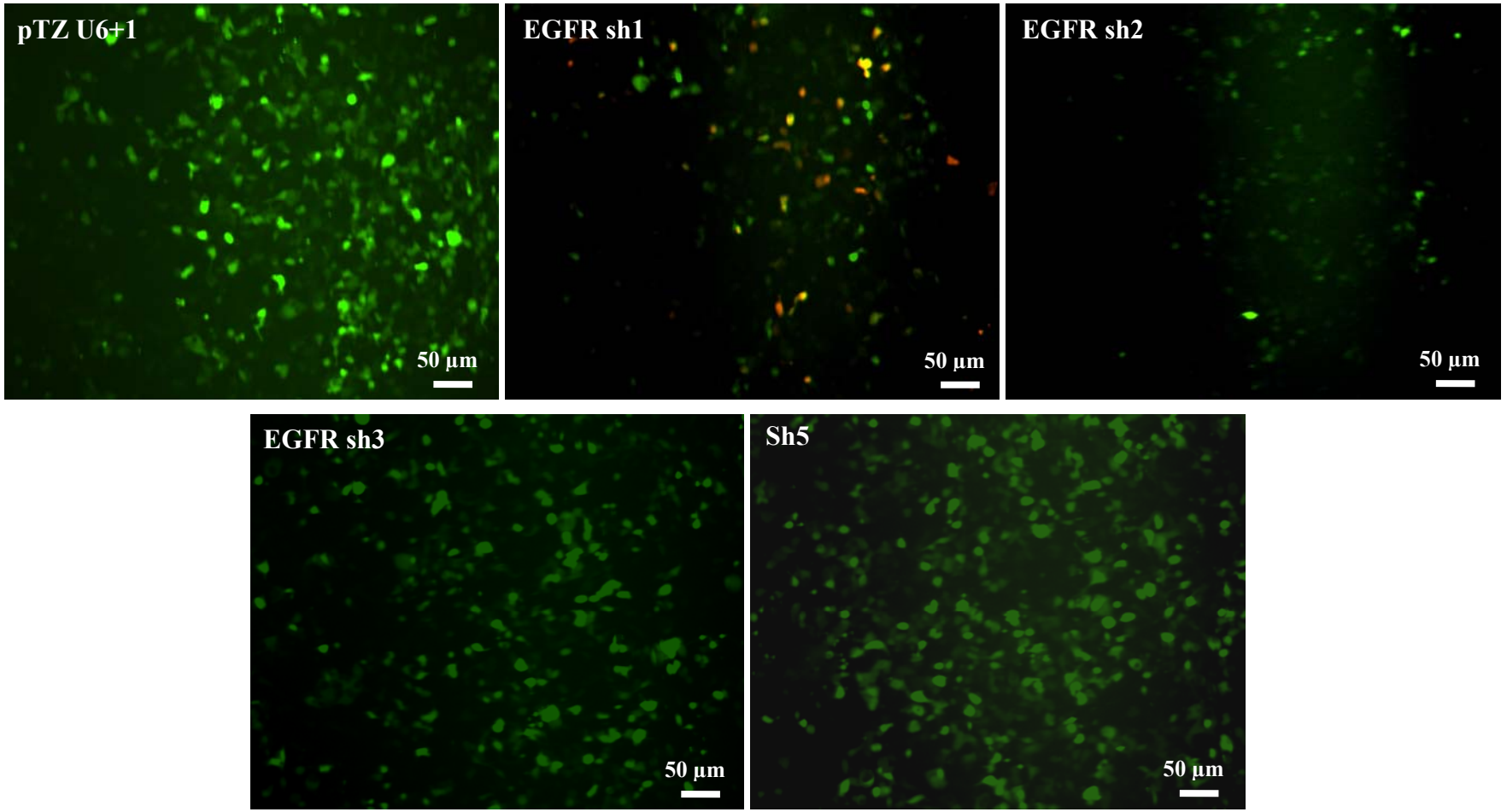


Figure 4.4: Knockdown efficiency determined through co-transfection of the pCIeGFP 3xEGFR target and either pTZ U6+1, EGFR sh1, EGFR sh2, EGFR sh3 or sh5. Fluorescent images were taken 24 hours after transfection using confocal microscope. EGFR sh1 shows an overlay of eGFP image and background control plasmid DsRED, while the other just show eGFP. Absence of the enhanced green fluorescent protein (eGFP) at wavelength of 488nm, indicates a high knockdown efficienc as seen in co-tranfections done with EGFR sh1 and EGFR sh2. Co-transfection using pTZ U6+1 and sh5 were as controls, while EGFR sh3 showed a low knockdown efficiency, reminiscent of the the controls.

The knockdown efficiency of the three EGFR shRNAs of the pCIeGFP 3xEGFR target reporter system was determine visually using the confocal microscope. Knockdown was characterized by the decrease in amount of green fluorescent protein (eGFP), relative to the controls. Figures 4.3.1 and 4.3.5 show the co-transfections of target (pCIeGFP 3xEGFR target), background control (pDsRed) and pTZ U6+1 or sh5 respectively. Figures 4.3.2 and 4.3.3 indicate knockdown of the eGFP by the EGFR sh1 and EGFR sh2 hairpins. The results obtained for EGFR sh3 show high levels of eGFP and are reminiscent of the results observed in the control transfections with pTZ U6+1 and sh5 (Figures 4.3.1 and 4.3.5). The pDsRed and transmission images are background transfection efficiency control.

4.4 Discussion

The knockdown efficiency of the three EGFR shRNAs was determined against two independent knockdown efficiency reporter assay systems psiCHECK and pCIeGFP, containing the target sequence for each of the three designed shRNAs. The target sequence (3xEGFR target) was cloned into the knockdown assay reporter systems, following the reporter gene so that the target sequence was sequentially arranged downstream to the mRNA transcript of the reporter gene but does not affect the reporter protein.

The three generated EGFR specific shRNAs demonstrated a high efficiency knockdown of the *Renilla* luciferase gene, with respect to Firefly luciferase in the dual luciferase psiCHECK 3xEGFR target reporter plasmid. This was determined by the decrease of the *Renilla* luciferase signal in those co-transfection experiments containing the psiCHECK 3xEGFR target and either EGFR sh1, EGFR sh2 or EGFR sh3 (Figure 4.2).

The knockdown efficiency obtained for the three EGFR shRNAs against the pCIeGFP 3xEGFR target did exhibit similar results with regards to EGFR sh1 and EGFR sh2. However the knockdown efficiency of EGFR sh3 was more representative of the results obtained when the pCIeGFP 3xEGFR target plasmid was co-transfected with control plasmids pTZ U6+1 and sh5 (Figures 4.3.1 and 4.3.5). The images showing eGFP fluorescence for each co-transfection of

pCIeGFP and the effector cassette pTZ U6+1, EGFR sh1, EGFR sh2, EGFR sh3 and sh5 are depicted together in figure 4.4, for comparability.

Comparison of the knockdown efficiency data for the three EGFR shRNAs obtained from the two different knockdown efficiency reporter systems (psiCHECK 3xEGFR target and pCIeGFP 3xEGFR target), shows a difference in knockdown efficiency for EGFR sh3. This contrasting result observed between the two reporter systems highlights the advantage of the psiCHECK 2.2 dual luciferase reporter plasmid over the pCIeGFP reporter plasmid. The results obtained from the psiCHECK dual luciferase target knockdown assay is quantitative since the luminometer quantifies the signal generate by the luciferase enzymes. In contrast the pCIeGFP reporter knockdown efficiency reporter assay is determined visually. pCIeGFP reporter system, however can be quantified using flow cytometery. In this case the pCIeGFP reporter results were not quantified. Nevertheless, the results obtained from the two knockdown efficiency reporter systems provided essential information for the progression of this knockdown experiment. The reporter systems show that there is efficient knockdown of the target sequence by EGFR sh1 and EGFR sh2, however a discrepancy was observed in the knockdown efficiency results obtained from the two knockdown reporter assay system, for EGFR sh3.

Although transcription of the three EGFR shRNAs was not directly tested for, the knockdown efficiency of the reporter systems, containing the target sequence, indicates that the three EGFR shRNA expression cassettes were able to generate products that resulted in the specific knockdown of the target according to the predicted outcome of the shRNA activation of the RNAi pathway.

CHAPTER 5

5.1 The potential use of RNAi in cancer therapy

RNAi offers a highly efficient and specific technique in the down regulation of target gene expression, making RNAi a possible therapeutic or treatment of human cancer. A number of potential cancer associated genes have been targeted using this technique. The genes include oncogenes, anti-apoptotic proteins and growth factor receptor genes. A summary of targeted genes in various tumour types is reviewed in Gartel and Kandel, 2006. The EGFR receptor has been found to be over expressed in a variety of tumour types including non-small lung cancer, breast, head and neck, gastric, colorectal, oesophageal, prostrate, bladder, renal, pancreatic and ovarian cancers (Salmon *et al.,* 1995). Targeting of EGFR using RNAi has only been done in lung cancer models (Zhang *et al*., 2005).

5.2 Knockdown of EGFR in HOSCC

Over expression of EGFR in human oesophageal squamous cell carcinoma (HOSCC) occurs in 29-92% of all HOSCC tumours (Lam, 2000). The HOSCC cell lines cultured in the Witwatersrand University Cell Biology Laboratory have been shown to have heightened expression of the low-affinity EGFR (Veale and Thornley, 1989). Once activated by EGF, the EGFR activates downstream signaling pathways such as the mitogenic protein kinase (MAPK) and phosphatidylinositol-3 kinase (PI3K) pathways (Artega, 2002). Activation of the MAPK pathway results in phosphorylation and activation of nuclear transcription factors such as Jun, FOS and Myc, which results in cellular proliferation. The activation of the PI3K pathway promotes cell survival, through active forms of Bcl-2 and Bcl-x which possess anti-apototic effects (Vanhaesebroek and Waterfield, 1999). Over expression of EGFR in cancer increases the sensitivity of the affected cells to low concentration of the appropriate growth factors. Thus increased EGFR expression results in increased proliferative potential, resistance to apoptotic signals as well as increased invasive and metastatic potential, all characteristic features of the cancerous state (Wujcik, 2006).

Three shRNAs were designed against human EGFR (Chapter 2) and their efficiency was tested using synthetically produced target sequence (3xEGFR target) cloned into two separate knockdown efficiency assay systems (Chapter 3). The efficiency of each shRNA was determined by its *in vivo* knockdown ability of the plasmid based reporter system containing the synthetically produced target sequence (Chapter 4).

Based on the results obtained from the previous chapters the aim was to knockdown EGFR using the specific EGFR shRNAs that were designed.

5.3 Materials and methods

5.3.1 HOSCC cell culture and transfection

The HOSCC cell lines WHCO1, WHCO3, WHCO5, WHCO6 and SNO were derived from moderately differentiated squamous cell carcinoma tumours (Veale and Thornley, 1989). The cell lines were maintained in a 3:1 ratio of Dulbecco's modified Eagles medium (Highveld Biological) to Hams F12 (Highveld Biological), supplemented with 10% fetal calf serum and cultured at 37°C in an atmosphere of 5% CO_2.

Forty eight hours prior transfection cells were rinsed with 1 x phosphate buffer saline (PBS), (136.8 mM NaCl, 2.68 mM KCl, 10.1 mM $Na_2HPO_4 \cdot 12H_2O$, 1.76 mM KH_2PO_4 pH 7.2). Cells were trypsinized with 2 ml of trypsin/ EDTA (0.01% trypsin, 0.004% EDTA) and 500 000 cells seeded into 3.5 cm tissue culture dishes (Falcon). Transfections of plasmid DNA were carried out using Lipofectamine 2000 (Invitrogen), according to manufacturers specifications, however the following alteration was made, 4 µl of Lipofectamine 2000 per 4 µg of total DNA in a final volume of 750 µl serum free medium per 3.5 cm dish. Plasmids EGFR sh1, EGFR sh2 and EGFR sh3 were used for knockdown assays, pTZ U6+1 and sh5 were used as controls. pCIeGFP was used for transfection efficiency. Plasmid stocks were diluted to working concentration of 0.5 µg/µl, confirmed using the NanoDrop (ND-100), UV-VIS spectrophotometer. On the day of transfection the media was aspirated and cells were gently rinsed with a minimal volume of 1 x PBS. The 750 µl of Lipofectamine 2000, plasmid DNA and serum free media was added to the cells and allowed to incubate for 6 hours at 37°C in an atmosphere of 5% CO_2. After 6 hours an

additional 1 ml of complete medium was added to the each 3.5 cm dish and incubated overnight at 37°C in an atmosphere of 5% CO_2. The following morning the media was aspirated and 2 ml complete media was added to each of the 3.5 cm dishes. Whole cell extractions of the transfected cell lines were performed 48 hours post-transfection.

5.3.2 Whole cell extraction

Cells were removed from the incubator and the media discarded. The cells were washed 4 times with ice cold modified Tris buffered saline (TBS) (50 mM Tris-HCl, pH 7.8, 8.6 g/l NaCl, 1 mM EDTA, 10 µl/ml Aprotinin and 0.1 mM PMSF final concentration). Cells were scraped off the dish in final volume of 1 ml TBS and placed in microfuge tube. Cells were harvested by centrifugation using the bench top mini-centrifuge at maxium speed for 2 minutes. The supernatant was discarded and the pellet of cells resuspended in 160 µl 1x Laemili Sample buffer (0.0625 M Tris-HCl pH 6.8, 2% SDS, 10% Glycerol, 5% β-Mercaptoethanol) from a 10 cm dish and 40 µl of 1x Laemili Sample buffer (0.0625 M Tris-HCl pH 6.8, 2% SDS, 10% Glycerol, 5% β-Mercaptoethanol) from a 3.5 cm tissue culture dish. Samples were then boiled for 10 minutes and then centrifuged at 10 000 g for 20 minutes at 4°C. Whole cell protein samples were stored at -20°C.

5.3.3 Protein estimation

The protein estimation was performed according to Bramhall *et al.* 1969 and is described briefly below: A 15 cm diameter sheet of filter paper (Whatman) was washed for 20 minutes in dH_2O, 95% ethanol for 5 minutes, 99% ethanol for 5 minutes followed by a 5 minute acetone wash. The filter paper was placed in the fume hood until dry. Bovine serum albumin (BSA) standard was prepared by weighing out the correct weight re-suspending it in final volume of 1x Laemili sample buffer (0.0625 M Tris-HCl pH 6.8, 2% SDS, 10% Glycerol, 5% β-Mercaptoethanol) to yield a final concentration of 2 µg/µl. Standard range of concentrations between 1 and 20 µg of BSA and 2-5 µl of protein sample were loaded onto the filter paper and allowed to dry. Protein samples were fixed by washing for 40 minutes in 7.5% TCA and then stained with Coomasie blue stain solution for further 45 minutes. The filter paper was destained in 12% ethanol, 10% glacial acetic acid solution until only protein spots remained. The protein spots were individually

excised and the protein stain eluted in 5 mls of elution solution (66% methanol, 0.0125% ammonia) overnight in the dark. The absorbance was measured at a wavelength of 596 nm. Protein concentration of the whole cell extraction was determined based upon the BSA standards (see appendix).

5.3.4 SDS-PAGE

SDS-PAGE was done according to Laemili, 1970 and is briefy described below. Between 30-40 µg of total protein was resolved using an 8% separating polyacrylamide gel (8% acrylamide, 0.1% NN' – methylenebisacrylamide, 375 mM Tris-HCl, pH 8.8, 0.2% SDS) and 5% stacking polyacryamide gel (5% acrylamide, 0.1% NN-methylenebisarcylamide, 125 mM Tris-HCl, pH 6.8, 0.2% SDS). Polymerization of the gels was done through the addition of 1 mM ammonium persulphate and 0.25% TEMED, prior to the gel being poured between the silicon and glass backing plate (Mighty Small™ dual gel caster, Hoefër). Gels were allowed to set at room temperature, and then electrophoresed at constant current of 25 mAmps in running buffer (25 mM Tris-HCl, pH 8.3, 3.74 mM SDS, 192.5 mM glycine). Gels that were not used for Western blotting analysis were stained with Coomassie blue staining solution (0.25% Coomassie brilliant blue powder, 50% methanol, 10% glacial acetic acid) and the background was destained until clear using destaining solution (12% glacial acetic acid, 10% methanol).

5.3.5 Western Blotting

Samples were transferred from polyacrylamide gels to nitrocellulose membrane using the Biorad transfer system, filled with transfer buffer (25 mM Tri-HCl, pH 8.3, 1.14% glycine, 20% methanol). Protein samples were transferred for 3 hours at 400 mAmps. Once the protein samples had been transferred onto the nitrocellulose membrane, the membrane was rinsed 3 times with Tris buffered saline (TBS) (6.06 g/l Tris-HCl pH 7.8, 8.6 g/l NaCl, 0.294 g/l $CaCl_2 \cdot 2H_2O$). The TBS was discarded and the nitrocellulose was placed in Blotto (50 mM Tris-HCl, pH 7.8, 2 mM $CaCl_2 \cdot 2H_2O$, 5% non-fat milk powder, 0.01% anti-foam, 0.05% Triton-X 100) for 1 hour at slow rotation. The Blotto solution was discarded and the membrane washed 6 times with TBS. The nitrocellulose membrane was incubated in primary monoclonal anti-EGFR clone F4 (Sigma) using a 4000x dilution in PBS. The primary antibody solution was discarded

and the nitrocellulose membrane was washed for 30 minutes in TBS, replacing the TBS at 5 minute intervals. This was followed by a further 1 hour incubation in anti-mouse IgG horseradish peroxidase (HRP) conjugate secondary antibody developed in goat (Sigma). After the hour incubation the secondary antibody was discarded and the nitrocellulose membrane washed for a further 30 minutes in TBS, with replacement of the TBS at 5 minute intervals. Chemiluminescent signal detection was performed using the SuperSignal West Pico Chemiluminescent Substrate Kit (Pierce), using a minimal volume 1:1 ratio of luminal/enhancer solution to stable peroxide buffer. The nitrocellulose membrane was then exposed to Hyperfilm (Separations). Film exposure times varied from 10 minutes to overnight. The film was developed using developer (appendix), rinsed with water then placed in fixer (appendix) for 5 minutes.

5.3.6 Densitometery

Densitometery analysis was performed following the Western blot analysis, using LabWorks™ Image acquiation and analysis software (LabWorks version 4.5). The band intensities corresponding to the EGFR band (170 kDa) were quantified using the computer software and analysed.

5.4 Results

Initially the EGFR expression levels of the 5 HOSCC cell lines were determined by western blotting. The elctrophoresis of the 5 whole cell extraction samples obtained for each cell line was standardized by loading 40 µg of total protein for cell line. The western blot showing the expression levels of EGFR between SNO, WHCO1, WHCO3, WHCO5 and WHCO6 is evident in Figure 5.1.

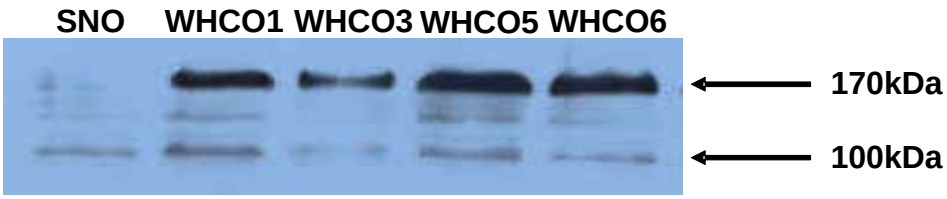


Figure 5.1: Western blot analysis of human epidermal growth factor receptor (EGFR) in 5 human oesophageal squamous cell carcinoma (HOSCC) cell lines.

Whole cell extraction procedure was performed on five HOSCC cell lines. The total protein was estimated using a colorimetric assay. Total protein amounts were standardized before loading on an 8% SDS-PAGE. EGFR (170 kDa) was analyzed by Western blot, probed with anti-EGFR monoclonal antibody followed by HRP-conjugate Goat, anti-mouse secondary antibody. Molecular weight of the protein bands are shown on the right.

A variation in EGFR expression levels was observed between the five HOSCC cell lines (Figure 5.1). WHCO1, WHCO5 and WHCO6 show elevated EGFR expression levels when compared to SNO and WHCO3. Due to the high expression levels of EGFR in these three cell lines they were subsequently selected for the knockdown assay using the three EGFR shRNAs. Figure 5.1 also shows lighter bands of lower molecular weight (≥100 kDa). These bands are thought to be EGFR degradation products, as a result of proteolytic cleavage, despite the whole cell extraction procedure been carried out on ice and in the presence of protease inhibitors (see also Panayotou and Gregoriou 1990).

Knockdown assessment of endogenously expressed EGFR was performed on each cell line, using each EGFR shRNAs individually. The plasmids pTZ U6+1 and the non-specific hairpin sh5 were used as controls. Following transfection, whole cell extractions were performed and total protein concentration estimated. The whole cell protein concentrations were standardized and 35 µg of each sample was loaded on 8% SDS-PAGE and the protein bands were stained using coomassie blue followed by a destaining of the background (Figure 5.2). This was necessary to ensure that the protein estimation method provided visual confirmation of protein estimation and that the knockdown seen in Figure 5.3 was not as a result of variation in loaded a specific amount of total protein for each sample.

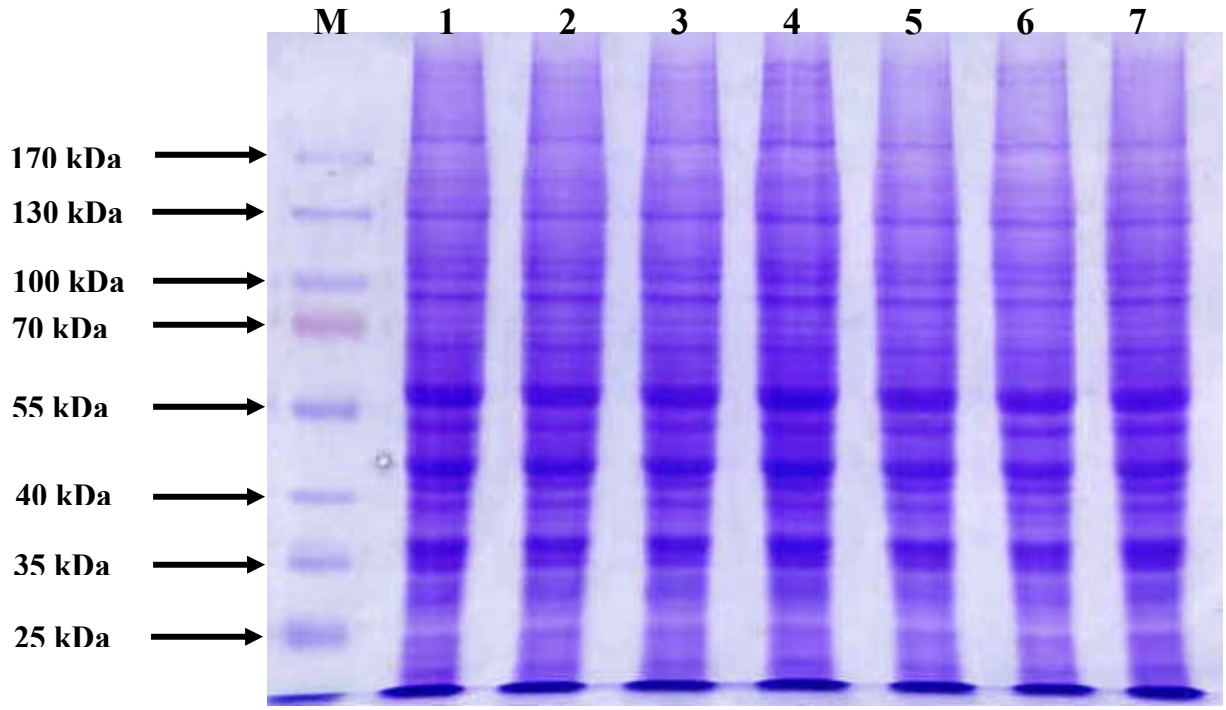


Figure 5.2: SDS-PAGE of samples obtained from transfection of cell line.WHCO1.

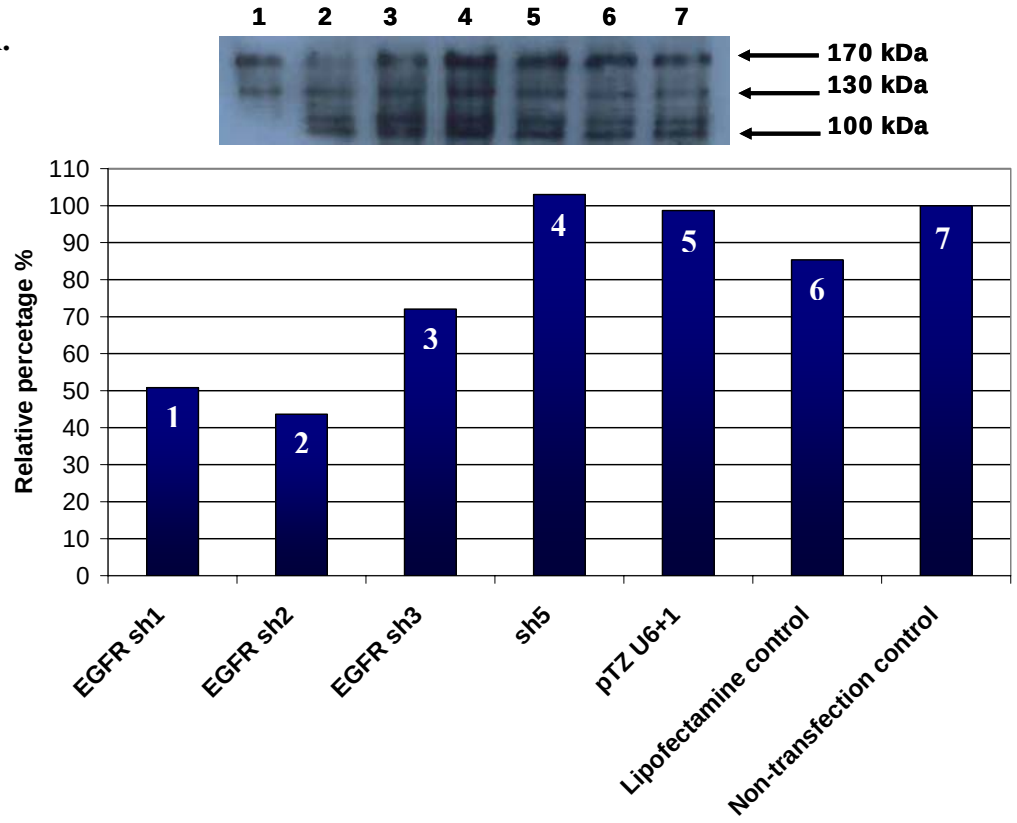
48 hrs following the transfection, whole cell extraction was done and total protein estimated. 35 µg of total protein was loaded in each lane. Lanes M. Marker; lane 1, EGFR sh1; lane 2. EGFR sh2; lane 3. EGFR sh3; lane 4. sh5; lane 5. pTZ U6+1; lane 6. Lipofectamine control; and lane 7. non-transfection control.

5.4.1 Knockdown of endogeous expressed EGFR

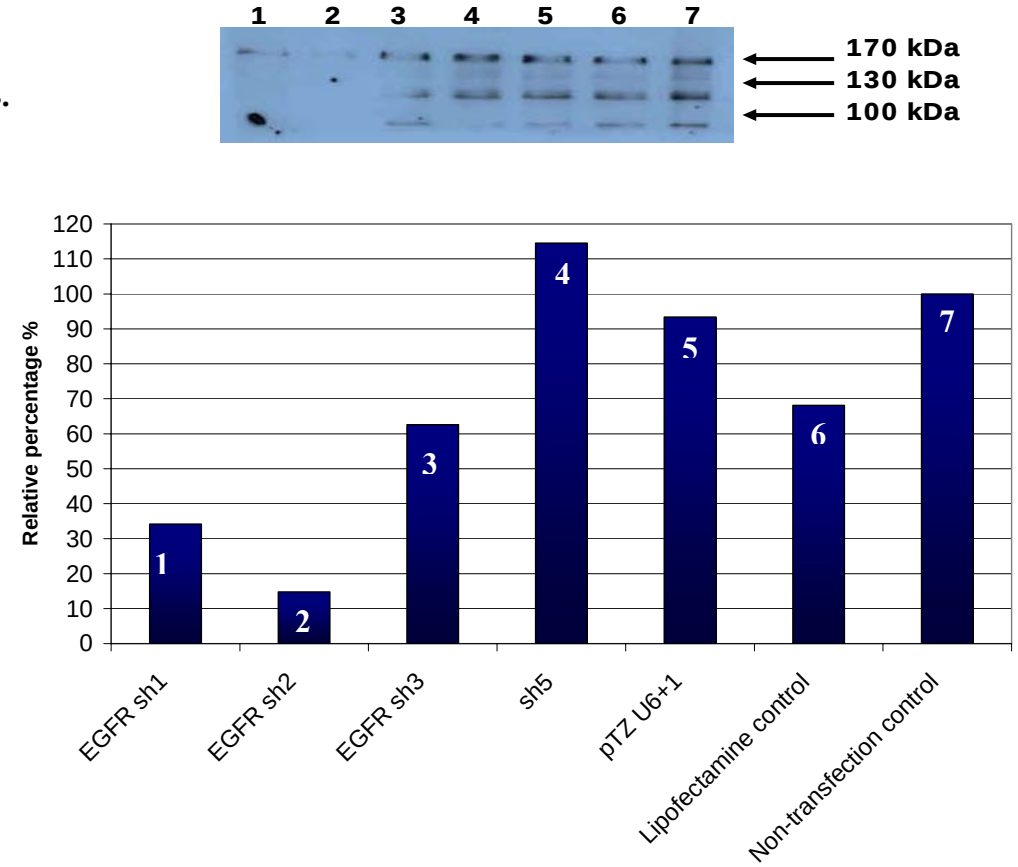
Relative percentage knockdown was determined through densitometric analysis of the EGFR 170 kDa band on the Western blot. Note that the cell transfections were all standardized according to the non-transfection control.

The relative percentage knockdown of EGFR varied not only between the three EGFR shRNAs but also amongst the three HOSCC cell lines transfected. Transfection of the WHCO1 cell line, showed 50% and 56% knockdown of endogenous EGFR by EGFR sh1 and EGFR sh2 respectively (Figure 5.3A). EGFR sh3 was less efficient resulting in relative percentage knockdown of EGFR of 27.95%. The control plasmids pTZ U6+1 and the non-specific hairpin sh5, showed no knockdown of EGFR relative to the non-transfection control.

A.


1 2 3 4 5 6 7
170 kDa
130 kDa
100 kDa
Relative percetage %
110
100
90
80
70
60
50
40
30
20
10
0
1
2
3
4
5
6
7
EGFR sh1
EGFR sh2
EGFR sh3
sh5
pTZ U6+1
Lipofectamine control
Non-transfection control

B.


1 2 3 4 5 6 7
170 kDa
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120
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0
1
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3
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6
7
EGFR sh1
EGFR sh2
EGFR sh3
sh5
pTZ U6+1
Lipofectamine control
Non-transfection control

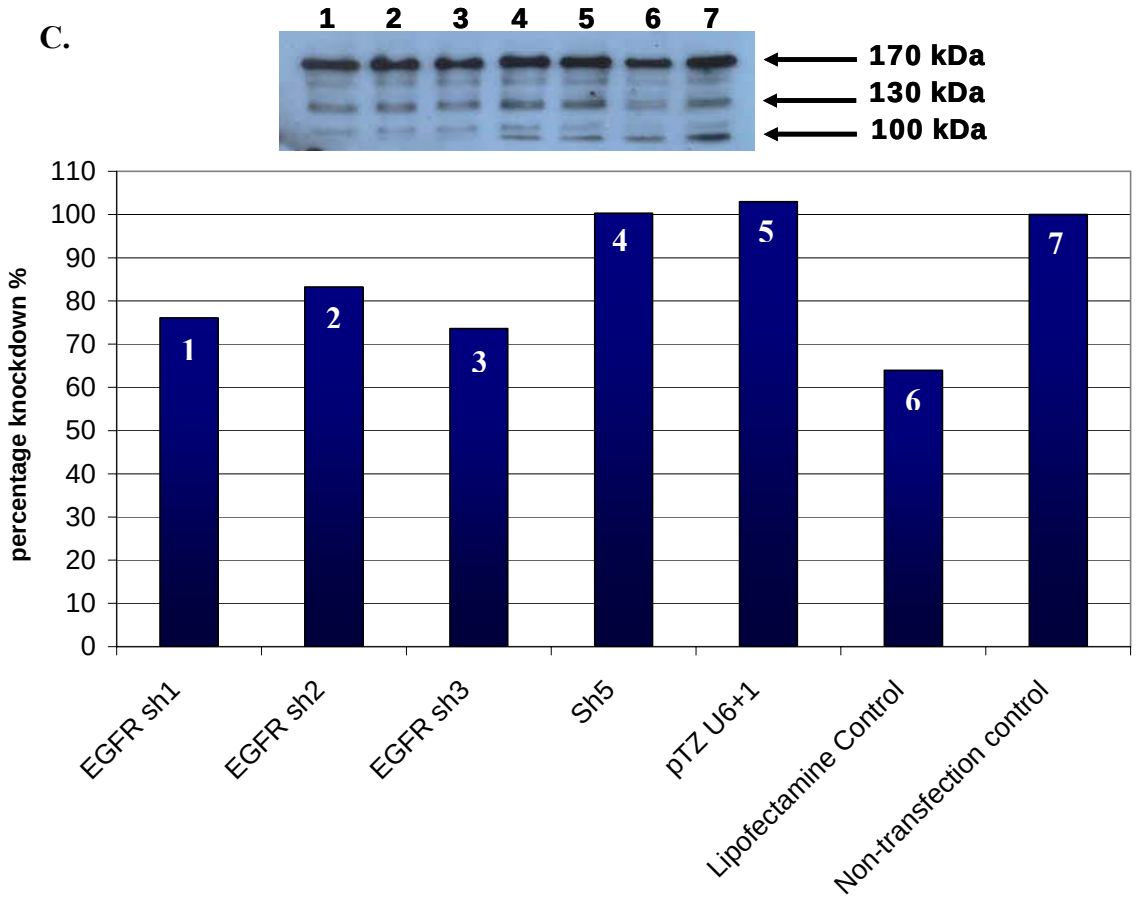


Figure 5.3: Quantitaive analysis of EGFR knockdown in three HOSCC cell lines A. WHCO1, B. WHCO5 and C. WHCO6.

Transfections were done using Lipofectamine 2000, using EGFR sh1, EGFR sh2 and EGFR sh3. pTZ U6+1 and sh5 were control plasmids. Whole cell extractions were performed 48 hours following transfection, and Western blot analysis was performed using primary anti-EGFR monoclonal antibody, followed by secondary goat, anti-mouse antibody incubation. Densitometry was performed on the 170 kDa EGFR band. Molecular weight shown to the right of the Western blot. Numbers above the Western Blot correspond to the respective transfections illustrated in the respective bar graph. Standard error bars are not displayed due to replicates being insufficient for proper stastical analysis.

Transfection of the WHCO5 cell line showed 65% knockdown of endogenous EGFR by EGFR sh1, 85% knockdown was achieved by EGFR sh2 and 40% knockdown was observed by EGFR sh3. The control plasmids used in the transfection again correspond to those results obtained for the non-transfection control. Overall the WHCO5 cell line shows the greatest relative

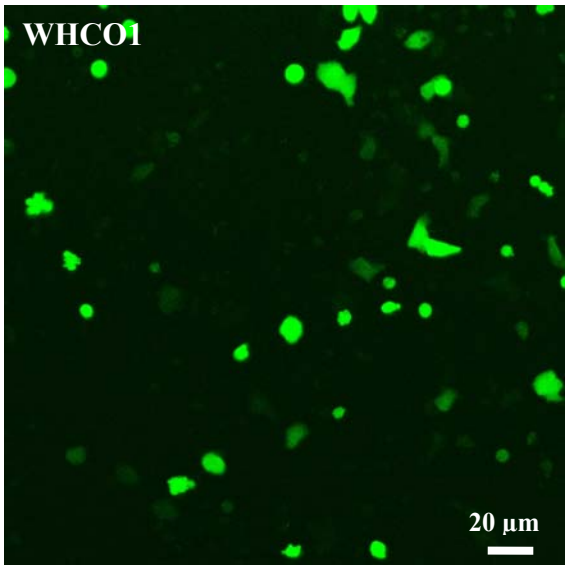
knockdown for each of the three EGFR shRNAs, from the three HOSCC transfected cell lines (Figure 5.3B).

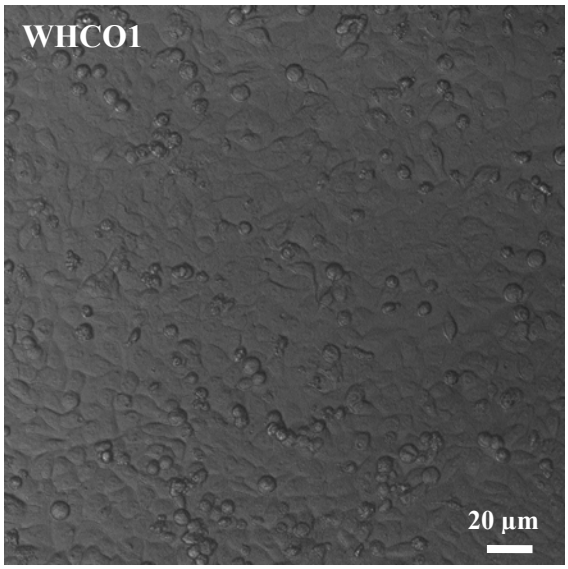
Transfection of the WHCO6 cell line showed the weakest knockdown of endogenous EGFR by all three EGFR shRNAs when compared to WHCO1 and WHCO5 cell lines. The level of EGFR knockdown obtained by three EGFR shRNAs was similar (15-30%), relative to the non-transfection, pTZ U6+1 and sh5 (Figure 5.3C).

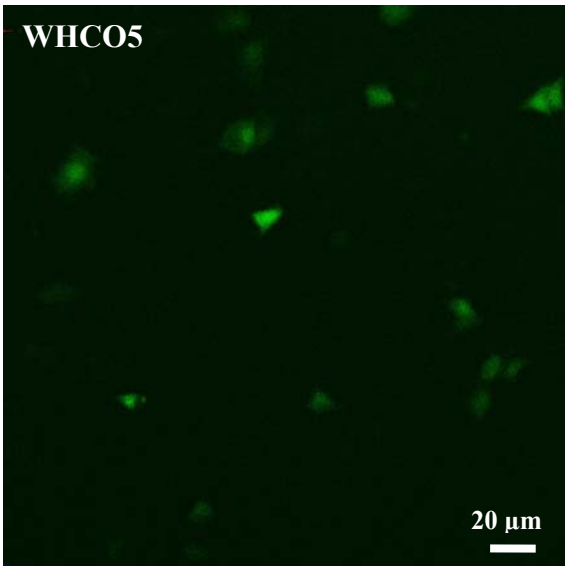
The Lipofectamine control, included the addition of Lipofectamine 2000 but no DNA to the transfection medium. The Lipofectamine control produced a perplexing result in all three of the HOSCC cell lines tested. As seen in Figure 5.3, the lipofectamine seems to cause a reduction in the of EGFR in varying degrees amoungst the three cell lines WHCO1, WHCO5 and WHCO6. The reduction in EGFR varies from 15% (WHCO1) to as much as 35% (WHCO6).

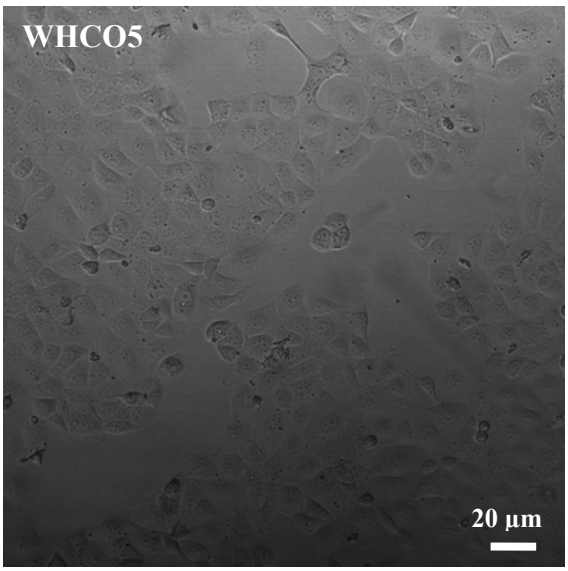
5.4.2 Estimation of transfection efficiency

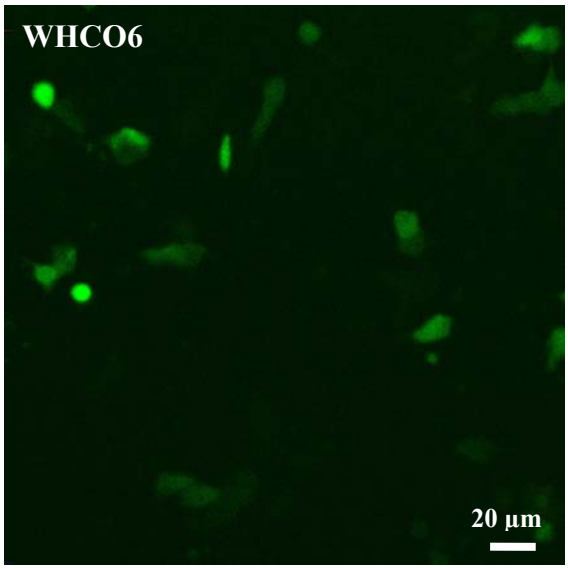
Transfection efficiency of the HOSCC cell lines was determined by transfection with the pCIeGFP plasmid which was viewed 24 hour post-transfection at 488 nm using the confocal microscope (Figure 5.4). The transfection efficiency for each cell line was as follows: WHCO1 12.5%; WHCO5 6.5%; WHCO6 8.2% and HEK293 67.9%. The transfection efficiency of the HOSCC cell lines was shown to be on average 5 times less efficient than that obtained in the HEK293 cell line.

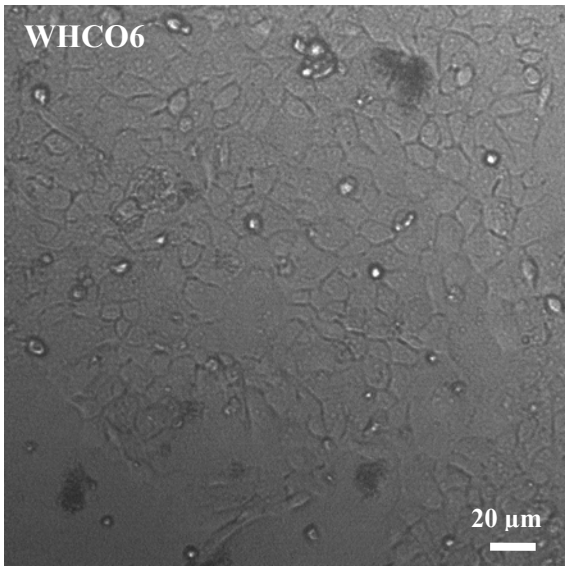

WHCO1
20 µm


WHCO1
20 µm


WHCO5
20 µm


WHCO5
20 µm


WHCO6
20 µm


WHCO6
20 µm

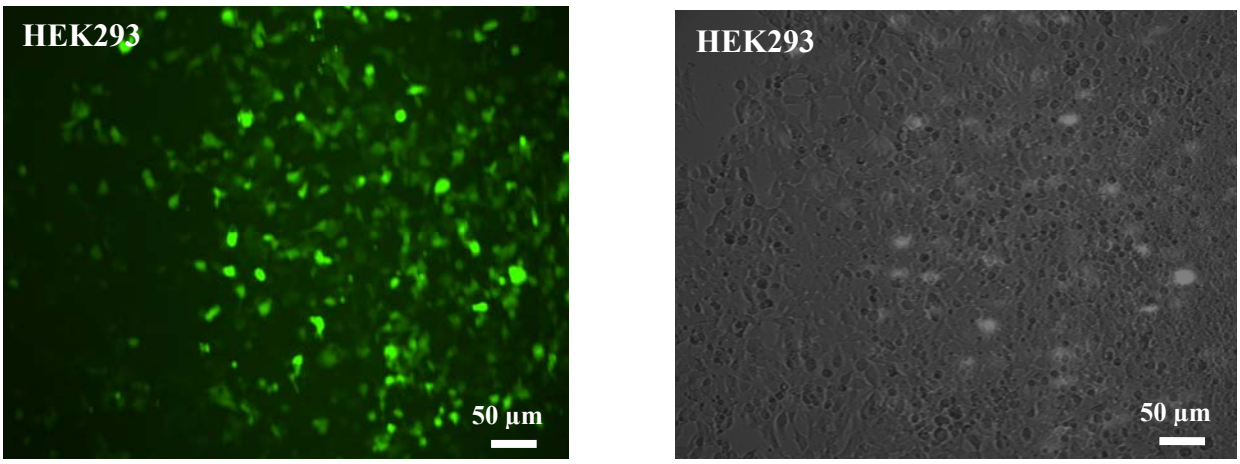


Figure 5.4: Visual representation of transfection efficiency of the HOSCC cell lines WHCO1, WHCO5, WHCO6 and HEK293 using pCIeGFP.

Cell lines were transfected with eGFP using Lipofectamine 2000 and viewed 24 hours post- transfection at 488 nm excitation wavelength, using the Zeiss LSM confocal microscope. Transfection efficiency was calculated by the number of fluorescing cells divided by total number of cells in field view. Transfection efficiency obtained in HEK293 cell (67.9%) line was 5 fold greater than average transfection efficiency obtained in WHCO1, WHCO5 and WHCO6 (~10%).

5.5 Discussion

The human EGFR has been found to be overexpressed in a number of malignancies resulting in increased proliferation, increased tissue invasion potential, angiogenesis and thus has emerged as a potential target in the development of novel cancer therapeutics (Wujcik, 2006). The specifically designed shRNAs (Chapter 2) were used to knockdown endogenous expression of EGFR in HOSCC cell lines.

The levels of EGFR protein expression of the 5 HOSCC cell lines were initially determined by Western blot analysis (Figure 5.1). WHCO1, WHCO5 and WHCO6 cell lines were selected for ttransfection with the EGFR shRNAs, as these 3 cell lines showed high levels of EGFR expression. Knockdown of EGFR was determined by Western blot followed by densitometry analysis of the EGFR 170 kDa band, and comparison of intensity between the cells transfected with the EGFR shRNAs, the control plasmids (pTZ U6+1 and sh5) and the non-transfection control. The difference in expression of EGFR observed between the non-transfection control

and the transfected treated cells is a measure of the knockdown obtained. The cell lines, WHCO1, WHCO5 and WHCO6, were selected over the other two cell lines (WHCO3 and SNO) since the latter showed lower levels of EGFR expression. Following transfection the determination of whether knockdown of EGFR occurred in these cell lines (WHCO3 and SNO) would be difficult as they already demonstrate intrinsically low EGFR expression levels.

In order to confirm that it is indeed specific knockdown of EGFR and not a result of different amounts of total protein loaded on the SDS-PAGE, total protein sample from the whole cell extraction following the WHCO1 transfection was separated on a SDS-PAGE (Figure 5.2). This protein gel confirms visually the successful protein estimation and the knockdown observed in the Western blot analysis was not a result of different total amounts of protein loaded.

Knockdown of endogenous EGFR was achieved in all three cell lines tested, with varying efficiencies. Variation in knockdown percentage was not only observed according to the EGFR shRNA used i.e. EGFR sh1, EGFR sh2 and EGFR sh3, but also between the three cell lines, WHCO1, WHCO5 and WHCO6. The greatest percentage knockdown of endogenous EGFR was observed in the WHCO5 cell line when transfected with EGFR sh2, yielding 85% knockdown relative to the non-transfection control. Moderate knockdown (30-65%) was observed by EGFR sh1, EGFR sh2 and EGFR sh3 in WHCO1 transfected cells and EGFR sh1 and EGFR sh3 in WHCO5 transfected cell line.

WHCO6 transfection with the three EGFR shRNAs showed low knockdown of endogenous EGFR when compared to the knockdown percentage obtained in the WHCO1 and WHCO5 cell lines (Figure 5.3). The control plasmids pTZ U6+1 and sh5 showed no decrease in EGFR expression levels, when compared to the non-transfection control. Even though the knockdown percentage of EGFR is low (15-30%) by each of the three EGFR shRNAs, it reveals definite knockdown when compared to the EGFR expression controls (pTZ U6+1, sh5 and non-transfection control).

The fact that the lipofectamine control produced decreased levels of endogenous expressed EGFR was unexpected, yet occurred in all three HOSCC cell lines tested. The decrease in EGFR was most prominent in the WHCO6 and WHCO5 cell lines but was also observed to a lesser extent in the WHCO1 cell line (Figure 5.3). When comparing the relative knockdown percentage of endogenous EGFR the better control is to compare the EGFR shRNAs with the plasmid

controls, pTZ U6+1 and the non-specific hairpin sh5. This is due to the fact that transfections were carried out using a 1:1 ratio of Lipofectamine 2000 to plasmid DNA. The lipofectamine control transfection contains no plasmid DNA in the reaction.

As mentioned previously the decrease observed in the expression of EGFR was not a result of loading different amounts of protein (Figure 5.2). This also indicates that total protein samples from the lipofectamine control are also accurate, however the reduction in EGFR expression obtained from the lipofectamine control can be explained by the following: Lipofectamine is a cationic lipid, with positively charged head groups that interact with the phosphate backbone of DNA, forming the transfection complex. The positively charged groups also facilitate the association of the transfection complex with the negatively charged cell membrane facilitating uptake of the transfection complex through endocytosis by the cell. The cationic lipid destabilizes the endosomal membrane resulting in the release of the DNA into the cell.
Pramfalk *et al.* 2004 reported the activation of membrane insulin receptor by cationic transfection reagents Fugene 6 (Roche) and Lipofectamine 2000 (Invitrogen). The insulin receptor is a heterodimeric protein with an internal tyrosine kinase. The cytoplasmic region is autophosphorylated upon insulin binding to the receptor, resulting in the internalization and subsequent down regulation of the receptor. Epidermal growth factor receptor is also a receptor tyrosine kinase (RTK), as it possesses a cytoplasmic tyrosine kinase domain. It is postulated that the mechanism by which EGFR is down regulated by treatment with Lipofectamine 2000 in this study is similar to that reported by Pramfalk *et al*. 2004. It is possible that the Lipofectamine 2000, cationic lipid is able to activate the kinase of the internal receptor kinase portion of the receptor. This activation of the kinase domain results in the autophosphorylation of the tyrosine residues, followed by internalization and subsequent down regulation of the EGFR.

The transfection efficiency assay is important in determining not only the efficiency that the EGFR shRNAs and control plasmids were transfected into the target cells, but the subsequent efficiency of these shRNA effector molecules against the endogenous EGFR. From the results obtained (Figure 5.4), the HOSCC cell lines WHCO1, WHCO5 and WHCO6, which were transfected with eGFP show an approximate 5 fold decrease in transfection efficiency when compared to the HEK293 cell line. Low transfection efficiency indicates that a small percentage of the cell population contains the EGFR shRNA effector molecules (10% in HOSCC cell lines).

Despite this, knockdown of EGFR was observed indicating that the EGFR shRNAs were indeed efficient in knocking down EGFR (Figure 5.3). The efficiency of the EGFR shRNAs is likely to be greatly under estimated. Moderate knockdown of 30-65% obtained in EGFR expression, from only 10% of the affected cell population indicates great efficiency of the designed EGFR shRNAs against EGFR. This also suggests that poor knockdown of EGFR observed in WHCO6 transfection by the three EGFR shRNAs, which only showed a 8.2% transfection efficiency with eGFP, should show substantial improvement with an improved transfection efficiency.

The greatest knockdown efficiency of EGFR obtained for EGFR sh1, EGFR sh2 and EGFR sh3, was all shown in the WHCO5 cell line. This cell line also showed the lowest transfection efficiency with eGFP (6.5%). This suggests that knockdown efficiency is not entirely determined by transfection efficiency and that the cell line may play a role in the difference in knockdown levels of endogenous EGFR.

A knockdown efficiency trend was observed between the three EGFR shRNAs. In figure 5.3 EGFR sh2 shows the greatest percentage decrease when transfected into cell lines WHCO1 andWHCO5. EGFR sh1 is the next efficient while EGFR sh3 consistently demonstrates the weakest EGFR knockdown (not referring to WHCO6 transfection). During the siRNA/shRNA design phase the importance of secondary structure formation of the mRNA target was introduced. This could be the reasoning behind the difference in efficiency between the three EGFR shRNAs. The mRNA secondary structure of the EGFR such that the target sequences for EGFR sh1 and EGFR sh2 are more accessible for the RISC complex than that of EGFR sh3 target site. This difference in knockdown efficiencies was not observed in the shRNA efficiency reporter system experiment and was perhaps due to the fact that the 3xEGFR target was too short and therefore did not have the same secondary structure as the endogenous EGFR mRNA transcript.

Unfortunately, due to time constraints, the transfections could not be repeated and the average percentage knockdown for each EGFR shRNA could not be determined. However, based on the data presented the EGFR specifically designed shRNAs (Chapter 2), showed not only efficient knockdown of a synthetically produced target sequence in siRNA/ shRNA efficiency system but

also showed knockdown of endogenously over expressed EGFR in three tested oesophageal squamous cell carcinomas, WHCO1, WHCO5 and WHCO6, despite low transfection efficiency.

CHAPTER 6

6.1 General discussion

Abnormalities or alterations affecting the function of EGFR, or members of the ErbB1 family of receptor tyrosine kinases, have been shown to influence many of the key features relating to tumour growth and development (Wujcik, 2006). These features include, autonomous cell growth, enhanced metastatic potential, tissue invasion and more recently, over expression of EGFR in certain solid tumours, have resulted in an increased resistance to current cancer therapeutics as well as poor prognosis (Nicholson et al., 2001; Ciardiello and Tortora, 2003).

Epidermal growth factor receptor is over expressed in a number solid tumours such as colon, head and neck, pancreatic, non-small cell lung cancer, breast, renal carcinoma, ovarian, glioma, bladder and oesophageal (Salmon et al., 1995). Over expression of EGFR results in an increase of receptors on the cell surface, providing these cells with increased sensitivity to low levels of appropriate growth factors. Thus, over expression mutations such as these, are refered to as gain-of-function mutations.

Human oesophageal squamous cell carcinoma (HOSCC) does show a world wide distribution, however, displays high incidence regions that include countries belonging to the former Soviet Union, China, Japan, Brazil, Bermuda and the Transkei region of South Africa (Lam, 2000). The poor prognosis and high mortality associated with this type of aggressive cancer makes it the sixth most common cause of death related cancer (Parkin et al., 2005). Over expression of EGFR is a common molecular characteristic of this cancer type occurring up to 92% of all HOSCC tumours (Lam, 2000).

6.2 Knockdown of endogeneously over expressed EGFR

This research report describes the methodology, and the process of designing and testing effective shRNAs molecules that target and knockdown human EGFR. Three shRNAs were designed specifically against human EGFR gene sequence. The shRNAs design criteria ensured

that there was specific knockdown of human EGFR and no off target effects and that the shRNAs were transcribed correctly from the U6 promoter by RNA polymerase III. The shRNA's knockdown efficiency was determined against plasmid born reporter systems containing a target sequence to which all three of the designed EGFR shRNAs were responsive. Once the knockdown efficiency of each EGFR shRNAs had been determined experimentally, then using the same EGFR shRNA constructs a knockdown assay of endogenous, over expressed EGFR was performed in three HOSCC cell lines.

In this report it has been demonstrated that specific EGFR knockdown was achieved in three of the HOSCC tested, unfortunately due to time constraints the necessary repetition of the experiment was not achieved. An extensive search of the literature highlighted that although this is the first account of RNAi based strategy used to knockdown EGFR in oesophageal cancer cells, RNAi has been used to specifically knockdown EGFR in other cancer cell lines.
Nagy *et al.* 2003, achieved significant knockdown of EGFR (erbB1) in the A431 epidermoid carcinoma cell line. The A431 cell line is characterized by over expression of EGFR. Nagy *et al.* 2003 report knockdown efficiencies of endogenous EGFR in A431 cell line by 68% (± 5) and 48% (± 4) by their designed siRNAs erbB1-1 and erbB1-3 respectively. These results are similar to the knockdown observed following transfection of WHCO1 and WHCO5 cell lines with EGFR sh1 and sh2. Using flow cytometery Nagy *et al.* 2003, were able to show the knockdown efficiency of their siRNAs within transfected population of cells. Nagy *et al.* 2003 reported that the Western blot analysis reveals knockdown percentage of EGFR of roughly 70%, whereas when the determined the knockdown percentage within the transfected population of cells, it was closer to 90%. Unfortunately the transfection efficiency is not given for the A431 cells by Nagy *et al*. however it is advised that, in lower transfection efficient cells lines, knockdown measurements should be performed on the transfected population of cells. Based upon this result greater efficiency would be expected by all three of the EGFR shRNAs tested here, if the transfected cell population is analyzed. It is difficult to predict the improvement of knockdown efficiency seen based upon the observation by Nagy *et al.* 2003, as the transfection efficiency of A431 cells was not given.

Research performed on non-small cell lung cancer (NSCLC) tumours by Zhang *et al.* 2005, revealed knockdown of endogenous over expressed EGFR of 71.31% and 71.78% in A549 and

SPC-A1 NSCLC cells respectively. Epidermal growth factor receptor expression levels were quantified using an immuno-fluorescent assay, by treating the cells with a primary anti-EGFR monoclonal antibody followed by FITC-conjugated anti-mouse secondary antibody, and EGFR values calculated as the percentage positive cells x mean intensity of fluorescence. The knockdown percentage obtained using this siRNA against NSCLC cell lines A549 and SPC-A1, is higher than the general knockdown that we obtained in the HOSCC cell lines. Transfection of WHCO1 with EGFR sh1 and sh2 which obtained knockdown percentages of approximately 50%. Knockdown percentage of EGFR in the WHCO5 cell line using EGFR sh1 was 65%. However, EGFR sh2 transfection of the WHCO5 cell line did show higher knockdown percentage (85%) than that attained by Zhang *et al.* 2005.

6.3 Vision for the future

6.3.1 Avenues for Exploration

Repetition of the transfection experiments will provide a better measurement of average knockdown on which statistical analysis can be performed to identify an expected knockdown efficiency. Another hurdle that was encountered during the study was the low transfection efficiency of the HOSCC cell lines. Future work would need to include the effect that EGFR knockdown has on these HOSCC cell lines. As one would expect a decrease in endogenous EGF expression levels would also affect cell proliferation, by decreasing the EGFR numbers there is a direct affect on the MAPK signaling pathway downstream of receptor activation.

In order to accurately determine the effect that EGFR knockdown has on these cell lines transient transfection is somewhat inadequate. Over time, transient transfection results in the target shRNAs becoming diluted out as a result of cell division (Leung and Whittaker, 2005). Short hairpin RNAs can be stably introduced into cells through either a selectable plasmid system or through integration into the genome through the use of viral vectors. Van de Wetering *et al.* 2003 produced an inducible system under the control of a tetracycline operator, which results in the expression of the designed shRNAs upon addition of doxycycline to the media. The use of this strategy eliminates two of the problems highlighted here. This enables a selection of transfected population of cells, giving a better estimation of percentage knockdown. The inducible shRNA

transcription system means that the shRNA is not expressed constantly, indicating that gene targets essential for cell survival can be selected and knockdown can be induced upon addition of doxycycline.

6.3.2 Consideration for clinical application

RNAi has proven to be an extremely useful tool in determining particular gene function and due to the highly specific down regulation of target genes has the potential to usher in a new era of molecular therapeutics against cancer, infectious diseases and dominant genetic diseases (Leung and Whittaker, 2005). An important obstacle that needs to be addressed is target tissue specific, delivery of these the RNAi effector molecules.

In terms of developing RNAi as a therapeutic against over expressed EGFR, Nagy *et al.* 2003, suggested that cancer cells showing a down regulation of EGFR (erbB1) expression, demonstrate an increased sensitivity to EGFR-targeted therapies such as antibodies or tyrosine kinase inhibitors and thus perhaps RNAi will form part of a combination treatment for over expression of EGFR in cancer.

6.4 Conclusion

Over expression of endogenous EGFR is prevalent in many different cancer types and is associated with progression of the disease. The use of carefully designed shRNA's can specifically and efficiently decrease the expression levels of endogeneous expressed EGFR in oesophageal squamous cancer cell lines by as much as 85%. These data highlight the potential use of RNAi as a possible therapeutic against over expression of EGFR associated with cancer cells.

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APPENDIX

Tissue culture

Tissue culture medium

DMEM medium solution

1.37%	DMEM
0.37%	Sodium bicarbonate
2%	Penicillin/Streptomycin solution (Penicillin: 500 U/ml; Streptomycin: 0.5%)

Make up to final volume with dH2O

Hams F12 medium solution

1.07%	Hams F12 medium
0.118%	Sodium bicarbonate
2%	Penicillin/Streptomycin solution (Penicillin: 500 U/ml; Streptomycin: 0.5%)

Make up to final volume with dH2O

Mix:
3 volumes DMEM medium solution
1 volume Hams F12 medium solution
Filter sterilize
Store at 4°C

Phosphate buffer saline (1x)

136.9 mM	Sodium chloride
2.68 mM	Potassium chloride
10.1 mM	Disodium hydrogen phosphate dodecahydrate
1.76 mM	Potassium dihydrogen phosphate

Adjust pH between 7.2 to 7.3
Make up to final volume with dH2O
Sterilise for 20 min at 151 lbq
Store at 4°C

Trypsin:Ethylenediaminetetra-acetic acid (EDTA) solution
Trypsin solution

0.01%	Trypsin

Make up to final volume with PBS

EDTA solution

0.004%	EDTA

Make up to final volume with PBS

Mix:

50%	Trypsin solution
50%	EDTA solution

Store at 4°C

Protein estimation

Coomassie Blue solution (0.25%)

0.25%	Coomassie brilliant blue powder
50%	Methanol
10%	Glacial acetic acid

Make up to final volume with dH2O

Destaining solution

12%	Glacial acetic acid
10%	Methanol

Make up to final volume with dH2O

Elution solution

66%	Methanol
33%	dH2O
1%	Ammonia

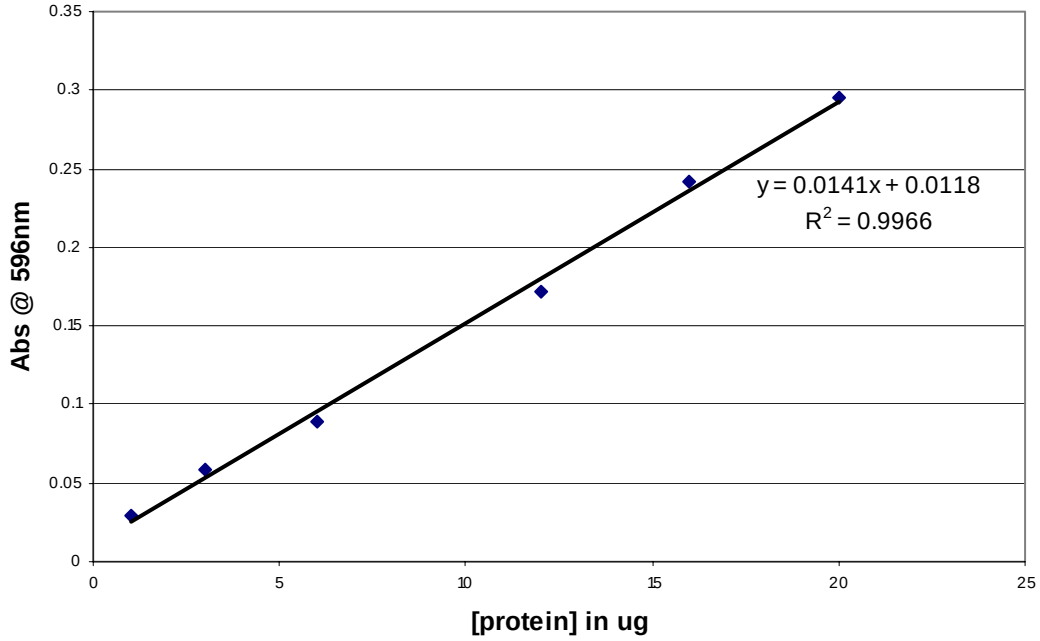

Figure a. Graphical representation of a protein estimation standard curve.
Standard curve showing the specific BSA standards, at varying concentrations from 1-20 µg, and their respective absorbance value at 596 nm were used to create the curve. The equation of the curve was used to calculate the unknown protein concentration in a given sample. R^2 = linear regression.

Sample preparation for SDS-PAGE

Double lysis buffer

123.8 mM	Tris-HCl, pH 6.8
4%	SDS
20%	glycerol
10%	β-mercaptoethanol

Make up to final volume with dH2O
Store at 4°C

SDS-PAGE

Separating gel

10%	Acrylamide
0.1%	NN'-methylenebisacrylamide
375 mM	Tris-HCl, pH 8.8

0.2%	SDS

Make up to final volume with dH2O
Just before use add:

1mM	Ammonium persulphate
0.25%	N,N,N'N'-tetramethylenediamine (TEMED)

Stacking gel

10%	Acrylamide
0.1%	NN'-methylenebisacrylamide
125 mM	Tris-HCl, pH 6.8
0.2%	SDS

Make up to final volume with dH2O
Just before use add:

1mM	Ammonium persulphate
0.25%	TEMED

Running buffer

3.74 mM	SDS
25 mM	Tris-HCl, pH 8.3
192.5 mM	Glycine

Make up to final volume with dH2O

Destain solution

10%	Acetic acid
10%	Methanol

Make up to final volume with dH2O

Western Blot

Transfer buffer

25 mM	Tris-HCl, pH 8.3
1.41%	Glycine
20%	Methanol

Make up to final volume with dH2O
Store at 4°C

Blocking solution

50 mM	Tris-HCl, pH 7.8
2 mM	Calcium chloride dihydrate
5%	non-fat milk powder
0.01%	anti-foam
0.05%	Triton-X 100

Make up to final volume with dH2O
Store at 4°C

SuperSignal West Pico Chemiluminescent Substrate kit

Before use **mix:**

50%	Luminol/Enhancer solution
50%	Stable peroxide buffer

Store in the dark

Developer

6.4 M	Metol
0.6 M	Sodium sulphite (anhydrous)
80 mM	Hydroquinine
0.45 mM	Sodium carbonate (anhydrous)
34 mM	Potassium bromide

Make up to final volume with dH2O
Store in the dark

Fixer

0.8 M	Sodium trisulphate
0.2 M	Sodium metasulphite

Make up to final volume with dH2O
Store in the dark